

Role of cyclo-oxygenase and related pathways in vascular health and disease

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Declaration

I, Martina Helena Lundberg, hereby declare that the work presented in this thesis is my own. Information derived from other sources and work done in collaboration has been appropriately cited.

Dedication

I dedicate this thesis to my father, Professor Jan M Lundberg,
who has always inspired me to be curious, patient and persistent.
And to my mother, Ingeborg A Lundberg, for her everlasting enthusiasm.

Abstract

The cardioprotective hormone prostacyclin is a potent inhibitor of platelet activation and is produced in vascular endothelium by the concerted actions of cyclo-oxygenase and prostacyclin synthase. There is an ongoing, heated debate about which COX isoform (COX-1 or COX-2) regulates endothelial prostacyclin production. This is an important area of study since non-steroidal anti-inflammatory drugs (NSAIDs) or COX-2 selective inhibitors, used to treat arthritis, are associated with an increased risk of cardiovascular events, which has been linked to a reduction of urinary markers of prostacyclin. Thus, the aim of my thesis was to investigate the relative contribution of COX-1 versus COX-2 to endothelial prostacyclin production and vascular function.

Using *en face* confocal imaging, I found that COX-1 immunoreactivity predominates over COX-2 in the endothelium of all blood vessels studied, including aortic arches from healthy wild type (WT, C57BL/6J) mice, in WT mice injected with endotoxin, ApoE^{-/-} mice, rats with chronic heart failure and in human pulmonary artery. I have performed bioassays with blood vessels from WT versus COX-1^{-/-} and COX-2^{-/-} mice to show that COX-1 not COX-2 drives the majority of vascular prostacyclin production in both young (3 month old) and mature (12 month old) mice of both sexes. Phenotypic differences in the COX-1^{-/-} and COX-2^{-/-} mice included elevated levels of nuclear 'primed' NF-κB in the endothelium of both the protected (greater) and atherosclerosis-susceptible (lesser) curvature of the aortic arch. Despite COX-2^{-/-} mice having normal endothelial prostacyclin production, more platelets adhered to the lesser curvature of the aortic arch.

The techniques and validation protocols employed have shown COX-1 as the predominant endothelial COX isoform, responsible for vascular prostacyclin production. Low levels of constitutive COX-2 was found localised to susceptible areas of the vasculature where it may contribute to prostacyclin production on a local level to limit inflammation, atherosclerosis and platelet adhesion.

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Publications and abstracts

Published papers

Joint first authorship:

"Role of Shear Stress in Endothelial Cell Morphology and Expression of Cyclooxygenase Isoforms"

Potter CMF; **Lundberg MH**; Harrington LS; Warboys CM; Warner TD; Berson RE; Moshkov AV; Gorelik J; et al. (*Arteriosclerosis, Thrombosis, and Vascular Biology*, December 2010)

Co-author:

"Despite profound dominance of COX-1, regions of vascular COX-2 expression are associated with protection against atherosclerosis"

Nicholas S Kirkby, **Martina H Lundberg**, Louise S Harrington, Lucy K. Bailey, Simon Cuhlmann, Paul C Evans, Ivana Vojnovic, Timothy D. Warner and Jane A. Mitchell. (Submitted to *Circulation Research*, January 2012)

"Shape and compliance of endothelial cells exposed to different shear stress patterns in vitro or selected from different regions of the aorta: assessment by scanning ion conductance microscopy"

Claire M.F. Potter, Sophie Schobesberger, **Martina H Lundberg**, Peter D. Weinberg, Jane A. Mitchell, Julia Gorelik. (In press, *PlosOne*, October 2011)

"Reduced endothelial dependent vasodilation in vessels from TLR4^{-/-} mice is associated with increased superoxide generation"

Harrington LS; **Lundberg MH**; Waight M; Rozario A; Mitchell JA.

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First author abstracts presented at conferences

“Role of cyclo-oxygenase and related pathways in endothelium of the aortic arch”

(Oral presentation, National Heart and Lung Institute postgraduate day, London, June 2011)

“Role of COX-1, COX-2 and phospholipase A₂ in prostacyclin release from aortic tissue of mature mice in vitro”

(Oral presentation, British Pharmacological Society conference, London, December 2010)

“COX isoforms in aortic endothelial cells from young adult and mature adult mice”

(Oral presentation, British Pharmacological Society Conference, London, December 2009)

“COX-1 or COX-2 deletion is associated with increased nuclear NF-κB in the endothelium of the aortic arch”

(Poster, WorldPharma, 16th IUPHAR World Congress of Basis and Clinical Pharmacology, Copenhagen, July 2010)

“Analysis of regional distribution of COX-isoforms in endothelial cells of the aortic arch of the mouse reveals a predominance of COX-1 in healthy blood vessels”

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Authorship on other abstracts presented at conferences

“Evaluation of aspirin-triggered 15-epi-lipoxin A4 as a biomarker of COX2 expression in vivo”

(Poster, British Pharmacological Society Conference, London, December 2011)

“Contribution of COX1 and COX2 to prostacyclin release by healthy mouse tissues”

(Poster, 10th World Congress on Inflammation, Paris, June 2011)

“Role of COX-1 and COX-2 in the release of prostanoids in murine lung and isolated lung fibroblasts”

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“Relationship between shear stress profile and endothelial cell morphology, physiology and structural responses”

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- Facility for live imaging at Imperial College (FILM) and Invitrogen image of the year competition (runner up with images included in a published calendar 2009, 2010 and 2011)

List of abbreviations

(+/+)	Wild Type; two functional copies of target gene (no mutant gene)
(+/-)	Heterozygote knockout; one copy of the functional target gene and one of the mutant gene
(-/-)	Homozygote knockout; no functional copies of the target gene, two copies of the mutant gene
APC	Adenoma Prevention with Celecoxib (clinical trial)
ApoE	Apolipoprotein
ApoE^{-/-}	Apolipoprotein homozygote knockout mice
APPROVe	Adenomatous Polyp Prevention On Vioxx (clinical trial)
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary DNA
COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
COX-1^{-/-}	Cyclo-oxygenase 1 homozygote knockout mice
COX-2^{-/-}	Cyclo-oxygenase 2 homozygote knockout mice
CLASS	Celecoxib Long term Arthritis Study (clinical trial)
cPLA₂	Cytosolic Phospholipase A ₂
DAG	Diacylglycerol
DAPI	4', 6'-diamidino-2-phenylindole
dH2O	Distilled Water
EDHRF	Endothelium-Derived Hyperpolarizing Factor
EGF domain	Epidermal Growth Factor Domain
EIA	Enzyme Immunoassay
ET-1	Endothelin-1
ER	Endoplasmic Reticulum
FDA	American Food and Drug Administration
FeCl₃	Iron (III) Chloride
FRET	Fluorescence Resonance Energy Transfer
GANC	Ganciclovir (anti-viral drug)
GPCRs	G-protein Coupled Receptors
G418	Genecitin (antibiotic)
HDL	High Density Lipoprotein
HTRF	Homogeneous Time-Resolved Fluorescence
IL-1	Interleukin-1
iPLA₂	Calcium independent Phospholipase A ₂
IP3	Inositol Triphosphate
6-ketoPGF_{1α}	Prostacyclin breakdown (hydrolysis) product
LAS AF	Leica Application Suite Advanced Fluorescence Lite Confocal software program
LDL	Low Density Lipoprotein
LDLR^{-/-}	Low Density Lipoprotein Receptor homozygote knockout mice
LPS	Lipopolysaccharide

List of Abbreviations

mPGES-1	Microsomal Prostaglandin E ₂ Synthase-1
mRNA	messenger RNA
NC3Rs	National Centre for the Replacement, Refinement and Reduction of animal research
Neo	Neomycin resistance gene insert
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
O/N	Overnight
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGES	Prostaglandin E ₂ Synthase
PGHS	Prostaglandin H Synthase
PGH₂	Prostaglandin endoperoxide
PGI-M	Prostacyclin metabolite (2,3-dinor-6-ketoprostaglandin F _{1α})
PGX	Initial name of prostacyclin
PLA₂	Phospholipase A ₂
PMT	Photo-Multiplier Detector
POX	Peroxidase
PPARs	Peroxisome Proliferator Activated Receptors
PPREs	Peroxisome Proliferator-Responsive Elements
Precision	Prospective Randomized Evaluation Of Celecoxib Integrated Safety Vs Ibuprofen Or Naproxen (clinical trial)
Ptgs1 gene	Prostaglandin-Endoperoxide Synthase 1 (COX-1) gene
Ptgs2 gene	Prostaglandin-Endoperoxidase Synthase 2 (COX-2) gene
RIA	Radio Immunoassay
ROI	Region Of Interest
RT	Room Temperature
Ser 530	Serine 530
sPLA₂	Secretory Phospholipase A ₂
SUCCESS IV	SUCcessive Celecoxib Efficacy and Safety Study (clinical trial)
TARGET	Therapeutic Arthritis Research and Gastrointestinal Event Trial (clinical trial)
TPA	Tetradecanoyl Phorbol Acetate
TRF	Time-Resolved Fluorescence
TXB₂	Thromboxane breakdown (hydrolysis) product
TX-M	Thromboxane metabolite (11-dihydro thromboxane B ₂)
TX-M	Thromboxane metabolite (2,3-dinor-thromboxane B ₂)
VIGOR	Vioxx Gastrointestinal Outcomes Research Study (clinical trial)
VLDL	Very Low Density Lipoprotein
WT	Wild Type

Chapter 1

General Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, diclofenac, naproxen and ibuprofen are some of the most common and widely available over-the-counter drugs worldwide. NSAIDs are generally indicated for the symptomatic relief of common pain conditions such as headaches, fever, dysmenorrhea and the cardinal signs of acute inflammation following tissue injury (swelling, redness, heat, pain and loss of function). For short term use, NSAIDs are generally highly effective, well tolerated and relatively safe with few side effects, hence their widespread use for common aches and pains. However, there is a large group of patients with chronic inflammatory conditions (autoimmune diseases) such as rheumatoid arthritis, osteoarthritis and ankylosing spondylitis that often rely on daily dosing of NSAIDs for symptomatic relief from debilitating stiffness and pain in affected joints. For this kind of long term, chronic NSAID use, there are a number of well recognised side effects that need to be considered and weighed against the benefit of pain relief.

The three main organ systems affected by chronic NSAID use are: 1) the gastro intestinal tract, 2) the kidneys and 3) the cardiovascular system. The most common side effect is on the gastrointestinal tract, where long term NSAID use can lead to erosions, perforations, bleeding and ulcers (Perini *et al.*, 2004; Wallace *et al.*, 1990; Wallace *et al.*, 2000). Another side effect is on the renal system where NSAIDs commonly induce a small (1-5 mmHg) rise in systemic blood pressure in normotensive as well as hypertensive patients which may increase hypertension related morbidity (Singh *et al.*, 2003). These gastrointestinal and renal side effects will be briefly introduced later on in this chapter.

A more recent area of concern is the association of chronic NSAID use with an increased risk of thrombotic cardiovascular events including myocardial infarction and stroke (Bluhm *et al.*, 2011; Bombardier *et al.*, 2000; Bresalier *et al.*, 2005). The focus of my PhD has been to investigate some of the basic mechanisms that relate to these cardiovascular side effects of NSAIDs.

Before exploring these cardiovascular side effects in greater detail and the specific aims of my thesis, I will first give some general background about the pharmacology of NSAIDs.

The mode of action of NSAIDs

Even though different forms of NSAIDs, such as salicylate from willow bark, have been used for thousands of years, it was not until the 1970s that the pharmacology behind the analgesic, anti-pyretic and anti-inflammatory effects of these drugs could be explained. Aspirin (acetylsalicylic acid) is often used as a class example for NSAIDs. For this reason NSAIDs are sometimes referred to as 'aspirin-like drugs'. In 1971, Sir John Vane published the paper 'Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs' (Vane, 1971), which along with 3 other papers (Collier *et al.*, 1971; Ferreira *et al.*, 1971; Smith *et al.*, 1971), for the first time identified that 'aspirin-like' drugs inhibit prostaglandin (PG) synthesis by interfering with the active site of PGH synthase (PGHS). As detailed below, PGHS is an integral membrane protein comprised of two enzymatic components, a cyclo-oxygenase (COX) and a peroxidase (POX) site. PGHS is, however, colloquially referred to as COX and this will also be the case throughout the thesis.

Prostanoids

Prostanoids (PGs, thromboxane and prostacyclin) are a subclass of the eicosanoid family of oxygenated derivatives of 20-carbon essential fatty acids. This thesis will focus on the prostanoids derived from arachidonic acid which is the most abundant precursor fatty acid source in mammals (Narumiya *et al.*, 1999). Arachidonic acid is a poly unsaturated omega 6 fatty acid with the first double (carbon-carbon) bond in the n-6 position in the 20 carbon chain linked to the carboxylic acid. The nomenclature for prostanoids derived from arachidonic acid is denoted by the number ₂ in subscript to denote 2 double bonds (i.e. PGE₂). All the prostanoids contain 20 carbon atoms divided up in a cyclopentane ring and 2 side chains (prostacyclin has got two cyclopentane rings). Their nomenclature is further decided based on modifications of this cyclopentane ring by the alphabetic letters A-I, where PG A-C only occur during artificial extraction procedures and PG D-I occur naturally in the body (Narumiya *et al.*, 1999). PGs were first isolated from seminal fluid and therefore the name prostaglandin comes from the association with the prostate gland which was first believed to be the tissue source of PGs (Goldblatt, 1935; V Euler, 1934). All prostanoids are short lived, biologically active lipid mediators crucial to many physiological and inflammatory processes. Due to their unstable nature, prostanoids cannot be stored within the cell, and are therefore produced on demand following any chemical or mechanical

stimuli leading to an increase in intracellular calcium. They are then released from the stimulated cell immediately after synthesis. Due to their chemical instability, they are rapidly degraded or metabolised with a half-life in the range of 30 seconds to a few minutes. Hence, the activity of prostanoids remain mainly local to the vicinity of their production (Narumiya *et al.*, 1999).

Prostanoid synthesis pathway

When hormones or physical forces activate cells, intracellular calcium increases and activates calcium activated forms of calmodulin, which in turn activates cytosolic forms of phospholipase A₂ (PLA₂). Activated PLA₂ cleaves arachidonic acid from the membrane phospholipids. The free arachidonic acid is then subject to enzymatic conversion by various enzymes, including the integral membrane enzyme PGHS. PGHS has two adjacent but spatially distinct active sites; the COX site and the POX site. The arachidonic acid gains access to the COX site through the interior of the lipid bilayer, where it undergoes bi-oxidation (+O₂) to form the unstable endoperoxidase PGG₂ (Figure 1.1). PGG₂ is then reduced (-e) to PGH₂ at the POX site (Figure 1.1 and 1.2). Various synthases and isomerases then convert the unstable PGH₂ into PGs (D₂, E₂, and F₂), prostacyclin and thromboxane (FitzGerald, 2003) (Figure 1.1). The NSAID aspirin irreversibly acetylates a key amino acid residue in the COX catalytic site (Serine-530) which interferes with arachidonic acid (substrate) binding and thereby renders the COX enzyme inactive (DeWitt *et al.*, 1990). While the NSAIDs diclofenac, ibuprofen and naproxen, are reversible competitive inhibitors that compete with the substrate (arachidonic acid) for the COX catalytic site (Figure 1.1).

Figure 1.1 Prostanoid synthesis pathway

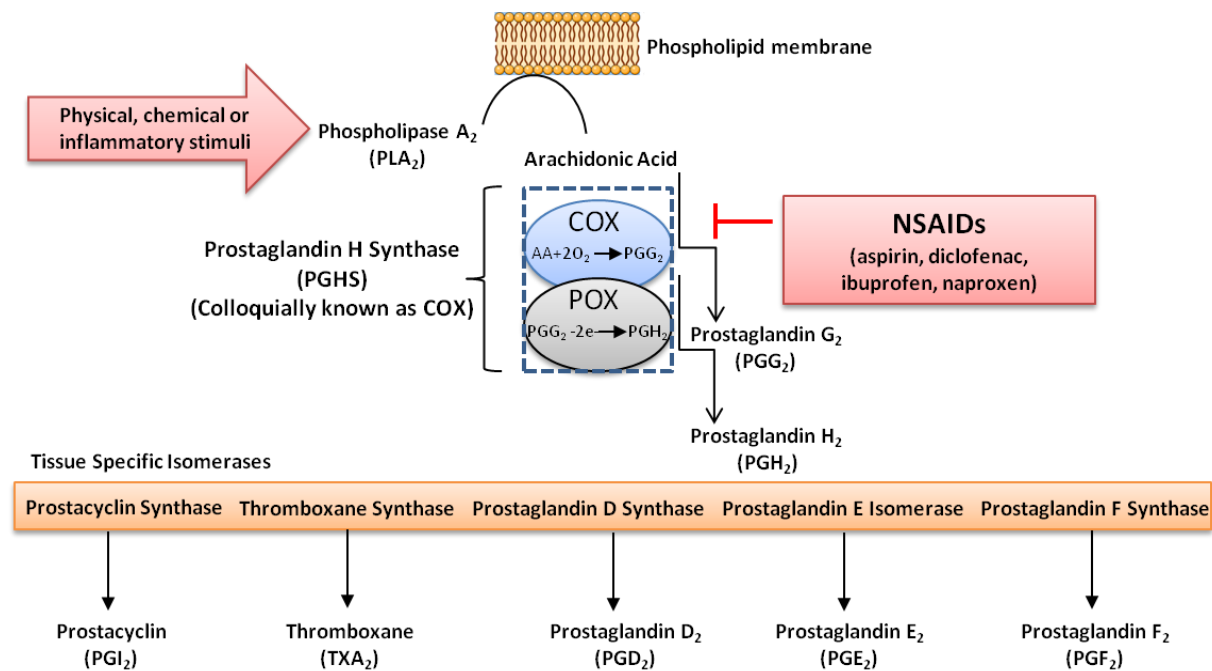


Figure 1.1 Prostanoid synthesis pathway. Following a mechanical, chemical or inflammatory stimuli, arachidonic acid is cleaved from the membrane phospholipids by phospholipase A₂ (PLA₂), then oxidised by the catalytic COX site, forming prostaglandin G₂ (PGG₂) which is then reduced at the POX site to form prostaglandin H₂ (PGH₂). PGH₂ is then further metabolised by various downstream tissue specific isomerases, to form (prostaglandin (D₂, E₂ and F₂), prostacyclin and thromboxane (figure modified from (FitzGerald, 2003).

The initiation of COX catalytic activity depends on a priming event from the POX site, where a free radical (Tyr385) formed by POX activity oxidises the COX site to the active form (Ouellet et al., 2001) (Figure 1.2). The two sites are thereby mechanistically interrelated and it is held that one priming event from the POX site can sustain many cycles of COX enzymatic activity (Gupta *et al.*, 2004).

Figure 1.2 Schematic of the priming event required for activation of COX oxidizing activity

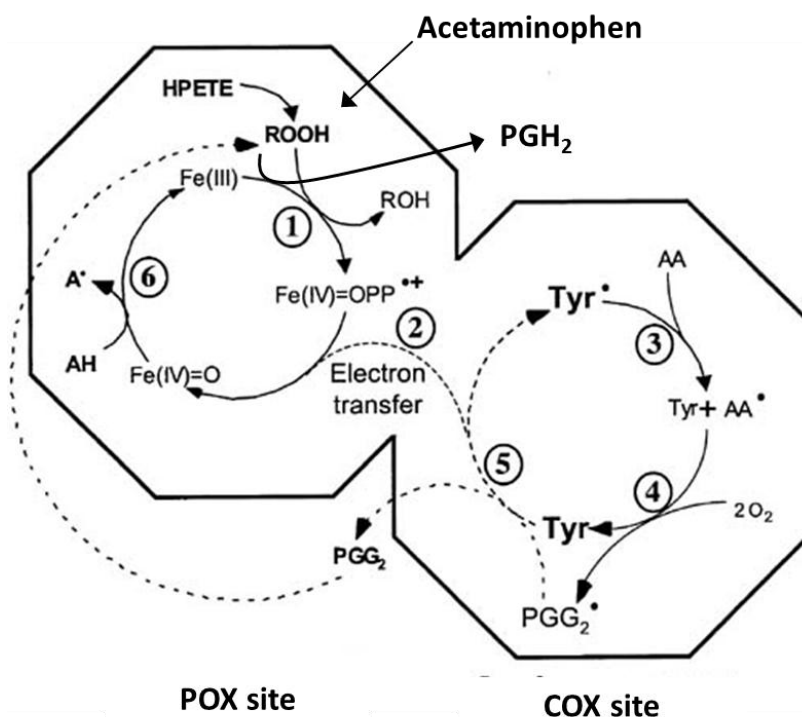


Figure 1.2 Schematic of the priming event required for activation of COX oxidizing activity. Heme iron oxidation states are indicated by roman numerals (Fe^{III} and Fe^{IV}). PP=protoporphyrin π -cation radical. AA = Arachidonic Acid. AA^{*} = Arachidonyl radical. Tyr= Tyrosine 385. Tyr^{*}= Tyrosine radical. AH= reducing substrate. A^{*}= oxidised co-substrate. PGG₂ = hydroperoxyendoperoxide (prostaglandin G₂). PGG₂^{*}= with a peroxy radical on carbon 15. ROOH= hydroperoxide substrate. ROH=alcohol product. HPETE= hydroperoxide of fatty acids. **1)** A lipid hydroperoxide dependent electron oxidation transforms the resting Fe^{III} heme enzyme in the POX site, into a $\text{Fe}^{\text{IV}}\text{OPP}$ heme ^{*} radical. **2)** Two electrons are transferred via intramolecular electron transfer from the $\text{Fe}^{\text{IV}}\text{OPP}$ heme ^{*} radical to the Tyrosine 385 (Tyr-385) in the COX active site, creating a tyrosine radical (Tyr^{*}). **3)** Following AA substrate binding into the COX active site, the Tyr^{*} extracts a hydrogen atom from the arachidonyl radical (AA^{*}) and initiates the COX reaction. **4)** The AA^{*} is oxidised with 2 O₂ forming the hydroperoxyendoperoxide PGG₂^{*}. **5)** The PGG₂^{*} transfers the radical back to Tyr. **6)** PGG₂ is then reduced (-2 electrons) to PGH₂ by the POX site. This reduction yields the oxidised heme radical ($\text{Fe}^{\text{IV}}\text{OPP}$ heme ^{*} radical) which starts the cycle over again creating the Tyr^{*} required for the COX catalytic activity. Figure modified from (Aronoff et al., 2006).

Even when the COX site is inhibited (non-covalently by NSAIDs and covalently by aspirin), the POX site can still continue to operate to form various free radicals species from endogenous peroxide substrates (Gupta et al., 2004). There are currently no available selective inhibitors of the POX site. However, the analgesic and anti-pyretic properties of paracetamol (acetaminophen) have been suggested to be linked to interference with the

POX site, thereby affecting prostaglandin synthesis by preventing the essential priming event required for COX activity (Figure 1.2) (Boutaud *et al.*, 2002; Lucas *et al.*, 2005). Paracetamol differs from NSAIDs in the way that it exhibits no or little anti-inflammatory effects and has limited effects on platelets at anti-pyretic doses in humans (Boutaud *et al.*, 2002). This cellular specificity may be explained by differences in cellular environments in regards to endogenous concentrations of hydroperoxides, which may override the POX site interference by acetaminophen in platelets and at sites of inflammation (Boutaud *et al.*, 2002). The low hydroperoxide tone in nerves may explain the analgesic and anti-pyretic effects of acetaminophen in the central nervous system (Boutaud *et al.*, 2002).

As mentioned above, even though PGHS contains both the COX site and the POX site, it is colloquially known simply as COX, and will be referred to as COX throughout the thesis.

Arachidonic acid liberation by PLA₂

Arachidonic acid can be cleaved from membrane phospholipids by various phospholipase enzymes, but PLA₂ is the only enzyme that can catalyse the liberation of arachidonic acid (by hydrolysis of membrane glucerophospholipids) in one single step and is considered the primary phospholipase enzyme involved in prostanoid synthesis. There are many different forms of PLA₂ including calcium regulated cytosolic PLA₂ (cPLA₂), secretory PLA₂ (sPLA₂) and calcium independent PLA₂ (iPLA₂) (Murakami *et al.*, 2002). However, calcium dependent activation of cPLA₂ and sPLA₂ seems to be the most common route for arachidonic acid release during prostanoid synthesis, whilst the calcium independent iPLA₂ seems to be mainly involved in phospholipid remodeling rather than in signaling (Murakami *et al.*, 1999). In contrast to cPLA₂ which can only contribute to prostanoid synthesis when COX is co-expressed within a particular cell, sPLA₂ has been reported to be excreted extracellularly and bind to the cell surface of neighboring COX expressing cells. In this way, a remote stimulus can induce transcellular PLA₂ activity leading to prostanoid synthesis in a separate cell to the initial stimulus and thereby overall prostanoid production can be amplified (Murakami *et al.*, 1999). Activation of the calcium dependent cPLA₂ and sPLA₂ along with the COX enzymatic conversion of arachidonic acid to PGH₂ are considered the rate limiting steps of prostaglandin synthesis (Cirino, 1998). In inflammatory conditions when prostanoid production is dramatically increased, there seem to be a two component system, where two

stimuli are required (activation of PLA₂ and induction of COX), to synergistically render more substrate available (arachidonic acid via PLA₂ activity) for COX driven enzymatic conversion into PGH₂ (Hamilton *et al.*, 1999; Saunders *et al.*, 1999). Basal PG production is generally low but increases immediately following an inflammatory response, before the recruitment of leukocytes and infiltration of immune cells into the affected tissue (Ricciotti *et al.*, 2011). Both COX (induction) and PLA₂ activity during inflammation is inhibited by the anti-inflammatory drugs corticosteroids. Indeed the therapeutic benefits of corticosteroids (such as prednisone in rheumatoid arthritis) are thought to be in part due to inhibition of PG production (Flower *et al.*, 1979).

Tissue specific production of prostanoids

The profile of PGs produced within a particular tissue or cell type will vary depending on the distribution of PG isomerases and synthase enzymes, substrate availability (arachidonic acid release via PLA₂ activity) as well as the oxidative state (hydroperoxide concentration) of the cell (Mitchell *et al.*, 1999; Ueno *et al.*, 2001). Kinetic parameters of the various PG synthases enzymes may also influence the ratio of prostanoids formed, in terms of substrate sensitivity and saturation (Ueno *et al.*, 2001). Endothelial cells are highly enriched with prostacyclin synthase and prostacyclin is therefore the major COX product of vascular endothelium (Kawka *et al.*, 2007; Liou *et al.*, 2000; Mitchell *et al.*, 2008; Moncada *et al.*, 1977). On the other hand, thromboxane is mainly produced by platelets due to the high expression of thromboxane synthase (Hamberg *et al.*, 1975). PGE₂ is formed by PGE synthases (PGES). It was previously held that PGE₂ could be formed non-enzymatically during times of COX induction and increased substrate (PGH₂) supply; which could saturate the other isomerases and synthases and result in proportionally higher PGE₂ production as seen inflammation (Mitchell *et al.*, 1999). However, high levels of PGE₂ at the site of inflammation has more recently been explained by the action of an inducible microsomal PGES-1 (mPGES-1), which is upregulated by pro-inflammatory cytokines, and could therefore drive part of the increased PGE₂ formation (Jakobsson *et al.*, 1999). This is further supported by reports from mice lacking functional mPGES-1 activity, which have impaired pain and inflammatory responses compared to normal mice and mPGES-1 is therefore a potential new drug target in the NSAID area (Trebino *et al.*, 2003).

There are also reports of prostacyclin synthase being inducible for instance following cyclic mechanical stretch in the myometrium leading to increased prostacyclin levels during pregnancy and labor (Korita *et al.*, 2002), or by cytokines and tumor promoters (Smyth *et al.*, 2009), but it has been highlighted that the endogenous regulation of prostacyclin synthase requires further investigation (Wu *et al.*, 2005). For example, prostacyclin synthase has been shown to be a constitutively expressed enzyme in human endothelial cells and substrate availability (PGH₂), appears to be the rate limiting step of prostacyclin synthase (Spisni *et al.*, 1995).

Biological effects of prostanoids- role of prostanoid receptors

In contrast to circulating systemic hormones (such as cortisone), prostanoids are autocrine or paracrine mediators that are released from a wide range of tissues in the body to influence signaling in the immediate environment of their release (Dubois *et al.*, 1998). The biological effects of prostanoids will depend on the distribution of prostanoid receptors (7 trans-membrane G-protein coupled receptors, GPCRs) and/or peroxisome proliferator activated receptors (PPARs) on local target tissues as well as the half-life of the prostanoids before they are broken down or metabolised (Dubois *et al.*, 1998). There are five major subdivisions of prostanoid receptors that have been defined pharmacologically and are named based on the first letter of their primary ligands (prostaglandin D₂: DP receptors, prostaglandin E₂: EP receptors, prostaglandin F_{2α}: FP receptors, Prostacyclin: IP receptors, thromboxane: TP receptors). Although, all prostanoids can activate different prostanoid receptors to various degree, their primary ligand show the highest affinity (Breyer *et al.*, 2001). The main signal transduction pathways and physiological relevance associated with the activation of these receptors are briefly summarised in Table 1.3, but mainly involves: contraction or relaxation of vascular smooth muscle, vascular homeostasis through platelet activation and inhibition, sensitizing sensory nerves to noxious stimuli, modulating neuronal activity including fever and sleep induction, transport of ions and water in the kidneys, secretion and motility in the gastro intestinal tract and cytoprotection of the gastric mucosa and regulating cell differentiation and apoptosis (Narumiya *et al.*, 1999).

Table 1.3 Prostanoid receptor signal transduction

Prostanoid	Receptor(s)	Signal Transduction	Main Activity
PGE ₂	EP ₁	[Ca ²⁺]↑ (Calcium channel dependent influx of extracellular Ca ²⁺) (Watabe <i>et al.</i> , 1993)	Bronchoconstriction (modulates adrenergic receptor responses) (McGraw <i>et al.</i> , 2006) Pain signalling (sensitisation of TRPV1) (Moriyama <i>et al.</i> , 2005)
	EP ₂	Coupled to G _s Activates adenylate cyclase →[cAMP] ↑ (Narumiya <i>et al.</i> , 1999)	Mast cell deactivation (Kay <i>et al.</i> , 2006) Bronchodilation (Maher <i>et al.</i> , 2010) Relax smooth muscle Vasodilatation (Breyer <i>et al.</i> , 2001)
	EP ₃	Coupled to G _i Inhibits adenylate cyclase →[cAMP] ↓ (Breyer <i>et al.</i> , 2001)	Constricts smooth muscle Vasoconstriction (Breyer <i>et al.</i> , 2001) Bronchoconstriction+Cough (Maher <i>et al.</i> , 2009) Fever (Ushikubi <i>et al.</i> , 1998)

Table 1.3 Prostanoid receptor signal transduction.

Table 1.3 Prostanoid receptor signal transduction (continued)

Prostanoid	Receptors	Signal Transduction	Main Activity
PGE ₂	EP ₄	Coupled to G _s Activates adenylate cyclase →[cAMP] ↑ (Narumiya <i>et al.</i> , 1999)	Relax smooth muscle Vasodilatation (Breyer <i>et al.</i> , 2001) Closure of ductus arteriosus (Nguyen <i>et al.</i> , 1997) Bronchodilation (Buckley <i>et al.</i> , 2011) Pain signalling (Clark <i>et al.</i> , 2008)
PGF _{2α}	FP _A FP _B	Coupled to G _q Activates Phospholipase C [IP ₃] →[Ca ²⁺] ↑ (Narumiya <i>et al.</i> , 1999)	Parturition+ Uterine contraction (Sugimoto <i>et al.</i> , 1997) Intraocular pressure (Lee <i>et al.</i> , 1988)
PGD ₂	DP1	Coupled to G _s Activates adenylate cyclase →[cAMP] ↑ (Narumiya <i>et al.</i> , 1999)	Sleep (Hayaishi, 1991) Mast cells- allergy (Narumiya <i>et al.</i> , 1999)
	DP2/ CRTH2	Coupled to G _i Inhibits adenylate cyclase →[cAMP] ↓ (Hirai <i>et al.</i> , 2001)	TH2/eotaxin/basophil chemotaxis (Hirai <i>et al.</i> , 2001)

Table 1.3 Prostanoid receptor signal transduction.

Table 1.3 Prostanoid receptor signal transduction (continued)

PGI₂ Prostacyclin	IP	Coupled to G _s Activates adenylate cyclase → [cAMP] ↑ Coupled to G _q Activates Phospholipase C → [Ca ²⁺] ↑ (Narumiya <i>et al.</i> , 1999)	Vasodilatation (Moncada <i>et al.</i> , 1976) Platelet Inhibition (Moncada <i>et al.</i> , 1976) Oedema (Murata <i>et al.</i> , 1997) Pain signalling (Murata <i>et al.</i> , 1997)
	PPARβ/δ	Heterodimerize with retinoid X receptor to act as a regulatory transcription factor on target genes that contain peroxisome proliferator-responsive elements (PPREs). → Gene induction/transcription	Platelet inhibition (Ali <i>et al.</i> , 2006a) Implantation in uterus (Lim <i>et al.</i> , 2000)
TXA₂ Thromboxane	TP_α	Couples to G _q → Phospholipase C activation G _s → Activates adenylate cyclase → [cAMP] ↑	Platelet activation (Hamberg <i>et al.</i> , 1975) Vasoconstriction Smooth muscle cell proliferation (Mitchell <i>et al.</i> , 2006b)
	TP_β	Couples to G _q → Phospholipase C activation G _s → Inhibits adenylate cyclase → [cAMP] ↓	

Table 1.3 Prostanoid receptor signal transduction.

For the purpose of this thesis I will focus on the two prostanoids and their corresponding receptors that are most relevant for cardiovascular physiology; prostacyclin acting on IP/PPARβ/δ receptors (Ali *et al.*, 2006a; Boie *et al.*, 1994) and thromboxane acting on TP receptors (Hirata *et al.*, 1996).

Prostacyclin and IP receptors

Prostacyclin was discovered in 1976 by Salvador Moncada, John Vane and co-workers, when they demonstrated that an unstable COX derived product (PGX), generated from vascular tissue was a potent inhibitor of platelet aggregation (Moncada *et al.*, 1976). Moncada and colleagues also showed that PGX (prostacyclin) generated from the arterial vessel wall,

relaxed the smooth muscle of some isolated blood vessels which suggested it could play a part in regulating vascular tone and the integrity of vascular endothelium to resist platelet adhesion (Moncada *et al.*, 1976). It is now well established that prostacyclin is the main arachidonic acid product from all vascular tissue (Moncada *et al.*, 1976; Vane *et al.*, 1995). It has been demonstrated that the highest level of prostacyclin production occurs in the endothelium of the vessel wall and then the production progressively decreases further in the vessel towards the adventitia (Moncada *et al.*, 1977).

Prostacyclin has several cardioprotective properties including vasodilatation, inhibition of platelet adhesion and aggregation, reduction of cholesterol uptake and prevention smooth muscle remodeling and thereby plays an important part in vascular hemostasis (Mitchell *et al.*, 2006b). As mentioned above for other COX products, prostacyclin generation can be stimulated by any stimuli that leads to an increase in intracellular calcium in the endothelial cells; such as thrombin, bradykinin, serotonin, platelet derived growth factor, contact with activated leukocytes, interleukin 1 (IL-1) or mechanical stretching of the vessel due to pulsatile pressure from blood flow exerted of the arterial wall (Vane *et al.*, 1990). Activated platelets may also affect endothelial prostacyclin production by transcellular redirection of platelet PG endoperoxide (PGH₂), which can then be utilised by prostacyclin synthase in nearby endothelium and result in a rapid increase in prostacyclin production (Vane *et al.*, 2003). This has been demonstrated in cultured human endothelial cells (Marcus *et al.*, 1980) and in healthy volunteers (Nowak *et al.*, 1989).

Following its synthesis and release, prostacyclin has a short half-life of a few minutes before it is non-enzymatically hydrolysed to a much less active but more stable compound; 6-keto prostaglandin F_{1α} (6-ketoPGF_{1α}) (Vane *et al.*, 1990). This breakdown product is in turn hepatically metabolised (within 30 minutes) to 2,3-dinor-6-ketoprostaglandin F_{1α} and 6,15 diketo 2,3-dinor-prostaglandin F_{1α}, where 2,3-dinor-6-ketoprostaglandin F_{1α} (PGI-M) is the major urinary metabolite of prostacyclin in man (Rosenkranz *et al.*, 1980). As mentioned earlier about other prostanoids, due to its rapid breakdown, prostacyclin is typically considered to be a local hormone rather than a circulating one. Plasma prostacyclin levels (as the breakdown product 6-ketoPGF_{1α}) measures to about 3 ng/L, which is generally considered to be too low to mediate a systemic effect but may represent an overflow of

prostacyclin that has already acted locally (Vane *et al.*, 1990) and when prostacyclin is infused *in vivo*, the effects wear off within 30 minutes (Vane *et al.*, 1995). The unstable nature of prostacyclin makes it complicated to use experimentally or therapeutically. Therefore, synthetic prostacyclin mimetics (such as iloprost, cicaprost or treprostinil sodium) are commonly used when investigating prostacyclin biology. Prostacyclin mimetics have also been valuable clinically for reducing pulmonary resistance in people suffering from pulmonary hypertension (McLaughlin *et al.*, 1998) and for peripheral arterial disease (Mohler *et al.*, 2000).

As defined in table 1.3 the two best studied prostacyclin receptor pathways are (i) cell surface IP receptors and (ii) cytosolic PPAR β receptors. IP receptors are expressed throughout the vascular tree but an early study reported expression in the kidney (interlobular arteries and the glomerular arterioles), liver, lung and neurons (Boie *et al.*, 1994). A subsequent study showed expression of IP mRNA in smooth muscle from many arteries including the aorta, the coronary arteries of the heart, the pulmonary artery and in the cerebral arteries, but no expression was found in veins (Oida *et al.*, 1995).

The vasodilatory and anti-aggregatory effects of prostacyclin have been attributed to IP receptor coupling to G_s type G protein (Table 1.3) activating adenylate cyclase, which leads to increased levels of cyclic adenosine monophosphate (cAMP) (Hata *et al.*, 2004). During prostacyclin/ IP receptor induced vasodilatation, elevated levels in cAMP and protein kinase A in vascular smooth muscle leads to the phosphorylation of myosin light chain kinase and the subsequent dephosphorylation of myosin light chains which antagonizes contractile stimuli and results in smooth muscle relaxation. Other effects of prostacyclin involves G_q and G_i coupling (Hata *et al.*, 2004). A similar cAMP and protein kinase A dependent pathway is also responsible for the anti-aggregatory effects of prostacyclin where deactivated myosin light chain kinase reduces the myosin phosphorylation which decreases platelet aggregation (Jin *et al.*, 2005). Another route to how IP may limit platelet activation mediated specifically by thromboxane is potentially through formation of an IP/TP α heterodimers, which are constitutively formed when the two receptors were expressed *in vitro* which seemed to have a similar cAMP elevating effect as prostacyclin/IP interaction (Wilson *et al.*, 2004).

The IP receptor is believed to undergo agonist induced desensitisation as well as agonist induced internalisation after prolonged agonist exposure leading to tachyphylaxis (Hata *et al.*, 2004). Whilst most of our information on prostacyclin sensing is related to IP receptors and their signaling pathway, PPAR β receptors are increasingly recognised as intracellular prostacyclin receptors (Belvisi *et al.*, 2009). PPAR β receptors also mediate, in part, the effects of prostacyclin mimetics on fibroblast proliferation (Ali *et al.*, 2006b) and platelet aggregation (Ali *et al.*, 2006a; Ali *et al.*, 2009b). Whilst the physiological and pathophysiological relevance of new prostacyclin signaling pathways, such as PPAR β are debated, it is widely accepted that the majority of acute effects of prostacyclin are mediated by IP receptors and cAMP signaling pathways (Vane *et al.*, 1995).

General phenotype of IP knockout mice

When homozygote IP homozygote knockout ($^{-/-}$) (IP $^{-/-}$) mice were derived from a C57BL/6 background, they were reported to be viable, reproductive and normotensive (Murata *et al.*, 1997). This is contrary to reports from endothelial nitric oxide (NO) knockout (eNOS $^{-/-}$) mice which were hypertensive, indicating a greater role for NO than prostacyclin in the maintenance of vascular tone (Huang *et al.*, 1995). Loss of functional IP receptor responses was verified based on the lack of responses to different prostacyclin mimetics on platelet aggregation, vasodilatation, blood pressure and bleeding time (Murata *et al.*, 1997). To confirm that it was an IP receptor specific knockout, PGE $_2$ was injected into the IP $^{+/+}$ wild type (WT) and the IP $^{-/-}$ mice, which caused a transient hypotension in both types of mice (Murata *et al.*, 1997). It was also established that vessels from IP $^{-/-}$ mice released comparable levels of prostacyclin (to WT mice). Furthermore, the IP $^{-/-}$ mice had normal heart rate, blood pressure and bleeding time (compared to WT mice). However, the IP $^{-/-}$ mice were reported to have reduced oedema and nociceptive responses that were similar to NSAID treated animals in models of inflammation, indicating a role for prostacyclin in inflammatory swelling and pain (Murata *et al.*, 1997). The most dramatic effects were seen in a iron (III) chloride (FeCl $_3$) model of thrombosis, where about two thirds of the IP $^{-/-}$ mice had obstructive thrombi and 30% of mice died within 1 day (during prolonged exposure to FeCl $_3$). Death was due to bilateral occlusion of the carotid arteries and/or embolic stroke (compared to none of the WT mice) (Murata *et al.*, 1997). It is important to note however,

that although the $IP^{-/-}$ mice were clearly more responsive to thrombotic stimuli, there was no spontaneous thrombus formation.

General phenotype of PPAR β knockout mice

PPAR β null mice (PPAR $\beta^{-/-}$) were reported to be smaller than control mice and to have an altered myelination of the corpus callosum in the brain, lipid metabolism and epidermal cell proliferation (Peters *et al.*, 2000). Furthermore, heterozygote PPAR $\beta^{+/-}$ mice had impaired wound healing (Michalik *et al.*, 2001).

Thromboxane acting on TP receptors

Thromboxane was discovered one year earlier than prostacyclin (in 1975) by Samuelsson and colleagues. They demonstrated that human platelets stimulated with arachidonic acid, released an unstable intermediate (which they named thromboxane A_2), with a half-life of 30 seconds, before it was broken down to the more stable but less active hydration product thromboxane B_2 (Hamberg *et al.*, 1975). They showed that thromboxane A_2 induced serotonin release from platelets as well as causing platelet aggregation (Hamberg *et al.*, 1975). Since then, it has been well established that thromboxane is predominantly derived from platelets and acts via TP receptors (Hirata *et al.*, 1996) (Table 1.3), but can also be produced by other cell types such as endothelial cells (F         *et al.*, 2010). Following intravenous administration of thromboxane B_2 , the two major urinary metabolites (TX-M) are 11-dihydro-thromboxane B_2 and 2,3-dinor-thromboxane B_2 (Patrignani *et al.*, 1989) where 2,3-dinor-thromboxane B_2 appears to be the major urinary thromboxane metabolite in man (Roberts *et al.*, 1981).

In addition to inducing platelet shape change, aggregation, secretion and adhesion, thromboxane contracts vascular and bronchial smooth muscle, increases smooth muscle remodeling, facilitates cholesterol uptake, and promotes leukocyte-endothelial interactions through acceleration of endothelial surface expression of adhesion molecules (Nakahata, 2008). There are two splice variants of TP receptors; TP α (first identified in the placenta) and TP β (first cloned from the endothelium) (Nakahata, 2008) (Table 1.3). Platelets express

both TP α and TP β . Defective TP receptor signaling (due to a single nucleotide polymorphism) has been linked to bleeding disorders (Hirata *et al.*, 1996).

Both TP receptors couple to G_q which leads to phospholipase C activation and subsequent inositol triphosphate/diacylglycerol (IP3/DAG) generation and increase in intracellular calcium which facilitates platelet aggregation (Table 1.3) (Smyth *et al.*, 2009). The pathway responsible for the change in platelet shape appears to be mediated via G12/G13 Rho/Rho kinase dependent regulation of myosin light chain phosphorylation (Smyth *et al.*, 2009). Following activation with the TP agonist U46619, both TP receptors are rapidly phosphorylated and desensitised (Habib *et al.*, 1997). Furthermore, TP α has been reported to be desensitised in response to prostacyclin and therefore TP α has been postulated to be the most relevant TP receptor for the prostacyclin and thromboxane mediated vascular hemostasis (Walsh *et al.*, 2000).

General phenotype of TP^{-/-} mice

TP^{-/-} mice have been reported to have a mild bleeding disorder (with prolonged bleeding time and altered platelet responses to collagen and arachidonic acid stimuli) as well as altered vascular responses to thromboxane and arachidonic acid (Thomas *et al.*, 1998). These findings confirmed that most of the known functions of thromboxane are mediated by a single TP gene locus (Thomas *et al.*, 1998).

As outlined above, the effects of thromboxane directly opposes those of prostacyclin and a balance between the two has long been suggested to form a central role for cardiovascular hemostasis and regulation of platelet interaction with the vessel wall (Vane *et al.*, 1990). I will discuss more about the 'thromboxane/prostacyclin balance' in later sections of this chapter. First I will give some background information about two isoforms of COX and later on how these two COX isoforms drive thromboxane and prostacyclin production within the cardiovascular system.

Two isoforms of COX

In the 1990's two isoforms of COX were identified from separate genes; COX-1 and COX-2. COX-1 was generally found constitutively expressed in several tissues, whereas COX-2, also at the time referred to as mitogen inducible COX (Lee *et al.*, 1992; Xie *et al.*, 1991) from the TIS10 gene (Kujubu *et al.*, 1991; Lee *et al.*, 1992; Xie *et al.*, 1991) was not generally present constitutively but expressed after stimulation of cells *in vitro* or at sites of inflammation *in vivo*. This led to the well-known hypothesis that COX-1 is the 'housekeeping' isoform that is constitutively expressed and responsible for the basal production of prostanoids (Figure 1.4). Examples of housekeeping prostanoid production included COX-1 driven protective PGE₂/prostacyclin production in the stomach/kidneys, prostacyclin release from the endothelium and thromboxane production in platelets. COX-2 on the other hand was identified as the inducible isoform primarily expressed at inflammatory sites and responsible for inflammatory levels of PGE₂ (and prostacyclin) (Figure 1.4) (Mitchell *et al.*, 1993). At sites of inflammation, COX-2 products (e.g. PGE₂) facilitate edema and sensitize sensory neurons to pain stimuli. COX-2 can also be upregulated in the spinal cord where it may contribute to prolonged central sensitization (Smyth *et al.*, 2009). Since we know that NSAIDs inhibit both isoforms of COX, inhibition of COX-1 was linked to the detrimental gastrointestinal side effects of NSAIDs whereas inhibition of COX-2 was linked to the therapeutic (analgesic, anti-pyretic and anti-inflammatory) effects of NSAIDs (Mitchell *et al.*, 1993) (Figure 1.4). However, there are also situations where COX-2 can be anti-inflammatory. For instance, in situations where the basal protective activity of COX-1 has been diminished or lost, (i.e. following vascular injury or inflammation in large blood vessels), COX-2 has been reported to be upregulated and have a protective anti-inflammatory role in adhesion receptor expression, cytokine release and smooth muscle proliferation (Mitchell *et al.*, 1998).

Figure 1.4 Hypothesis of the 1990s: COX-1 is the constitutive 'housekeeping' isoform and COX-2 is the therapeutic target of NSAIDs

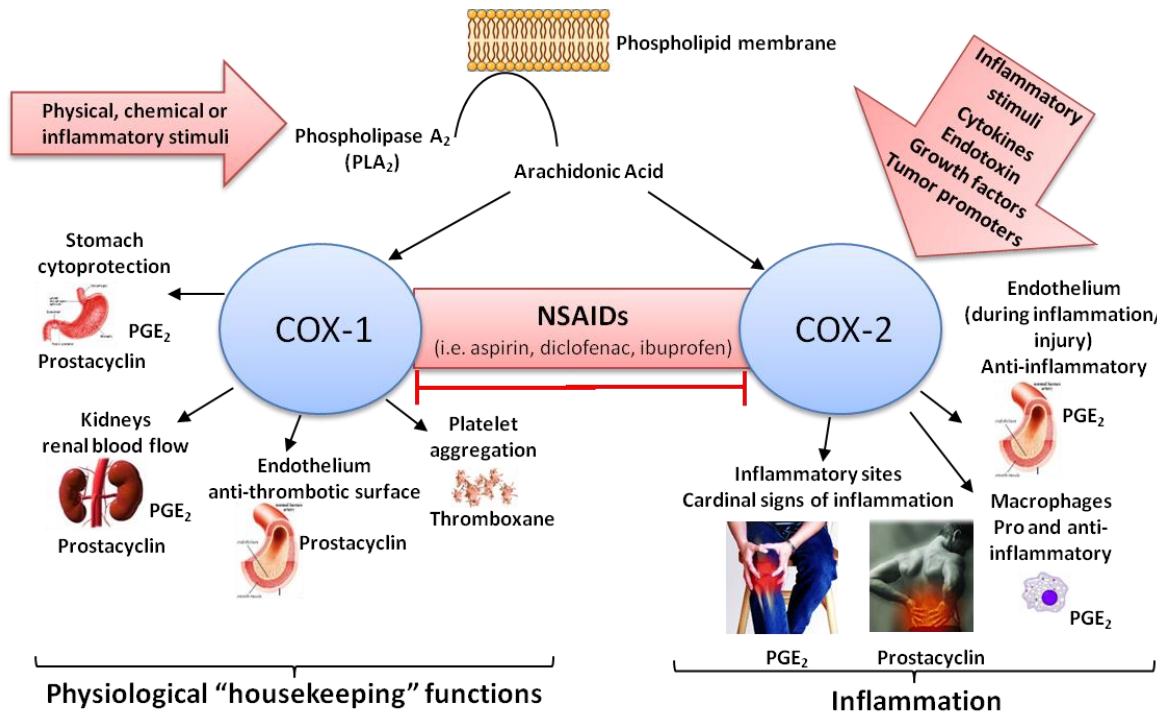


Figure 1.4 Hypothesis of the 1990s: COX-1 is the constitutive 'housekeeping' isoform and COX-2 is the therapeutic target of NSAIDs. COX-1 is highly expressed within the stomach (important for cytoprotection of the gastric mucosa), the kidneys (important for sodium balance and renal blood flow), within the endothelium (important for creating an anti-thrombotic surface) and within platelets (for platelet aggregation). COX-2 is mainly found induced at inflammatory sites within infiltrating macrophages where it is responsible for the production of pro-inflammatory (and sometimes anti-inflammatory) prostaglandins such as PGE₂ leading to swelling (oedema) and pain. COX-2 can also be induced in endothelial cells following vascular injury or by inflammatory stimuli where it seems to have a protective anti-inflammatory role (Mitchell *et al.*, 1998).

NSAIDs and side effects; relevance of COX-1 and COX-2

As mentioned earlier, NSAIDs have a number of well recognised side effects mainly affecting the 1) gastrointestinal tract, 2) kidney, 3) cardiovascular system. The area of NSAIDs and cardiovascular side effects will be discussed in detail later in the introduction. In this section I will briefly introduce the basics of the gastrointestinal and renal side effects of NSAIDs.

Gastrointestinal side effects of NSAIDs

The endoscopic evidence for NSAID induced gastric mucosal damage is well documented (Wolfe *et al.*, 1999). However, due to broad definitions of NSAID induced gastrointestinal side effects, variations in dosage and duration, it is difficult to accurately estimate the exact prevalence of these adverse gastrointestinal effects, but it appears that approximately 50% of NSAID users develop gastric erosions (small and superficial lesions confined to the mucosa) and that a small percentage of these patients go on to develop gastric ulcers (larger and deeper lesions) which could result in fatal hemorrhage (Wolfe *et al.*, 1999).

Dyspepsia is a common symptom from chronic NSAID use and is often a reason behind discontinuation of a certain NSAID treatment regime. Dyspeptic symptoms are not reliable warning signs since severe gastrointestinal complications are often asymptomatic and this leads to many thousands of hospitalisations and even some deaths each year (Wolfe *et al.*, 1999).

Risk factors for susceptibility to NSAID induced gastrointestinal side effects include increasing dose and duration of the NSAID use, advanced age, concomitant use of several NSAIDs (i.e. low dose aspirin with another NSAID) corticosteroids or anti-coagulants and a history of ulcers or gastrointestinal bleeds (Wolfe *et al.*, 1999). Prostaglandins, nitric oxide, hydrogen sulfide and neuropeptides are examples of autacoids mediating resistance to mucosal injury, and the term cytoprotection is often used to describe the ability of prostaglandins to reduce the injury from necrotizing agents (Wallace, 2008). Following exposure to gastrointestinal irritants, there is an acute inflammatory response which include an increase in blood flow and recruitment of leukocytes to minimize injury and prevent entry of foreign substances into the systemic circulation (Wallace, 2008).

NSAIDs have been reported to trigger part of the inflammatory response, including upregulation of intracellular adhesion molecule 1 (ICAM-1) leading to recruitment of circulating neutrophils to adhere to the gastric vascular endothelium which can cause mucosal injury due to the release of free radicals and proteases (Wallace *et al.*, 1990). NSAID induced gastrointestinal side effects can be divided into topical and systemic. Topical side effects include direct cytotoxic effects of the ingestion of NSAIDs on the gastrointestinal

epithelium. Systemic side effects are related to decreased synthesis of mucosal prostaglandins resulting in reduced mucosal blood flow, bicarbonate and mucus secretion, impaired platelet aggregation, reduced angiogenesis and increased leukocyte adherence (Wallace, 2008). At the therapeutic doses required to reduce inflammation and pain, NSAIDs inhibit both isoforms of COX and it is held that the gastrointestinal side effects of NSAIDs are caused by simultaneous inhibition of both COX isoforms (Wallace *et al.*, 2000). Thus, as discussed in later sections, COX-2 selective NSAIDs were developed to spare the gastrointestinal tract with some degree of success.

Renal side effects of NSAIDs

Reported renal side effects from NSAID use include hypertension, hyperkalemia, oedema, and nephrotoxicity, some of which are often associated with impaired sodium excretion and decreased glomerular filtration rate (Murray *et al.*, 1993). Considering the widespread use of NSAIDs, these side effects are relatively infrequent in people with normal and healthy kidneys, but remain a clinical concern in patients with a compromised renal system where inhibition of vasodilatory prostanoids (primarily prostacyclin and PGE₂) can lead to decreased renal perfusion and glomerular blood flow and even precipitate renal failure in susceptible individuals (Weir, 2002). Importantly, the kidney is an organ where COX-2 is recognised to be constitutively expressed and appears to be essential for renal homeostasis including salt retention, volume and blood pressure regulation (Yang *et al.*, 2000).

Expression patterns of COX isoforms have been reported to differ between species, but immunohistochemical and in situ mRNA analysis have shown that in normal human kidneys COX-1 is expressed in kidney endothelial cells, smooth muscle cells, interstitial cells and collecting duct cells of both pre and post glomerular vessels, whereas COX-2 is localised to the intraglomerular podocytes and endothelial and smooth muscle cells of renal arteries and veins (Kömhoff *et al.*, 1997). In general terms, COX-1 derived prostanoids appear to be important for hemodynamic control and glomerular filtration rate whereas COX-2 derived prostanoids are important for regulating salt and water excretion, but both these functions also overlap (Weir, 2002).

The role of renal COX-1 and COX-2 was also demonstrated in a study with elderly patients treated with NSAIDs, where NSAID induced acute sodium retention was concluded to be mediated by COX-2 inhibition, whereas the reduction in glomerular filtration rate was due to inhibition of COX-1 (Catella-Lawson *et al.*, 1999). Reduced secretion of sodium may lead to oedema, exacerbations of heart failure or elevated blood pressure. Critically NSAIDs induce a small (1-5 mmHg) rise in systemic blood pressure in normotensive as well as hypertensive patients; however, the relevance of NSAID induced blood pressure elevation will be covered in later sections.

Pharmaceutical quest for COX-2 selective or COX-1 sparing NSAIDs

The identification of COX-2 as the therapeutic target of NSAIDs, started a pharmacological quest to find COX-2 selective, or as Sir John Vane and colleagues thought was a more accurate and descriptive term: COX-1 sparing NSAIDs (Vane *et al.*, 2000). The overall rationale with the development of these drugs was to 'spare' the physiologically important 'house-keeping' isoform: COX-1 and thereby hopefully avoid the gastrointestinal and renal side effects of traditional NSAIDs. However, the aim was to maintain effective inhibition of the 'real' therapeutic target of NSAIDs: COX-2, in order to keep the therapeutic utility of these drugs, yet improve their overall safety profile. In order to succeed with the rational drug design, the two isoforms had to be thoroughly examined for any subtle differences that could be exploited in the pharmacological hunt for COX-2 selectivity.

Similarities and differences of COX-1 versus COX-2

COX-1 and COX-2 share 60% sequence homology but are encoded by different genes; COX-1 (22 kilo bases located on chromosome 9) and COX-2 (8 kilo bases on chromosome 1) (Smith *et al.*, 2000). Both isoforms are homodimeric, glycosylated, integral membrane bound proteins (Luong *et al.*, 1996), located in the lumen of the endoplasmic reticulum and in the nuclear envelope (Luong *et al.*, 1996; Morita *et al.*, 1995). Both isoforms are comprised of three main domains; the helical membrane binding domain, the catalytic domain (largest domain containing both the COX and the POX active sites) and the terminal epidermal growth factor (EGF) domain (Luong *et al.*, 1996).

Enzyme kinetics COX-1 versus COX-2

The gross enzymatic kinetics are very similar for both isoforms, however, there are differences during low substrate conditions, which may give COX-2 an advantage over COX-1 when competing for newly released substrate (arachidonic acid) (Murakami *et al.*, 1999; Smith *et al.*, 2000). Another subtle difference in kinetics is that COX-2 has been reported to have a lower threshold than COX-1 for hydroperoxide activation (required for the priming event from the POX site; see Figure 1.2) and can therefore oxygenate PGG₂ even during low peroxide conditions (Murakami *et al.*, 1999). As briefly mentioned earlier, low peroxide conditions are typical of endothelial cells and nerves, which are enriched with anti-oxidant pathways, whilst high peroxide conditions are typically found in platelets and inflammatory sites (Boutaud *et al.*, 2002). It has also been suggested that COX-1 and COX-2 can form heterodimers (Yu *et al.*, 2006) where COX-2 may provide the peroxide tone required for COX-1 activity in endothelial cells (Bolego *et al.*, 2009).

3D structures of COX-1 versus COX-2

Elucidation of the three dimensional structures of COX-1 and COX-2 revealed that Serine 530 (Ser 530), which has been identified as the acetylating site of aspirin and crucial for COX enzymatic activity, is conserved within both isoforms (yellow dashed circle, Figure 1.5). In addition, Tyr-385, described in earlier sections (see Figure 1.2) as the key residue for the priming event by the POX site, is also preserved (Rouzer *et al.*, 2008). However, importantly, the main exploitable difference between COX-1 and COX-2 is the amino acid residue 523 which is found in the active site (Luong *et al.*, 1996). This difference has consequences for the shape and size of the binding site for NSAIDs where the valine-523 gives COX-2 an additional hydrophobic side pocket within the substrate binding site (compared to COX-1 isoleucine-523). Valine-523 also results in slightly wider central channel (wider than COX-1 by approximately 17%), and this makes COX-2 more promiscuous in its substrate and NSAID selectivity (Figure 1.5). All together, the volume of the total COX-2 binding site is 394Å³ versus the 316Å³ binding site of COX-1 (Kujubu *et al.*, 1991). These structural differences between the COX-1 and COX-2 active sites were exploited in the development of COX-2 selective inhibitors.

Figure 1.5 Ribbon diagrams showing similarities and differences between COX-1 and COX-2

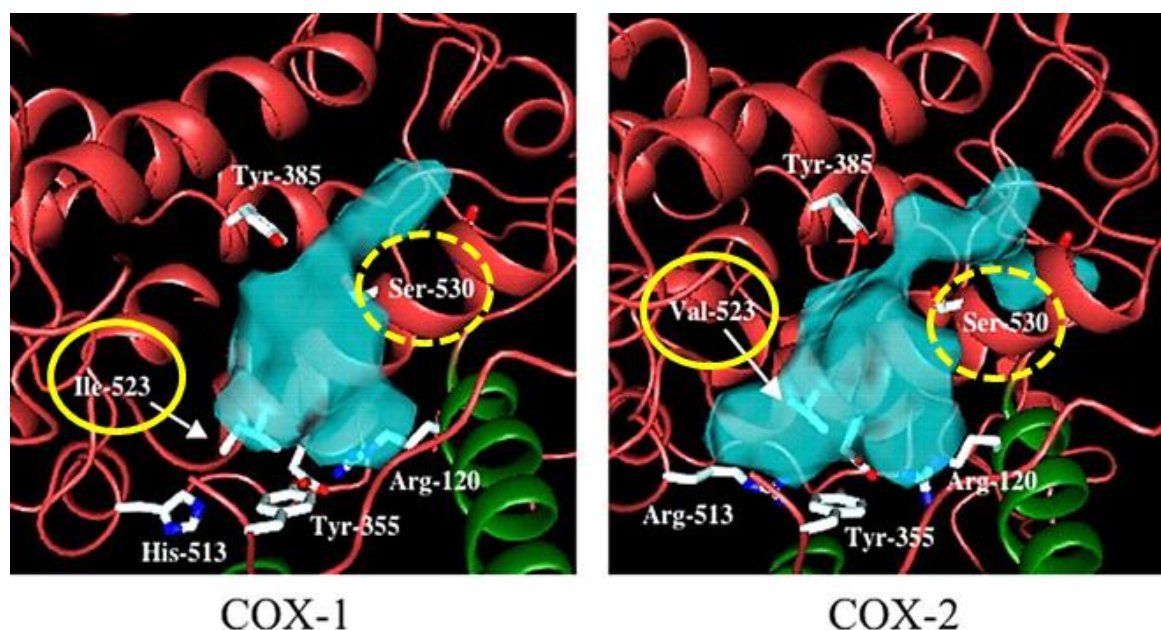


Figure 1.5 Ribbon diagrams showing similarities and differences between COX-1 and COX-2. 3D structure of COX-1 and COX-2 proteins. Yellow circle indicate isoleucine and valine at amino acid position 523, which is a main difference between the two isoforms, resulting in a larger side pocket in the active site of the COX-2 enzyme. Dashed yellow circle indicate the position of Serine 530 (Ser-530) which is the acetylating site of aspirin which is crucial for enzymatic activity within the active site of both COX-1 and COX-2. (Figure modified from (Rouzer *et al.*, 2008).

The launch of COX-2 selective (COX-1 sparing) NSAIDs

The COX-2 selective (or COX-1 sparing) NSAIDs; celecoxib (Celebrex™, developed by Searle/Pfizer) was approved by the FDA in December 1998 and rofecoxib (Vioxx™, developed by Merck) and was approved in January 1999. These drugs became very popular since they were associated with fewer gastrointestinal side effects (by sparing COX-1) compared to traditional non-selective (COX-1 and COX-2 inhibiting) NSAIDs but they still maintained their therapeutic anti-inflammatory and analgesic utility (by inhibiting COX-2) (Bombardier *et al.*, 2000; Silverstein *et al.*, 2000).

Celecoxib (Celebrex™)

Celecoxib was licensed in the United Kingdom in 2000 for osteoarthritis and rheumatoid arthritis (Flower, 2003). The Celecoxib Long term Arthritis Study (CLASS) was a double blind, randomised trial that compared celecoxib (400mg x2/day) with ibuprofen (800mg x3/day) or diclofenac (75mg x2/day) in 8059 patients with osteoarthritis or rheumatoid arthritis in

regards to gastrointestinal side effects as well as other adverse effects (Silverstein *et al.*, 2000). Concomitant low-dose aspirin use was permitted in the study. This study concluded that after 6 months on daily dosing of celecoxib (even at doses 2-4 times the maximum therapeutic dosage), there were fewer gastric ulcers, perforations, bleeds and associated complications compared with the NSAID group (ibuprofen or diclofenac) used at standard therapeutic doses (Silverstein *et al.*, 2000). In addition, celecoxib seemed to be associated with fewer adverse effects on the kidney compared to ibuprofen or diclofenac (Silverstein *et al.*, 2000).

Rofecoxib (Vioxx™)

Vioxx was licensed in the United Kingdom for osteoarthritis in 1999 and rheumatoid arthritis in 2001 (Flower, 2003). In the randomised and double blinded VIGOR trial (Vioxx Gastrointestinal Outcomes Research Study), the efficacy and adverse side effect profile of high dose rofecoxib (Vioxx) (50mg/day) was compared with standard dose naproxen (1000mg/day) during long term treatment in 8076 patients with rheumatoid arthritis (Bombardier *et al.*, 2000). Aspirin use was not permitted in the study; hence patients requiring low-dose aspirin for cardioprotection were excluded. It was shown that rofecoxib had a similar efficacy as naproxen in terms of therapeutic pain relief, but was associated with around half the gastrointestinal side effects compared with naproxen treatment (Bombardier *et al.*, 2000).

The launch of two COX-1 sparing COX-2 selective NSAIDs, celecoxib and rofecoxib, was therefore at first a pharmacological success story with convincing proof of concept data where both these drugs displayed an improved gastrointestinal side effect profile compared to traditional NSAIDs, confirming the triumph of the rational drug design.

However, there were also concerns raised about the COX-2 selective inhibitors. Although selective inhibition of COX-2 spared the gastrointestinal tract from disruption of the mucosal defense (seen with conventional NSAIDs), they were instead shown to interfere with ulcer healing (Perini *et al.*, 2004). Furthermore, even before the launch of COX-2 selective inhibitors, there were also concerns regarding the cardiovascular safety of these drugs.

Cardiovascular concerns

Concerns were raised early on in the COX-2 field after preclinical studies showed that COX-2 could have a protective function during vascular inflammation (Bishop-Bailey *et al.*, 1998; Bishop-Bailey *et al.*, 1997; Mitchell *et al.*, 1998). Furthermore, one of the ‘first to man’ papers showed that MK-966 (later launched as Vioxx), halved the urinary excretion of the prostacyclin metabolite PGI-M (2, 3-dinor-6-keto prostaglandin $F_{2\alpha}$) compared to placebo, in healthy older adults, after 2 weeks of treatment, without affecting the urinary thromboxane metabolite TX-M (11-dehydro TxB_2) (Catella-Lawson *et al.*, 1999).

Similar results were obtained in a study with celecoxib (McAdam *et al.*, 1999). This caused an upheaval of concern since it is a well-established idea that a balance between thromboxane and prostacyclin is required for vascular haemostasis, where reduced prostacyclin could tip the balance in favor of thromboxane and potentially lead to an increased thrombotic risk. With these two lines of concerns, the field was alerted to the possibility that COX-2 inhibitors might be associated with an increased cardiovascular risk.

Cardiovascular signal in the VIGOR study

This concern seemed, by some, to be realised when data from the VIGOR study showed an apparent increase in cardiovascular deaths associated with the rofecoxib arm compared to the naproxen arm of the now infamous study (Bombardier *et al.*, 2000). It was estimated that 45 out of 4047 rofecoxib treated patients versus 20 out of 4039 naproxen treated patients had suffered a serious thrombotic cardiovascular adverse event (myocardial infarction, cardiac thrombus, unstable angina, resuscitated cardiac arrest, sudden or unexplained death and ischemic stroke or transient ischemic attacks) (Mukherjee *et al.*, 2001). The incidence of these thrombotic cardiovascular event rates (myocardial infarction, stroke and cardiac death) were relatively low (~1%) but still significantly different between the rofecoxib and the naproxen treated groups with myocardial infarction having the most striking event rate (FDA, 2001). There were 37 deaths (from all causes) in the VIGOR trial, 22 rofecoxib treated patients and 15 naproxen treated patients, and 9 out of 22 cases in the rofecoxib treated group died from thrombosis/atherosclerosis related deaths versus 5 out of 15 in the naproxen treated group (FDA, 2001).

Overall, the relative risk in suffering a thrombotic cardiovascular event on rofecoxib treatment versus naproxen treatment was 2.8 (which was statistically significant) and this was considered to be a cautionary flag in terms of cardiovascular safety of COX-2 selective inhibitors (Mukherjee *et al.*, 2001). However, it was hypothesised that this could be explained by either a pro-thrombotic effect of rofecoxib or an anti-thrombotic effect of naproxen (or both) (Mukherjee *et al.*, 2001).

Cardiovascular outcomes in the CLASS trial

The CLASS trial however, did not demonstrate a significant difference in cardiovascular signal in the celecoxib arm compared to the ibuprofen or diclofenac arms of the study (Mukherjee *et al.*, 2001; Silverstein *et al.*, 2000).

No cardiovascular signal in the TARGET trial

The TARGET trial (Therapeutic Arthritis Research and Gastrointestinal Event Trial) was designed to test the gastrointestinal and cardiovascular safety of the COX-2 selective inhibitor lumiracoxib (Novartis) (at 400mg/day) compared with naproxen (500mg x2/day) and ibuprofen (800mg x3/day) in 18325 patients with osteoarthritis (Farkouh *et al.*, 2004). Lumiracoxib differs structurally from the other coxibs and has a shorter half-life (2-6 hours) leading to less systemic drug exposure. Myocardial infarction (which was one of the primary endpoints), did not differ between the treatment groups (irrespective of aspirin use) (Farkouh *et al.*, 2004).

Arguments concerning the cardiovascular risk of COX-2 selective inhibitors

1. CLASS and VIGOR trials had not been powered to detect cardiovascular events

It was argued at the time that any cardiovascular risk associated with COX-2 selective drugs had not been conclusively investigated since the CLASS and the VIGOR studies were designed to identify gastrointestinal events and hence had not been 'powered' to detect cardiovascular events since these effects were unexpected and as a result, evaluation of cardiovascular endpoints had not been pre-specified (Bombardier *et al.*, 2000; Mukherjee *et al.*, 2001; Silverstein *et al.*, 2000). Furthermore, due to ethical reasons implicated in clinical

trials involving patients with arthritis (where patients often depend on pain relief for daily functioning), neither of these studies had a placebo arm.

2. Cases occurred in patients that should have been on aspirin for cardioprotection

Even though aspirin use for cardiac reasons was an exclusion criterion for the VIGOR study, the study still included 'aspirin-indicated' patients (with a past medical history of stroke or myocardial infarction etc). It was therefore first argued that some of the cases in the rofecoxib treated group occurred in patients that should have been on aspirin for cardioprotection (hence had underlying cardiovascular disease) (FDA, 2001; Mukherjee *et al.*, 2001). However, there were also events in the rofecoxib treated group that did not fall into this 'aspirin-indicated' subgroup and the number of 'aspirin indicated patients' were similar between the rofecoxib and the naproxen treatment groups (FDA, 2001; Mukherjee *et al.*, 2001).

3. Different dosing

Other arguments included that the cardiovascular signal could be attributed to that supra-clinical doses of COX-2 inhibitors (50mg/day rofecoxib and 400mg/day celecoxib) were compared to therapeutic doses of the comparative NSAIDs (Mukherjee *et al.*, 2001).

4. The 'naproxen argument'

The 'naproxen argument' stated that the difference in myocardial infarction between the rofecoxib and the naproxen treated groups could be explained by anti-thrombotic effects of naproxen due to potent inhibition of COX-1 in platelets (Rahme *et al.*, 2002; Van Hecken *et al.*, 2000). Ex-vivo whole blood assays from 76 healthy volunteers showed that rofecoxib, meloxicam, diclofenac and naproxen all inhibited COX-2 (endotoxin induced COX-2 generated PGE₂) compared to placebo, whereas the corresponding inhibition of COX-1 (thromboxane generation in whole blood) was most potently inhibited by naproxen and least by the 'COX-1 sparing' inhibitor rofecoxib (similar to placebo) (Van Hecken *et al.*, 2000). These results were supported by a population based matched case control study comparing naproxen and other non-aspirin NSAIDs in protection against acute myocardial infarction, where naproxen had a protective effect compared to the other NSAIDs (Rahme *et al.*, 2002). The incidence of myocardial infarction was similar in patients with

osteoarthritis when rofecoxib was directly compared to ibuprofen (Day *et al.*, 2000) or to diclofenac (Cannon *et al.*, 2000).

5. Patients with rheumatoid arthritis have an underlying increased risk of cardiovascular disease

Patients with rheumatoid arthritis have 1.5-3 fold increased risk of cardiovascular events compared to control subjects, where both traditional risk factors as well as disease severity are important predictors of future cardiovascular events (Solomon *et al.*, 2010). However, there was no measure of cardiovascular risk markers (such as C-reactive protein), in the VIGOR study which makes it difficult to differentiate between patients groups. Even if there is an increased cardiovascular risk in this group of patients, this would have affected both the naproxen group and the rofecoxib group and therefore does not explain the difference in cardiovascular events between the two treatments (FDA, 2001).

One of the arguments for why there was a cardiovascular signal with rofecoxib in the VIGOR trial and not with celecoxib in the CLASS trial was that the VIGOR trial was focused on patients with rheumatoid arthritis whereas the CLASS trial also included patients with osteoarthritis. This was considered important since it had been shown that the relative risk of a myocardial infarction is elevated (1.4) in patients with rheumatoid arthritis versus osteoarthritis or placebo (DeMaria, 2002; FitzGerald, 2003). It is however also important to consider that in the 'real world', a wide range of patients are taking these drugs many of whom may have a medical history or lifestyle associated elevated risk for cardiovascular disease.

Each of these arguments was valid with the main issue being that, at that time, no placebo controlled study had been performed and no study at all had been designed specifically to evaluate the cardiovascular safety of COX-2 selective inhibitors (placebo or otherwise). Over the following 4-5 years, several randomised clinical trial and small scale observational studies were published which continued to indicate a cardiovascular signal with rofecoxib (Graham *et al.*, 2005; Hippisley-Cox *et al.*, 2005; Jüni *et al.*, 2004).

The withdrawal of rofecoxib/Vioxx™

Finally, two trials, one with rofecoxib; APPROVe (Adenomatous Polyp Prevention On Vioxx) (Bresalier *et al.*, 2005) and one with celecoxib: APC (Adenoma Prevention with Celecoxib) (Solomon *et al.*, 2005) designed to show utility of COX-2 selective inhibitors in the prevention of familial colon cancer, were performed with placebo arms. The APPROVe trial was a randomised, double blinded trial with 2586 patients followed over 3 years: 1287 patients were randomly assigned 25mg/day rofecoxib (this is the maximal recommended long-term daily dose) and 1299 patients took an identical looking placebo pill once a day. Potential thrombotic adverse events were reviewed in a blinded fashion by an external committee (Bresalier *et al.*, 2005). 46 out of 1287 patients in the rofecoxib treated group suffered a thrombotic adverse event versus 26 out of 1299 patients in the placebo group and the corresponding relative risk was 1.92 (Bresalier *et al.*, 2005). Out of these thrombotic adverse events it was non-fatal heart attack (21 in the rofecoxib treated group versus 9 in the placebo group) or ischemic stroke (11 versus 6) that stood out (Bresalier *et al.*, 2005).

The overall cardiovascular mortality was however similar between the two groups. The increased risk did not seem to be associated by a higher baseline or use of low dose aspirin; however, the relative risk in the rofecoxib treated group was particularly high amongst patients with a history of symptomatic atherosclerosis or diabetes (Bresalier *et al.*, 2005). Post-hoc analysis revealed that the thrombotic event rates were similar in the first 18 months of study (19 events in each group), but the increased relative risk with rofecoxib became evident after that (26 events with rofecoxib versus 6 events in the placebo group) (Bresalier *et al.*, 2005). However, an increased rate of investigator reported hypertension, oedema and congestive heart failure occurred earlier in the study (after around 5 months) and the incidence of these adverse events were once again higher in the rofecoxib treated group (Bresalier *et al.*, 2005). With this data in hand, the APPROVe study was terminated 2 months ahead of the planned date of completion after recommendation from the external safety monitoring board and the steering committee, and on the same day (30th September 2004), Merck announced that they had decided to voluntarily withdraw rofecoxib (Vioxx™) from the market worldwide (Merck, 2004).

APC trial (celecoxib versus placebo)

The APC trial was also discontinued early due to reported cardiovascular events in the celecoxib treated groups (Solomon *et al.*, 2005). There was an apparent dose related increased risk of myocardial infarction, stroke or heart failure in the celecoxib treated groups (200mg x2/day: 16 patients out of 685) and (400mg x2/day: 23 patients out of 671) compared to placebo (7 patients out of 679) (Solomon *et al.*, 2005).

The results from the placebo controlled APPROVe and APC trials, suggested that the increased cardiovascular risk associated with these two structurally distinct COX-2 selective inhibitors reflected a class effect that was increased with dose and duration of therapy. It is however important to remember that these two drugs were also associated with a reduction in serious gastrointestinal adverse events compared to traditional NSAIDs and that the risk of an adverse cardiovascular events was still very low, especially in patients with a low cardiovascular risk profile (Wang *et al.*, 2005). This allowed celecoxib to stay on the market, to conserve the benefit of 'COX-1 sparing' inhibition, in a segmented patient market at low cardiovascular risk (but at high risk of gastrointestinal complications) where the benefit of reduced gastrointestinal side effects may still outweigh the risk of cardiovascular adverse events (Wang *et al.*, 2005). The American food and drug administration (FDA) are still closely monitoring celecoxib and Pfizer are currently doing a large randomized clinical trial: Prospective Randomized Evaluation Of Celecoxib Integrated Safety Vs Ibuprofen Or Naproxen (PRECISION) (ClinicalTrials.gov, 2006-2014). Two doses of celecoxib (100-200mg x2/day) are compared to the two most commonly prescribed traditional NSAIDs naproxen (375-500mg x2/day) and ibuprofen (600-800 x3/day), in patients with osteoarthritis and rheumatoid arthritis (18 years and older) with (or at risk of developing) cardiovascular disease, assessing cardiovascular, renal and gastrointestinal safety (as well as therapeutic symptomatic benefit), to answer the question if celecoxib (just like rofecoxib) has a riskier cardiovascular profile compared to naproxen or ibuprofen.

Traditional NSAIDs also carries cardiovascular risk

The evidence supporting the cardiovascular risk profile of COX-2 selective inhibitors were mainly derived from clinical trials whereas data about traditional NSAID's had to be based on observational and retrospective studies and meta-analysis (Chan *et al.*, 2006; Johnsen *et*

al., 2005; McGettigan *et al.*, 2006). This is mainly due to the fact that traditional NSAIDs were developed long before the requirement of today's rigorous safety studies, and therefore these drugs do not have the same amount of placebo controlled large scale clinical trial data expected of new drugs today. The 'Vioxx story' served as a whistleblower to the whole field, including the 'traditional NSAIDs' and raised a cautionary flag that all of these drugs may be associated with cardiovascular side effects, since at therapeutic clinically relevant doses, all NSAIDs inhibit COX-2 (hence their therapeutic anti-pyretic, analgesic and anti-inflammatory effects).

The distinguishing factor between traditional NSAIDs and COX-2 selective inhibitors is, of course the level of COX-1 inhibition (Van Hecken *et al.*, 2000; Warner *et al.*, 2008). We know that COX-2 selective drugs do not reduce urinary markers of thromboxane production, but do inhibit prostacyclin markers. If we consider the thromboxane versus prostacyclin balance as the only important factor in cardiovascular risks of these drugs, we would expect COX-2 selective drugs to be more dangerous than traditional NSAIDs. However, this does not seem to be the case and a number of articles now conclude that traditional NSAIDs have a similar cardiovascular risk as COX-2 selective drugs (Fosbøl *et al.*, 2010a; Fosbøl *et al.*, 2010b; Kearney *et al.*, 2006; McGettigan *et al.*, 2006; McGettigan *et al.*, 2011; Trelle *et al.*, 2011). For example, a recent network meta-analysis study reported that rofecoxib was associated with the highest risk of myocardial infarction, ibuprofen with the highest risk of stroke and diclofenac had a high risk of cardiovascular death, but the conclusion was still that cardiovascular risk needs to be considered when prescribing any COX-2 selective inhibitor or traditional NSAID (Trelle *et al.*, 2011).

The reasons why traditional and COX-2 selective NSAIDs carry similar risks of cardiovascular events is not clear, and research continues in this area. However, it should be noted that, as with COX-2 selective inhibitors, traditional NSAIDs reduce prostacyclin production, but debate surrounds the level of inhibition of platelet COX-1 and if this is sufficient, at standard doses, to inhibit platelet aggregation. It has been suggested that inhibition of platelet derived thromboxane and the persistence of thromboxane dependent platelet aggregation

may not be linear since and that pharmacologic inhibition of platelet COX-1 (and subsequent depression of serum thromboxane B₂) must be virtually complete before dramatic changes in platelet activity is seen (Reilly *et al.*, 1987). In addition, a residual 10% capacity to generate thromboxane is enough to sustain thromboxane dependent platelet aggregation (Di Minno *et al.*, 1983).

Black box warning

In 2005, the FDA concluded that the available data suggest that the increased risk of cardiovascular adverse events is a class effect shared by both traditional NSAIDs and COX-2 selective inhibitors. The general advice is to take the lowest effective dose for the shortest duration possible. In the US, all prescription NSAIDs and celecoxib now share the same cardiovascular black box warning:

'These drugs may increase the chance of heart attack or stroke, which can lead to death and that this chance increases when NSAIDs are taken for long periods and if you already have established heart disease or risk factors for it (such as high blood pressure).'

The current clinical situation suggests that most NSAIDs, including COX-2 selective inhibitors, are associated with an increased risk of cardiovascular events but that, as was predicted at the start of their development, COX-2 selective inhibitors carry lower risk of gastrointestinal events (Mitchell *et al.*, 2006b). These cardiovascular side effects are now part of the scientific press as well as the lay press; with headlines like: 'Painkiller heart alert' (DailyMail, 2011). These headlines came from the results from a recent systemic review study investigating NSAIDs at typical doses in community based observational studies and the associated cardiovascular events in populations with both low and high cardiovascular risk backgrounds (McGettigan *et al.*, 2011). This review concluded that naproxen was least likely to increase cardiovascular risk, whereas rofecoxib, diclofenac and celecoxib had a dose-related elevation in cardiovascular risk (McGettigan *et al.*, 2011).

However, since most studies concerning the cardiovascular safety profile of NSAIDs derive data from studies in patients with established disease (i.e. arthritis) and/or in patients with underlying cardiovascular disease and/or in studies with a non-cardiovascular endpoint, a

recent study instead focused on cardiovascular risk and NSAIDs in healthy individuals (Fosbøl *et al.*, 2010a). This study once again concluded that the use of rofecoxib and diclofenac (and high doses of ibuprofen) was associated with higher cardiovascular morbidity and mortality. Naproxen on the other hand was once again identified to have a safer cardiovascular risk profile (Fosbøl *et al.*, 2010a).

Underlying mechanism of the cardiovascular side effects?

It seems clear that NSAIDs, including COX-2 selective drugs, increase risk of cardiovascular (mainly thrombotic) events; however the underlying mechanism(s) behind these side effects are still debated. I will now present the two main arguments:

- 1) Inhibition of COX-1/COX-2 derived prostanoids in the kidney and associated renal and blood pressure effects.

And/or

- 2) Disruption of the thromboxane/prostacyclin balance through inhibition of COX-2 derived prostacyclin in vascular endothelium.

Elevation in blood pressure

The first argument involves how the renovascular side effects of NSAIDs/COX-2 selective inhibitors (primarily blood pressure elevation) may relate to the cardiovascular side effects. Critically NSAIDs, including COX-2 selective inhibitors, induce a small (1-5 mmHg) rise in systemic blood pressure in normotensive as well as hypertensive patients and antagonise and destabilize the blood pressure lowering effects of anti-hypertensive drugs to an extent that can increase hypertension related morbidity (Singh *et al.*, 2003). Considering that around 40% of patients with osteoarthritis or rheumatoid arthritis receive antihypertensive treatment, it is estimated that increases in systemic blood pressure of 1-5 mmHg results in thousands of additional strokes and ischemic heart disease events each year (Singh *et al.*, 2003).

Large clinical trials have shown that a sustained increase in systolic blood pressure of 3mmHg is associated with a 10-20% increased risk of congestive heart failure or stroke (ALLHAT, 2000; Whelton *et al.*, 2001). Therefore, whilst NSAID related increases in blood pressure are relatively small, it is precisely in line with the predicted rate of cardiovascular side effects associated with NSAIDs. In the VIGOR trial, the mean increase in systolic and diastolic blood pressure was 4.6mmHg and 1.7mmHg respectively in the rofecoxib treated group, versus 1.0mmHg and 0.1mmHg in the naproxen treated group (Mukherjee *et al.*, 2001). This effect was once again observed when rofecoxib was compared to placebo in the APPROVe trial where the mean change in blood pressure in the rofecoxib treated group was 3.4mmHg increase (systolic) and 0.9mmHg (diastolic) compared to respective changes of -0.5 and -0.8 in the placebo group (Bresalier *et al.*, 2005). The elevation in blood pressure following rofecoxib treatment was evident after 1 month in the APPROVE trial, which could have been one of the factors contributing to the acceleration of cardiovascular risk (heart attack or stroke) which appeared in the rofecoxib arm of the study after 18 months of treatment (Bresalier *et al.*, 2005).

On the contrary, hypertension was lower in the celecoxib group than the ibuprofen or diclofenac group in the CLASS study (Silverstein *et al.*, 2000). And the TARGET trial reported that lumiracoxib had a smaller mean change in blood pressure (both systolic and diastolic) compared to ibuprofen or naproxen (Farkouh *et al.*, 2004). Furthermore, in the double blinded SUCcessive Celecoxib Efficacy and Safety Study (SUCCESS IV), 810 elderly patients with osteoarthritis were randomised to receive either celecoxib (200mg/day) or rofecoxib (25mg/day), and after 6 weeks there was a significant increase in systolic blood pressure in the rofecoxib treated group compared to the celecoxib treated group (+2.3mmHg versus -0.5mmHg) (Whelton *et al.*, 2001). This study also reported a difference in other cardiorenal markers such as in the development of oedema, where almost twice as many rofecoxib treated patients developed clinical signs of oedema and even congestive heart failure and that these symptoms were coupled with disordered sodium retention, starting at day 5-7 of treatment and leading to the development of oedema by the first 1-2 weeks of treatment (Whelton *et al.*, 2001).

Overall these reports seem to match the cardiovascular outcome of the drugs studied, since in these particular trials, it was only rofecoxib that elevated blood pressure (and oedema) compared to the comparative NSAID/placebo and was associated with the increased risk of thrombotic events. It is also important to bear in mind that when blood pressure findings are expressed as group averages of mean increase in blood pressure, it may underestimate individual changes in blood pressure, and that may when sustained over time and alongside other risk factors have detrimental implications on cardiovascular health.

Hence, the renovascular and blood pressure elevating side effects may well play a significant part, contributing to an increased thrombotic risk in susceptible patients on chronic dosing with NSAIDs or COX-2 selective inhibitors. However, even though these renal side effects are important to consider, the focus of my thesis has been to more closely investigate how COX pharmacology in vascular endothelium may relate to the cardiovascular side effects of NSAIDs and COX-2 selective inhibitors.

Thromboxane/prostacyclin balance for vascular homeostasis-relevance of endothelial COX-2

As covered in earlier sections, the activities of the two short lived prostanoids thromboxane and prostacyclin counteracts one another and a balance between the two is crucial for vascular homeostasis (Bunting *et al.*, 1983). It has long been suggested that an imbalance in the ratio of pro-thrombotic thromboxane and anti-thrombotic prostacyclin underpins arterial thrombosis (Bunting *et al.*, 1983) (Figure 1.6). As outlined in earlier sections, thromboxane, which is a vasoconstrictor and a potent activator of platelet aggregation, is mainly formed through the concerted actions of COX-1 and thromboxane synthase in platelets (Bunting *et al.*, 1983; Reiter *et al.*, 2001), but can also, to a lesser degree be synthesised by endothelial cells, leukocytes and lung fibroblasts (Bunting *et al.*, 1983; Félétou *et al.*, 2010).

Prostacyclin, which is a vasodilator and one of the most potent naturally occurring inhibitors of platelet activation, is predominantly formed by macrovascular endothelium (Moncada *et al.*, 1977), and is the main arachidonic acid product from large arteries, veins and the microcirculation (Bunting *et al.*, 1983).

The anti-thrombotic and cardioprotective effects of low dose aspirin for cardiovascular disorders, is attributed the ability of aspirin to irreversibly acetylate/inhibit COX in anucleate platelets, leading to a tipping of the thromboxane/prostacyclin balance in favour of prostacyclin, since the nucleated endothelial cells can overcome the irreversible acetylation by synthesis of new COX within a few hours which alongside prostacyclin synthase can kick-start the production of prostacyclin again, whilst the acetylation of COX in the anucleate platelets is extended until new platelets (with new functional COX) are produced by megakaryocytic cells in the bone marrow (Vane *et al.*, 1990).

Considering that the half-life of platelets is around 8-11 days, regular low doses of aspirin results in accumulative inhibition of thromboxane production in platelets, but with little effect on endothelial prostacyclin production, which leads to a reduction in platelet reactivity and the favored anti-thrombotic cardioprotective environment (Figure 1.6) (Mitchell *et al.*, 2006b; Vane *et al.*, 1990). Due to gastrointestinal and increased bleeding time safety considerations, the lowest effective dose of aspirin is used in at-risk individuals as anti-platelet prophylaxis which is associated with a significantly reduced risk of thrombotic stroke or myocardial infarction (Patrono, 2001).

On the contrary, the increased thrombotic risk of traditional (non-aspirin) NSAIDs and COX-2 selective inhibitors has been postulated to be due to a tipping of the balance in favor of thromboxane (at the expense of reduced prostacyclin) thereby potentially increasing platelet reactivity (Figure 1.6). As described in earlier sections about the initial cardiovascular concerns about COX-2 selective inhibitors; this hypothesis was mainly derived from two early ‘first to man’ studies examining how daily dosing of rofecoxib and celecoxib (compared to other NSAIDs) affected urinary markers of thromboxane versus prostacyclin (Catella-Lawson *et al.*, 1999; McAdam *et al.*, 1999; Van Hecken *et al.*, 2000). The first study was designed to assess the renal effects of MK-966 (rofecoxib), compared to indomethacin or placebo, for 2 weeks of treatment, randomised and blinded in 26 healthy elderly patients (Catella-Lawson *et al.*, 1999). There was transient sodium retention with both active drugs, but only indomethacin was associated with a reduction in glomerular filtration rate. Furthermore, both rofecoxib and indomethacin decreased levels of the

urinary marker of the prostacyclin metabolite PGI-M, whilst the levels in placebo remained unchanged (Catella-Lawson *et al.*, 1999).

Importantly, the urinary marker of thromboxane biosynthesis TX-M was inhibited by indomethacin but not by rofecoxib, which indicated that the thromboxane/prostacyclin balance had been tipped in favour of thromboxane in the rofecoxib treated patients (Catella-Lawson *et al.*, 1999) (illustrated in Figure 1.6). A different study in 76 healthy volunteers also demonstrated that rofecoxib had no significant effects on urinary TX-M, despite lowering PGI-M and 6-keto PGF_{1α} to a comparable level with the other NSAIDs studied (diclofenac, ibuprofen, naproxen, meloxicam) (Van Hecken *et al.*, 2000).

Another study comparing celecoxib to ibuprofen or placebo, in 37 healthy young volunteers also obtained similar results as the two rofecoxib studies; celecoxib was shown to halve the urinary excretion of PGI-M compared to placebo, without affecting urinary TX-M (McAdam *et al.*, 1999). This paper concluded that it seems to be a class effect of COX-2 selective inhibitors to inhibit markers of prostacyclin (without affecting markers of thromboxane) and that this implies a major role for COX-2 in both renal and systemic prostacyclin biosynthesis in young and healthy volunteers (McAdam *et al.*, 1999).

Since vascular endothelium is the major source of prostacyclin production, this led to the assumption that COX-2 is constitutively expressed in vascular endothelium and responsible for the biosynthesis of cardioprotective prostacyclin (Figure 1.6) (Grosser *et al.*, 2006; Grosser *et al.*, 2010; Smyth *et al.*, 2009).

Figure 1.6 Thromboxane/prostacyclin balance

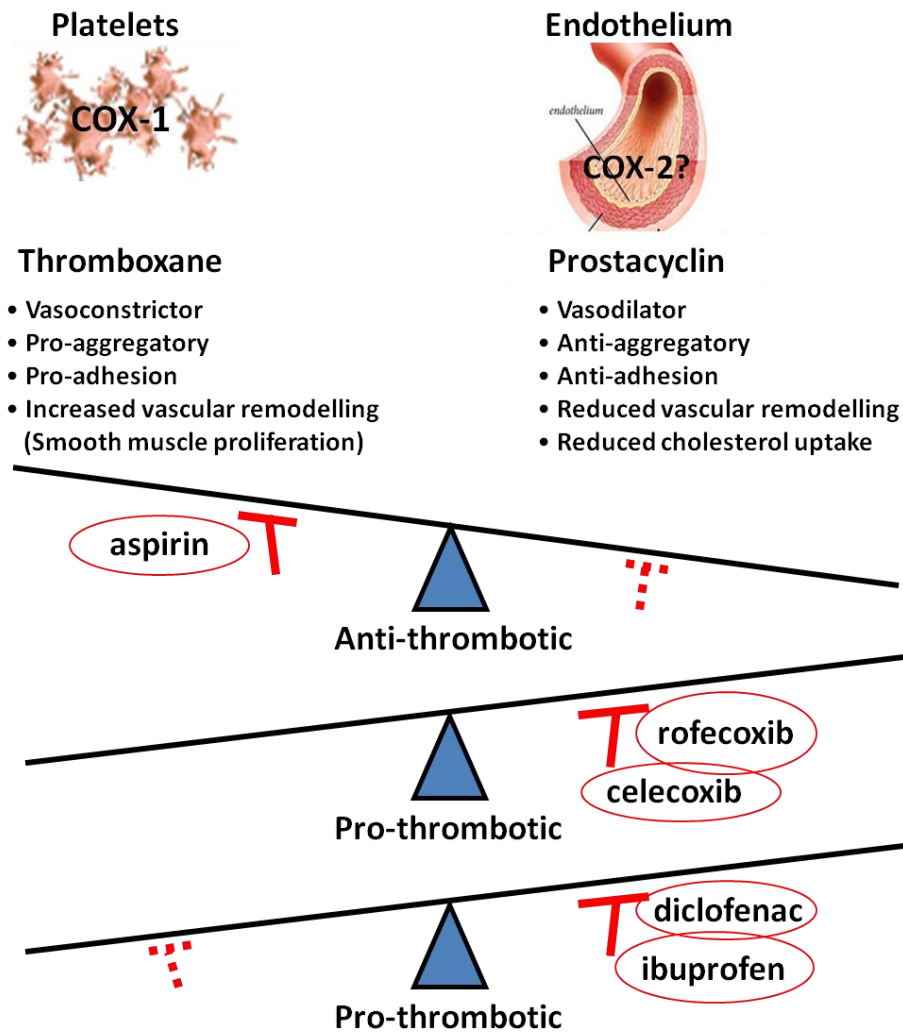
Thromboxane/prostacyclin balance:

Figure 1.6 Thromboxane/prostacyclin balance. Arterial thrombosis is dependent on a balance between pro-thrombotic thromboxane synthesised in platelets and anti-thrombotic prostacyclin produced in vascular endothelium. Filled red lines indicate strong inhibition, dotted red lines indicate relatively less inhibition. Traditional NSAIDs aspirin, ibuprofen and diclofenac have been shown to inhibit urinary markers of both thromboxane and prostacyclin, whereas the COX-1 sparing or COX-2 selective inhibitors have been shown to inhibit urinary markers of prostacyclin without concomitant inhibition of thromboxane (Catella-Lawson et al., 1999; McAdam et al., 1999). This has been taken as pharmacological evidence for a predominance of constitutively expressed COX-2 in vascular endothelium responsible for systemic prostacyclin biosynthesis. An imbalance in the ratio between systemic levels of COX-1 derived thromboxane from platelets and COX-2 derived prostacyclin in endothelium has been proposed as a hypothesis behind the increased thrombotic risk (via vasoconstriction, platelet activation and atherothrombosis) associated with COX-2 selective inhibitors (and NSAIDs).

In the remaining sections of my introduction chapter I will focus on information and questions directly relevant to my PhD thesis.

Fundamental questions about endothelial COX expression and prostacyclin biosynthesis

There are currently some key preclinical questions which require answering before we can make informed interpretations of the clinical data obtained using NSAIDs, including COX-2 selective inhibitors. First and foremost it is essential to establish which isoform of COX predominates in vascular endothelium and responsible for the production of prostacyclin and the extent to which this mediates cardioprotection and reduces thrombosis.

Traditionally, the ‘house-keeping’ isoform COX-1 was shown to predominate in endothelial cells and responsible for driving prostacyclin production in healthy blood vessels (Mitchell *et al.*, 1993). However, after the COX-1 sparing, or COX-2 selective inhibitors (rofecoxib and celecoxib) were surprisingly shown to reduce urinary markers of prostacyclin, this was taken as evidence that constitutively active COX-2 in endothelial cells (rather than COX-1), drives the systemic prostacyclin biosynthesis (Figure 1.6) (Funk *et al.*, 2007; McAdam *et al.*, 1999). The results from the clinical studies were also replicated in mice, where COX-2^{-/-} mice and celecoxib treated WT mice had significantly reduced levels of urinary PGI-M (without any alterations in urinary TX-M levels) (Cheng *et al.*, 2006). This together with the clinical data discussed above was used to substantiate the idea that COX-2 is the dominant COX isoform in endothelium (Fitzgerald, 2004).

The view about COX-2 predominance in vascular endothelium has been challenged (Mitchell *et al.*, 2006a; Mitchell *et al.*, 2006b) since immunohistological studies have invariably shown COX-1 constitutively expressed within the endothelium with COX-2 only expressed at sites of vascular inflammation or damage (Belton *et al.*, 2000; Kawka *et al.*, 2007; Mitchell *et al.*, 1998; Mitchell *et al.*, 2006a; Schönbeck *et al.*, 1999). Consequently, whilst there is a general consensus that COX-1 predominates in platelets (and that COX-2 is absent in mature platelets) (Mitchell *et al.*, 2006b; Reiter *et al.*, 2001) controversy surrounds the isoform present in endothelial cells. Understanding the expression pattern and activity of COX-1 and COX-2 in the vascular endothelium and how this potentially relates to the cardiovascular side effects of NSAIDs and COX-2 selective inhibitors is the key focus of my thesis.

I will now provide a brief overview of what is known about COX expression in vascular endothelium in health and disease.

COX pathways in the endothelium- during health and disease

A thin lining of endothelium covers the luminal surface of every blood vessel in the body. This lining is made up by a mosaic of individual endothelial cells, joined together forming a monolayer interface between the blood circulating in the lumen and the underlying structures of the vessel wall. A typical endothelial cell is typically thicker around the nuclear area and then flattens out towards the cell membrane periphery.

The endothelium was initially thought to be a simple cell lining that prevented the blood from accessing subendothelial tissues, but in 1966 Florey commented that the endothelium was more than a nucleated cellophane wrapper (Florey, 1966). A more active anti-thrombotic role of the endothelium became apparent when it was discovered that endothelial cells synthesise prostacyclin (Moncada *et al.*, 1976). The current, well established view of the endothelium is that it is a widely disseminated and dynamic organ composed of trillions of endothelial cells with a combined weight of ~1kg, with complex barrier functions, essential production of autocrine and paracrine vasoactive substances, key metabolic processes and important immunological functions (Cines *et al.*, 1998). Furthermore, the endothelial cell layer has anti-coagulant (non-stick) properties and plays an important part in vasoregulation, vascular permeability, acute and chronic inflammation, wound healing and atherogenesis. The key vasoactive substances released from the endothelium include the vasoconstrictor endothelin-1 (ET-1), the endothelium-derived hyperpolarizing factor (EDHRF) and the potent anti-platelet and vasodilators NO and prostacyclin (Mitchell *et al.*, 2008). However, for the purpose of my thesis, I will focus only on COX derived products, principally prostacyclin.

COX expression/activity in the endothelium and shear stress

A major disadvantage with studying endothelial cells in static culture conditions *in vitro* is that the endothelial cells are removed from their natural dynamic environment. In the body endothelial cells are chronically exposed to mechanical forces such as shear stress (frictional force per unit area) and cyclic strain caused by the action of blood flowing continuously over the vessel wall. Endothelial cells are very sensitive to their biomechanical environment and shear stress is known to affect gene expression, cell turnover, cell signaling, receptor expression, the endothelial membrane and cytoskeleton, wall permeability, inflammation, angiogenesis, development of atherosclerosis and the overall morphology of endothelial cells (Weinberg *et al.*, 2007).

One hypothesis that has been used to explain the lack of COX-2 in vascular tissue *in vitro* is that COX-2 may be 'constitutively induced' by hemodynamic shear forces in the endothelium (FitzGerald, 2002), and therefore not detectable when studied in static conditions. Various studies have reported an upregulation of COX-2 in endothelial cells following exposure to shear stress *in vitro* (Di Francesco *et al.*, 2009; Okahara *et al.*, 1998; Topper *et al.*, 1996), but there have also been conflicting results (Dancu *et al.*, 2004; Potter *et al.*, 2011) and there is by no means consensus on this point. Thus, one key area of investigation in my thesis has been to study expression of COX-1 and COX-2 *in vivo* at areas of polarised shear stress, most notably in the greater and lesser curvature regions of the aortic arch (see below).

The area of shear stress biology in the endothelial field is controversial in its own right and one which continues to evolve. It has been suggested, for example, that relative differences in magnitude and directionality of shear stress (measured as dynes/cm²) may be more relevant to study than absolute magnitude (Suo *et al.*, 2007). Indeed, early studies of effects of shear stress on COX expression were performed *in vitro* using cultured endothelial cells where generally laminar shear was applied and for relatively short periods (Di Francesco *et al.*, 2009; Okahara *et al.*, 1998; Topper *et al.*, 1996). Therefore the use of isolated cells in the study of COX and shear stress is inevitably complicated by disagreement on the physiological relevance of the model. It is therefore better in many respects to examine enzyme expression in blood vessels where shear patterns have been mapped. The aortic

arch (with its direct anatomical connection to the heart) is an ideal structure in this respect. Whilst on the whole, the aortic arch may experience shear stress of a high magnitude, there are relative differences in magnitude and directionality of the shear stress patterns in the greater versus lesser curvature respectively which categorically influence inflammatory responses (Ali *et al.*, 2009a; Hajra *et al.*, 2000; Iiyama *et al.*, 1999; Jongstra-Bilen *et al.*, 2006; Zakkar *et al.*, 2008). The endothelium in the greater curvature of the mouse aortic arch is subject to unidirectional and high wall shear stress with an estimated magnitude of 600 dynes/cm², whereas the lesser curvature is associated with wall shear stress of a relatively lower magnitude (of approximately 150 dynes/cm²) which is highly oscillatory (multidirectional) in direction during the cardiac cycle (Figure 1.7) (Suo *et al.*, 2007).

Figure 1.7 Map of the hemodynamic shear stress patterns in the mouse aortic arch

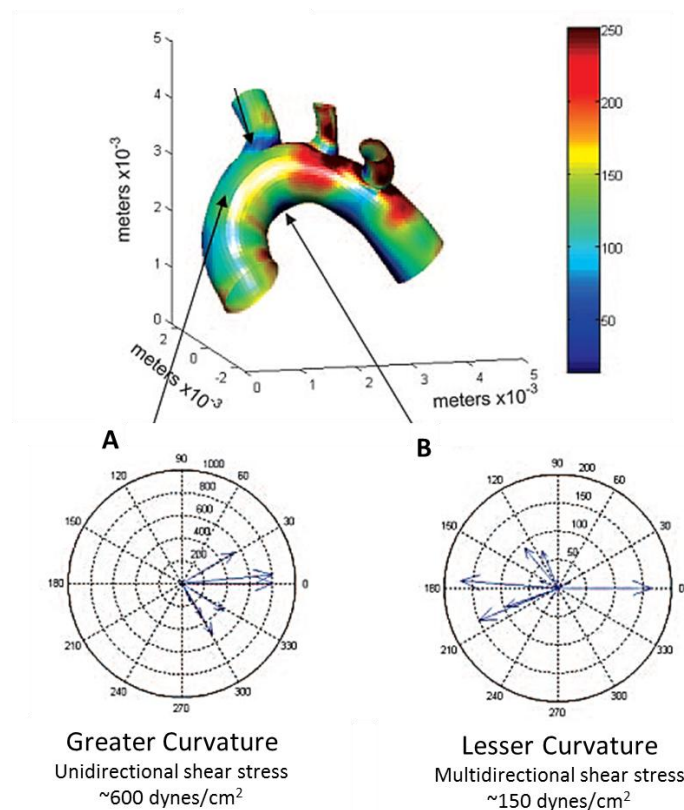


Figure 1.7 Map of the hemodynamic shear stress patterns in the mouse aortic arch. Computational fluid dynamics model of shear stress patterns in the mouse aortic arch. Since the flow is pulsatile the shear stress vector changes in both magnitude and direction during different stages of the cardiac cycle. **(A)** The shear stress pattern in the greater curvature is relatively high in magnitude (~ 600 dynes/cm²) and changes little in direction (unidirectional), whereas the shear stress pattern in **(B)** the lesser curvature have a relatively lower shear stress magnitude (~ 150 dynes/cm²), which is highly oscillatory during the cardiac cycle (multidirectional). Figure modified from (Suo *et al.*, 2007).

Endothelium of the greater and the lesser curvature of the aortic arch are referred to as 'protected' and 'susceptible' to atherosclerotic plaques respectively (Iiyama *et al.*, 1999). It has long been recognised that the pattern of atherosclerosis is site specific in laboratory animals and in humans and therefore follows a predictable pattern with higher susceptibility in bifurcations, curvatures and near arterial branches where there is a diversion away from the protective high and unidirectional shear stress to low and oscillating shear stress (Caro, 2009; Davies, 2009; Glagov *et al.*, 1988).

COX expression in the endothelium during inflammation

As mentioned in earlier sections, COX-2 is an immediate early gene that can be readily induced in cells by inflammatory or angiogenic agents (McAdam *et al.*, 2000) (see Figure 1.4). Thus, there are numerous papers showing that COX-2 is induced in vascular cells after stimulation with a range of inflammatory agents including endotoxin or lipopolysaccharide (LPS), IL-1, tumor necrosis factor α (TNF α) and growth factors (McAdam *et al.*, 2000). As mentioned in earlier sections, COX-2 has been reported to be upregulated following vascular injury or inflammation in large blood vessels where it seems to have a protective anti-inflammatory role in adhesion receptor expression, cytokine release and smooth muscle proliferation (Bishop-Bailey *et al.*, 1997; Mitchell *et al.*, 1998). Thus, the notion that COX-2 is induced at sites of inflammation by inflammatory mediators in vessels *in vivo* is well accepted and not in contention. However, the role of COX-2 in vascular inflammation remains incompletely understood. Hence, in my thesis I intentionally induced COX-2 *in vivo* using LPS in order to study COX-1 and COX-2 expression and activity during endothelial inflammation.

Gender differences

COX activity and isoform expression may differ between male versus females. Estrogen has been shown to be able to upregulate COX-2 expression and prostacyclin release in endothelial cells *in vitro* which has been linked to the cardioprotective effects of estrogen in pre-menopausal women (Akarasereenont *et al.*, 2000; Egan *et al.*, 2004). I have therefore considered potential gender differences in endothelial COX expression and activity in my thesis.

Age related endothelial dysfunction

Endothelial dysfunction is typically described as an impaired endothelial dependent dilation of blood vessels in response to physical or chemical stimuli, which could be due to an alteration in the endothelial release of vasoactive substances (Mombouli *et al.*, 1999). It is important to note that the literature suggests that COX-2 in the endothelium is increased as we age and/or with endothelial dysfunction associated with chronic cardiovascular conditions (Heymes *et al.*, 2000; Wu *et al.*, 1998). This is a relevant argument considering that the target patient group for chronic dosing of NSAIDs and COX-2 selective inhibitors are often elderly patients with arthritis. It has been shown that acetylcholine dependent dilation is impaired in mice at 12 month of age compared to 3 month of age which is one of the characteristics of age related endothelial dysfunction (Gendron *et al.*, 2007). I have therefore assessed where relevant the effect of age on COX expression and activity.

Heart failure

Heart failure is a common, disabling and deadly cardiovascular disease affecting 1-2% of the adult population (6-10% of the over 65s) in developed countries (McMurray *et al.*, 2005). It is characterised by the inability of the heart to supply enough blood flow to meet the needs of the body, mainly due to ventricular dysfunction, which is often caused by myocardial infarction or prolonged hypertension (McMurray *et al.*, 2005). It has been shown that plasma levels of prostacyclin and PGE₂ metabolites are significantly higher in patients with severe chronic heart failure compared to normal subjects (Dzau *et al.*, 1984). Furthermore, there has been reports about COX-2 expression in myocardial tissue from patients with end stage heart failure, that was absent in tissue from normal control hearts (Wong *et al.*, 1998). COX-2 expression was also reported to be higher in venous endothelial cells harvested from patients with chronic heart failure compared to healthy subjects (Colombo *et al.*, 2005). In this thesis I will present data from a rat model of chronic heart failure, where the aim was to investigate if the prostanoid profile and/or relative expression of endothelial COX-1 and COX-2 from major arterial vessels had changed in a compromised cardiovascular system.

Atherosclerosis

Atherosclerosis and subsequent cardiovascular disease is the leading cause of morbidity and mortality in the industrialised world. It is now thought that atherosclerosis starts in adolescence (Strong *et al.*, 1999) and silently progress but remains asymptomatic for decades until clinical symptoms typically manifests from middle age (Deanfield *et al.*, 2007). Atherosclerosis is an inflammatory disease that is potentiated by systemic risk factors such as high plasma levels of modified (oxidised) low density lipoprotein (LDL), free radicals from smoking, hypertension, obesity and diabetes.

It is characterized by lesions (or atheroma/atherosclerotic plaques) forming in large to middle sized arteries that consist of a fibrous cap and a lipid-rich core, accompanied by intense immunological activity and inflammation, cell death and fibrosis (Hansson *et al.*, 2006). Early plaques (also known as fatty streaks) are typically enriched in foam cells/ lipid-laden macrophages which have engulfed oxidised LDL and T-cells. These fatty streaks can either disappear with time or develop into intermediate plaques characterised by proliferating smooth muscle that becomes intermixed with the area of inflammation (Ross, 1999). Mature plaques (atheroma) are characterised by a core of lipids and debris from dead cells that are surrounded by activated macrophages, T-cells and mast cells producing cytokines, proteases and vasoactive substances. A mature plaque is covered with endothelium and stabilised by a fibrous cap of smooth muscle and collagen fibers. The most detrimental clinical outcome is plaque rupture when pro-thrombotic material in the plaque is exposed to the blood, leading to a thrombus formation, which in the coronary arteries can result in a myocardial infarction and heart failure or in case of the carotid arteries lead to an ischemic stroke (Hansson *et al.*, 2006).

Endothelial dysfunction plays a key role at all stages of atherogenesis and is characterised by alterations in the interactions between the endothelium with the circulating blood and the vessel wall including hyper-adhesiveness (adherence of monocytes and platelets to intact endothelium due to upregulation of adhesion molecules), enhanced permeability to lipoproteins and an imbalance in local vasoactive and pro- and anti-thrombotic factors (Ross, 1999).

There are numerous publications linking COX isoform expression, prostacyclin, thromboxane and NSAID usage to atherosclerosis, although again, there is no common consensus. Most studies are in agreement that both COX-1 and COX-2 are present in atherosclerotic plaques, but the role each isoform may play in disease protection or progression is not clear. Early studies showed COX-1 and COX-2 in human atheroma, mainly colocalising with macrophages in the shoulder region and in the lipid core periphery and lower levels detected in smooth muscle cells (Schönbeck *et al.*, 1999). Furthermore, urinary levels of PGI-M and TX-M were found to be elevated in patients with atherosclerosis compared to normal subjects (Belton *et al.*, 2000) suggesting increased COX activity and expression was significant. Given the association between prostacyclin and cardioprotection it has been postulated that accelerated atherogenesis, due to a reduction in prostacyclin may be fundamental to the development of an increased cardiovascular hazard in patients on long term chronic dosing with NSAIDs and COX-2 selective inhibitors (Grosser *et al.*, 2006) and could be a factor behind the 18 month 'latent period' in the APPROVe trial, before the cardiovascular risk profile of rofecoxib diverged from placebo (Rudic *et al.*, 2005). I have therefore specifically investigated the expression and activity of COX-1 and COX-2 in animal models of atherosclerosis.

NF-κB

Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor crucial regulator of cellular responses as well as innate and adaptive immune and inflammatory responses. The NF-κB/Rel family consists of homo or heterodimeric proteins, and the p50/p65 (Rel A) heterodimer is the predominant NF-κB subunit in endothelial cells (Read *et al.*, 1994). NF-κB is constitutively present in an inactivated state in the cytoplasm of almost all cell types, ready to be activated/translocated to the nucleus for control of transcription of DNA (Li *et al.*, 2002).

During the cytosolic inactivated state, NF-κB is bound to an inhibitory protein referred to as IκB, where IκB-α preferentially binds to the p65 subunit. Following activation (i.e. LPS stimulation), IκB kinases are activated which phosphorylates the IκB-α inhibitor leading to polyubiquitination and degradation whereas the p50/p65 heterodimer is transported to the

nucleus where it interacts with other transcription factors and coactivators to transactivate target genes. A negative feedback loop leads to upregulation of I κ B- α expression which associates with the DNA bound NF- κ B and a nuclear export sequence in the I κ B- α binds to exportins and brings back the NF- κ B to its inactivated state in the cytoplasm (Arenzana-Seisdedos *et al.*, 1995).

COX activity via prostacyclin release may influence NF- κ B since the prostacyclin analogues have been reported to inhibit NF- κ B translocation/activation in human alveolar macrophages (Raychaudhuri *et al.*, 2002), in CD4 positive T-cells (Zhou *et al.*, 2007a) and in dendritic cells (Zhou *et al.*, 2007b). Furthermore, activated NF- κ B is thought to play a key role in the development of vascular inflammation and atherosclerosis (Collins *et al.*, 2001). Interestingly, the endothelium lining the lesser curvature of the aortic arch has been shown to be 'primed for activation' with elevated levels of nuclear NF- κ B and cell adhesion molecules, which may have relevance to the increased susceptibility of atherosclerotic plaques forming in this area (Hajra *et al.*, 2000). I have therefore investigated how NF- κ B activity in the endothelium is affected by COX-1 or COX-2 gene deletion. For these studies I have used untreated and LPS treated mice in order to compare control versus inflammatory conditions.

Platelet adherence and interactions with the endothelium

As described in earlier sections, COX-2 selective inhibitors (and) NSAIDs depress urinary markers of prostacyclin substantially (but incompletely) in humans (Catella-Lawson *et al.*, 1999; McAdam *et al.*, 1999). Prostacyclin is one of the most potent, endogenous inhibitors of platelet activation and is also known to inhibit the interaction between platelets and the vascular wall and thereby contribute to the anti-thrombotic surface of the endothelium (Moncada *et al.*, 1977). After all, one of the main functional characteristics of intact endothelium is non-reactivity to platelets (Vane *et al.*, 1995). However, prostacyclin has been shown to inhibit platelet-platelet interactions more potently than inhibition of platelet adhesion (platelet/collagen interaction) (Higgs *et al.*, 1978), which allows for repair of a damaged vessel wall whilst limiting thrombus formation (Vane *et al.*, 1995). Data from mice completely deficient in the prostacyclin (IP) receptor, indicate that a loss in this prostacyclin

signaling pathway does not lead to spontaneous thrombosis, but instead is associated with an increased thrombotic tendency following thrombotic stimuli (Murata *et al.*, 1997). Since it has been reported that COX-1 and COX-2 knockout mice have altered urinary levels of TX-M and PGI-M (Cheng *et al.*, 2006), it is important to consider how this may affect platelet/endothelial interactions in vascular endothelium. I therefore investigated the presence of any adhered platelets in the greater and the lesser curvature of wild type (WT) versus COX-1^{-/-} and COX-2^{-/-} mice. For this study I used an antibody against glycoprotein IIb/IIIa; commonly referred to as CD41 (also known as integrin $\alpha_{IIb}\beta_3$, which is a cell surface receptor for fibrinogen on mature platelets) in order to selectively detect platelets (not endothelial cells/other nucleated cells) adhered to the vessel intima. CD41 is reported to be expressed on the platelet plasma membrane, intraluminal side of the platelet open canalicular system and on the platelet α -granule membrane (Schmitt *et al.*, 2001).

Summary

Almost a decade has passed since Vioxx was dramatically withdrawn from the market on a wave of publicity linking COX-2 inhibitors to cardiovascular deaths. We now know that regular NSAIDs, including over-the-counter drugs such as diclofenac and ibuprofen, share the potential to cause heart attack and strokes. However, we still do not understand some of the very basic concepts relating to COX-1 and COX-2 expression and activity in cardiovascular tissues.

The focus of my thesis is to study COX-1 and COX-2 expression and activity in blood vessels in health and in disease. I have focused on the study of relative expression patterns of COX-1 and COX-2 in endothelium of different anatomical regions relevant to shear stress responsive endothelial phenotypes, particularly those prone or protected to atherosclerosis.

Hypothesis

‘COX-2 is present in vascular endothelium and regulates prostacyclin release and functional responses in health and disease’

Aims

The aims and research questions of my thesis can be summarised as follows:

- 1) How is the morphology of the endothelium and the underlying smooth muscle defined in the gross area of the mouse aortic arch?***
- 2) Which COX isoform predominates in vascular endothelium and how does this relate to endothelial cell morphology?***
- 3) Which COX isoform drives endothelial prostacyclin production in vascular tissue?***
- 4) Does the relative expression pattern and/or bioactivity of endothelial COX-1 and COX-2 change following cardiovascular disease?***
- 5) What are the functional consequences of COX-1 and COX-2 gene deletion in the aortic arch endothelium in health and disease?***

Chapter 2

General Methods

Animals

All experiments were carried out in accordance with the United Kingdom Home Office Guide on the Animals (Scientific Procedures) Act 1986. All animals were housed in dedicated biomedical research husbandry facilities under the supervision of certified animal care technicians according to institutional guidelines and international laws. The regulated scientific procedures in this thesis include administration of substances by intraperitoneal injection (i.p.) and ear clipping (sampling small pieces of ear tissue) for genotyping. These procedures were carried out in accordance with the specified animal allowance in our project licence (70/6913) as well as my personal licence (70/21691). Throughout the course of my PhD, I have endeavoured where possible to share tissue from animals and optimise the powering of studies in order to use the minimum number of animals possible, in line with the direction and philosophy of the '3 Rs' at the National Centre for the Replacement, Refinement and Reduction of animal research guidelines (NC3Rs, 2012).

Genetically modified mice; COX-1 or COX-2 knockout mice

I have used genetically modified mice, where either the COX-1 or COX-2 gene has been deleted (COX-1^{-/-} and COX-2^{-/-} respectively). The COX-1^{-/-} and COX-2^{-/-} mice used in this thesis were from our own colonies that were derived from breeding pairs donated by Professor Ballou's lab at the University of Tennessee Health Science Centre (Memphis USA). The colonies are on a C57BL/6J background (Jackson labs, USA) and were originally created by Professor Oliver Smithies' group at the University of North Carolina, USA (Langenbach *et al.*, 1995; Morham *et al.*, 1995). The genotypes of the breeding pairs for the COX-1^{-/-} and COX-2^{-/-} colonies as well as their offspring were tested rigorously throughout the breeding process. The COX-1^{-/-} colony was bred by homozygote breeding; both male and female were homozygote for COX-1 (^{-/-}) generating 100% homozygote COX-1^{-/-} litters. The COX-2 colony however, required heterozygote breeding (homozygote male COX-2^{-/-} mice was bred with female heterozygote COX-2^{+/-} mice), since homozygote female COX-2^{-/-} mice are infertile (Morham *et al.*, 1995). The possible genotypes of offspring from these breeding pairs were therefore either wild type (WT) (COX-1^{+/+} COX-2^{+/+}), COX-2^{+/-} (heterozygotes) or COX-2^{-/-} (homozygotes); and mice were genotyped at breeding (Figure 2.1). The colonies I have used

in my thesis (WT, COX-1^{-/-} and COX-2^{-/-} mice) have been backcrossed on a regular basis, into their background C57BL/6J (Harlan, UK).

Genotyping procedure

The DNA from ear clips was extracted by using Dneasy Tissue Kit (Qiagen, 69056). In brief, the DNA from the ear clip samples was obtained by first digesting the tissue with (Qiagen proteinase K), followed by lysis (Qiagen lysis buffer and ethanol), multiple washing steps (Qiagen wash buffer) in mini-spin columns and then DNA bound to columns was eluted (using Qiagen elution buffer), ready for 3-primer polymerase chain reaction (PCR). The PCR protocol was developed according to (Ballou *et al.*, 2000; Reddy *et al.*, 1999). Extracted DNA was pipetted into tubes (GE healthcare, 27-9559-01) containing illustra PuReTaq Ready-To Go PCR beads with 3 primers (Invitrogen) for each gene. The PCR program on a Techgene PCR machine was set to: 5 minutes at 94°C (denaturing), 32-38 cycles (COX-1 PCR) and 35 cycles (COX-2 PCR) of 15 seconds at 94°C (denaturing), followed by 15 seconds 60°C (COX-1 PCR) and 15 seconds 55°C (COX-2 PCR) (annealing), 1 minutes 72°C (extension), 5-10 minutes 72°C (final extension), finished off at 4°C. Amplified DNA samples were then either stored in the freezer (-20°C) or run through electrophoresis on the same day.

The COX-1 primer for the WT allele was 5' AGGAGATGGCTGCTGAGTTGG (which yielded a fragment of 601bp to represent COX-1^{+/+}). The COX-1 null allele (with the neomycin insert) was identified with a primer for 5' GCAGCCTCTGTTCCACATACAC (which yielded a fragment of 646bp to represent COX-1^{-/-}). The reverse primer for both COX-1 WT (COX-1^{+/+}) and mutant allele (COX-1^{-/-}) was 3' AATCTGACTTTCTGAGTTGCC and yielded two fragments of 601bp and 646 respectively to represent COX-1^{+/-} (Figure 2.1) (Ballou *et al.*, 2000; Reddy *et al.*, 1999).

The COX-2 primer for the WT allele was 5' ACACACTCTATCACTGGCACC (which yielded a fragment of 760bp to represent COX-2^{+/+}). The COX-2 null allele (with the neomycin insert) was identified with a primer for 5' ACGCGTCACCTTAATATGCG (which yielded a fragment of 905bp to represent COX-2^{-/-}). The reverse primer for both COX-2 WT (COX-2^{+/+}) and mutant allele (COX-2^{-/-}) was 3' ATCCCTTCACTAAATGCCCTC and yielded two fragments of 760bp and 905bp respectively to represent COX-2^{+/-} (Figure 2.1) (Reddy *et al.*, 1999).

Figure 2.1 PCR blot of extracted DNA from genotyping of our mouse colony

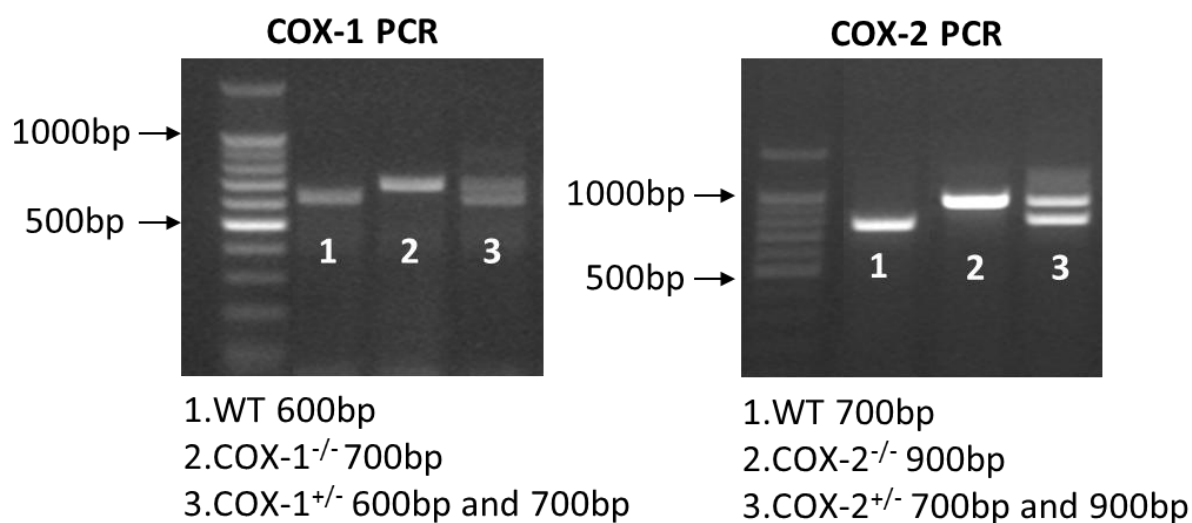


Figure 2.1 PCR blot of extracted DNA from genotyping of our mouse colony. (1) DNA from wild type (WT) mice ear clips from COX-1^{+/+} at 601bp (~600bp), and COX-2^{+/+} 760bp (~700bp) (2) DNA from COX-1^{-/-} at 646bp (~700bp) and COX-2^{-/-} at 905 (~900bp) (3) DNA bands from COX-1^{+/-} with two bands at 601bp and 646bp (~600bp and 700bp) and 760bp and 905bp (~700bp and 900bp) for COX-1^{+/-}.

WT mice

The WT control mice were generated by inter-crossing COX-1^{+/-} and COX-2^{+/-} mice, then crossing their COX-1^{+/+} and COX-2^{+/+} offspring in order to maintain a similar genetic background. However, for some studies, before viable colony offspring WTs were available, I performed some experiments (see individual figure legends) using standard C57BL/6J male mice (purchased from Harlan, UK) as controls.

The strategies for targeting COX-1 and COX-2 gene disruption

The following information is interpreted from the papers originally describing the strategies and practicalities of generating the genetically modified mice used in this thesis. I have included this amount of detail in my thesis as, although not my work, it is very relevant for the understanding of the results obtained in my PhD.

Strategic generation of COX-1^{-/-} mice

The strategy when creating the COX-1^{-/-} mice was to target the Serine 530 (Ser-530) amino acid located in the active catalytic site of COX-1 (Langenbach *et al.*, 1995). Ser-530 is located in a highly conserved area of the COX-1 gene, also known as the prostaglandin-endoperoxide synthase 1 (*Ptgs1*) gene and has been identified as the acetylating site of aspirin. Aspirin interaction with COX-1 involves a bulky acetyl group that forms a covalent bond with the Ser-530 residue which interferes with arachidonic acid (substrate) binding and thereby renders the COX-1 enzyme inactive (DeWitt *et al.*, 1990). The mouse *Ptgs1* complementary DNA (cDNA) sequence (DeWitt *et al.*, 1990) (Figure 2.7) revealed that the codon for Ser-530 is located in exon 11, hence this became the target for the COX-1 disruption (Langenbach *et al.*, 1995). A 357bp probe synthesised from 129 mouse strain embryonic stem (ES) cell DNA (confirmed by southern blot to be specific for a single gene), was used to screen E14TG2a mouse ES cells for the 5' end of exon 11 on the *Ptgs1* gene. This was followed by the isolation of a clone of a fragment from the 3' region of the *Ptgs1* gene of approximately 15kb (including exon 11) (Figure 2.2) (Langenbach *et al.*, 1995). The 15kb fragment clone was confirmed to be from the COX-1 (*Ptgs1*) isoform and not COX-2 (*Ptgs2*), by a multistep process that included the use of specific primer pairs for sequences of exon 11 in *Ptgs1* and established that 100bp of DNA from exon 11 matched the corresponding published sequence of *Ptgs1* (Langenbach *et al.*, 1995).

Figure 2.2 Strategic disruption of the COX-1 (*Ptgs1*) gene

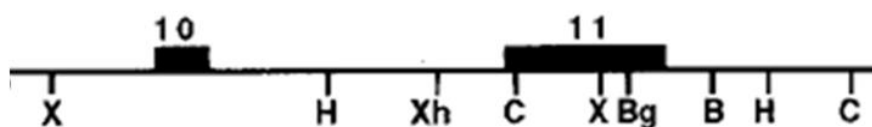


Figure 2.2 Strategic disruption of the COX-1 (*Ptgs1*) gene. A ~15kb fragment of the 3' region of the *Ptgs1* gene, showing the position of exon 11 (black box) which includes the codon for the acetylating target of aspirin (amino acid residue Ser-530). Black boxes represent intron 10 and exon 11 respectively. Restriction site associated DNA markers: X (*Xba*I), H (*Hind*III), Xh (*Xho*I), C (*Cla*I), Bg (*Bgl*II) and B (*Bam*HI). Figure modified from (Langenbach *et al.*, 1995).

The targeting vector construct included a 4.3kb NotI-XhoI fragment (Figure 2.3B, see N-Xh), a neomycin resistance gene (Neo) designed to replace ~ 1 kb of intron 10 and the first 44bp of exon 11 (Figure 2.3B, see Neo), a 2.3kb BamHI fragment (Figure 2.3B B+C) and a herpes simplex virus thymidine kinase insert (Figure 2.3B, see Tk) (Langenbach *et al.*, 1995). This targeted pPNT plasmid vector was then linearized with NotI (Figure 2.3B, see N) and electroporated into trypsinised 129 derived E14TG2a ES cells (Langenbach *et al.*, 1995).

Figure 2.3 Disruption of the *Ptgs1* gene by recombination with the vector construct

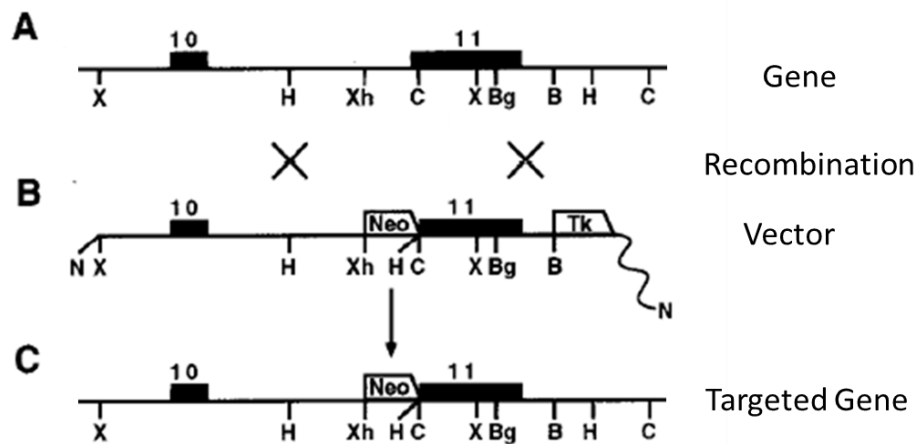


Figure 2.3 Disruption of the *Ptgs1* gene by recombination with the vector construct. (A) A ~15kb fragment of the 3' region of the *Ptgs1* gene. Black boxes represent intron 10 and exon 11 respectively. Restriction site associated DNA markers: X (XbaI), H (HindIII), Xh (XhoI), C (Clal), Bg (BglII) and B (BamHI). X indicates homologous recombination. (B) Linearized vector construct; a 4.3kb N (NotI) to Xh (XhoI) fragment, a neomycin resistance gene (Neo) inserted to replace ~1kb of intron 10 and the first 44bp of exon 11 and a 2.3kb BamHI fragment from C (Clal) to B (BamHI). Tk indicate a herpes simplex virus thymidine kinase insert. Wavy line indicates the plasmid part and N is the restricted associated DNA marker (NotI). (C) Successful homologous recombination of the vector construct into the targeted gene (from X to B) is Neo positive and Tk negative. Figure modified from (Langenbach *et al.*, 1995).

Positive selection was made by adding the antibiotic genecitin (G418) in order to harvest the vector targeted genes since the Neo gene insert is resistant to G418 (Langenbach *et al.*, 1995). Subsequently, the anti-viral drug ganciclovir (GANC) was added as a negative selection process, to provide a 10-fold enrichment of the correctly targeted genes compared to GANC alone (to discard of any herpes simplex Tk containing vectors indicating non-homologous insertion of the vector construct into the genome) (Mansour *et al.*, 1988) (Figure 2.4).

Figure 2.4 Positive/negative selection enrichment of ES cells with the targeted mutation

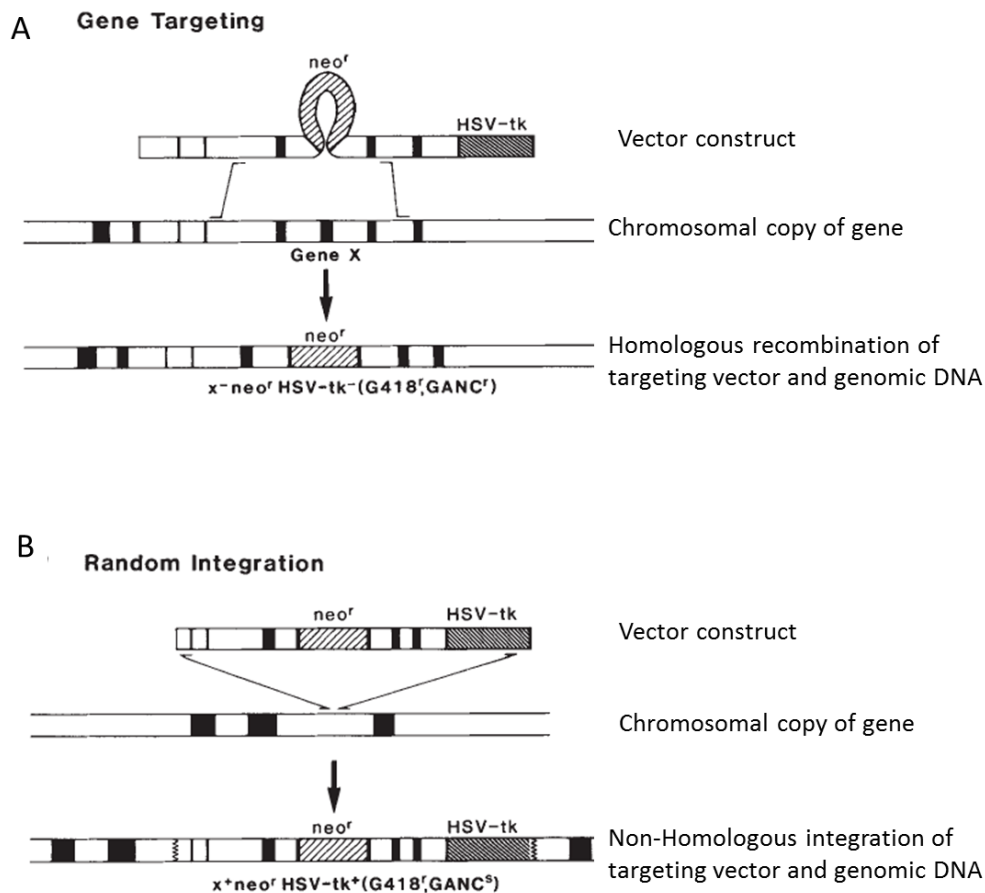


Figure 2.4 Positive/negative selection enrichment of ES cells with the targeted mutation. Closed black boxes indicate exons. Open boxes denote introns. **(A)** Gene targeting- the neomycin resistance gene (*neo*) in the vector construct will replace the target gene of interest (Gene X). Successful homologous recombination of the targeting vector with the genomic DNA results in the disruption of the targeted gene and loss of the herpes simplex virus thymidine kinase gene insert (HSV-tk). These cells will be resistant to both genecitin (G418) and the anti-viral drug ganciclovir (GANC) and would therefore pass through both the positive (G418) and negative (GANC) selection process. $x^{-} neo^{r} HSV-tk^{-} (G418^{r}, GANC^{r})$ means negative for gene X (x^{-}), contain the Neo resistance gene (neo^{r}), the HSV-tk gene is not present (HSV-tk⁻) and the targeted gene would be G418 and GANC resistant ($G418^{r}, GANC^{r}$) **(B)** Random integration or non-homologous integration of the vector construct through the ends of the linearised DNA allows for integration of both *neo* and HSV-tk inserts into the genome and therefore such cells would pass through the positive selection with G418 but would be discarded in the negative selection process since they are sensitive to GANC ($GANC^{s}$). All in all, this selection process favours higher yield for cells that contain the desired targeted mutation. Figure modified from (Mansour et al., 1988).

DNA from the remaining G418 and GANC resistant colonies (6 out of 96) were isolated and used for Southern blot analysis (Figure 2.5E) (Langenbach *et al.*, 1995). Cells from two of the successfully vector targeted clones were then injected into C57BL/6 blastocysts (Figure 2.5A).

Figure 2.5 Original breeding setup for generating $COX-1^{-/-}$ mice

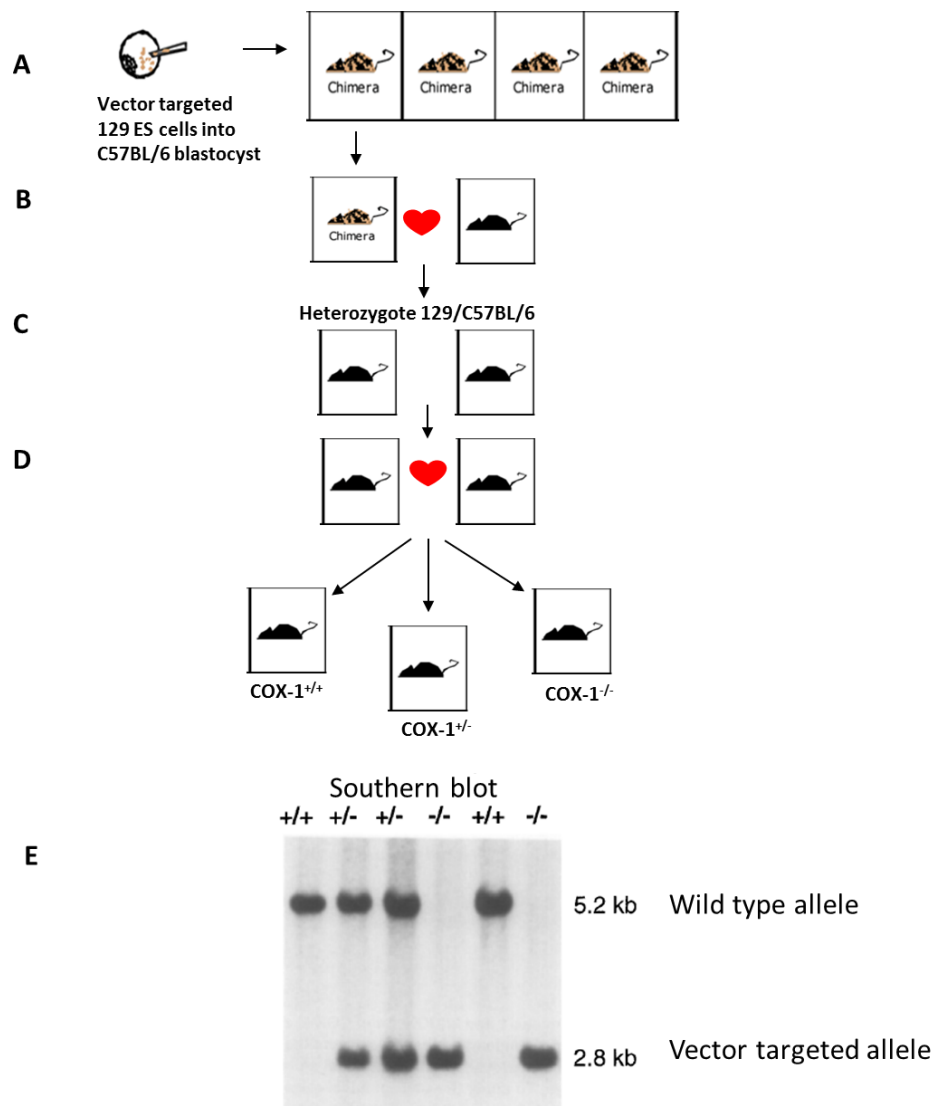


Figure 2.5 Original breeding setup for generating $COX-1^{-/-}$ mice. (A) Embryonic stem (ES) cells (from the 129 strain) carrying the targeted vector clone were injected into C57BL/6 blastocysts. Four male chimeras were born. (B) + (C) One of them produced heterozygote 129/C57BL/6 offspring when bred to a female C57BL/6 mouse. (D) These heterozygote siblings were then inbred to create wild type (WT), heterozygote and homozygote $COX-1$ genotypes. (E) Southern blot showing the Hind III digested wild type allele (5.2kb) and vector targeted allele (2.8kb) from tail clip DNA from $COX-1$ wild type (+/+), $COX-1$ heterozygote (+/-) and $COX-1$ homozygote (-/-) mice. Southern blot from (Langenbach *et al.*, 1995).

Details about the COX-1 antibodies used in this thesis

Two different COX-1 antibodies (160109 and 160108, both from Cayman antibodies with no reported cross reactivity with COX-2) were used in the experiments presented in this thesis (Figure 2.6).

Figure 2.6 Comparison of the two COX-1 antibodies used in this thesis

Murine COX-1 antibody (Cayman 160109)																													
Antigen:	Synthetic peptide from the murine COX-1 sequence, conjugated to KLH (amino acid 274-288; LMRYPPGVPPERQMA) ¹																												
Cross-reactivity:	(+) Murine and rat COX-1; (-) ovine and human COX-1 and COX-2 (all species) Homology of peptide antigen with COX from other species:																												
	<table> <tr> <td>Murine COX-1</td><td>LMRYPPGVPPERQMA</td></tr> <tr> <td>Rat COX-1</td><td>LMRYPPGVPEKQMA</td></tr> <tr> <td>Human COX-1</td><td>LMhYPrGiPPqsQMA</td></tr> <tr> <td>Ovine COX-1</td><td>LMhYPrGiPPqsQMA</td></tr> <tr> <td>Murine COX-2</td><td>eMiYPPhiPenlQfA</td></tr> </table>	Murine COX-1	LMRYPPGVPPERQMA	Rat COX-1	LMRYPPGVPEKQMA	Human COX-1	LMhYPrGiPPqsQMA	Ovine COX-1	LMhYPrGiPPqsQMA	Murine COX-2	eMiYPPhiPenlQfA																		
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Ovine COX-1 antibody (Cayman 160108)																													
Antigen:	Synthetic peptide, from the ovine seminal vesicle COX-1 sequence (amino acid 272-282; LMHYPRGIPPQ) ^{1,2}																												
Cross-reactivity:	(+) Ovine seminal vesicle, human, bovine endothelial, and porcine COX-1; (-) rat and murine COX-1 and ovine, human, and murine COX-2 Homology of peptide antigen with COX-1 from other species:																												
	<table> <tr> <td>Ovine</td><td>LMHYPRGIPPQ</td></tr> <tr> <td>Human</td><td>LMHYPRGIPPQ</td></tr> <tr> <td>Canine</td><td>LMHYPRGIIPQ</td></tr> <tr> <td>Rabbit</td><td>LMHYPRGvPPr</td></tr> <tr> <td>Rat</td><td>LMrYppGvPPe</td></tr> <tr> <td>Murine</td><td>LMrYppGvPPe</td></tr> </table>	Ovine	LMHYPRGIPPQ	Human	LMHYPRGIPPQ	Canine	LMHYPRGIIPQ	Rabbit	LMHYPRGvPPr	Rat	LMrYppGvPPe	Murine	LMrYppGvPPe																
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Figure 2.6 Comparison of the two COX-1 antibodies used in this thesis. Two COX-1 antibodies were used in this thesis; rabbit polyclonal anti-murine COX-1 (Cayman 190109) and rabbit polyclonal anti-ovine COX-1 (Cayman 160108). They both cover a relatively similar amino acid range; amino acid positions 274-288 (LMRYPPGVPPERQMA) and 272-282 (LMHYPRGIPPQ) respectively. The murine COX-1 antibody was used to detect mouse and rat COX-1 and the ovine COX-1 antibody was used to detect human COX-1 in this thesis.

Truncated COX-1 protein in COX-1^{-/-} mice

If a truncated version of the disrupted COX-1 protein was made it would lack 120 amino acids from the carboxy-terminal (including Ser-530). Furthermore, any alternative splicing to eliminate the Neo gene would affect the reading frame due to elimination of 14 amino acids (Langenbach *et al.*, 1995) (Figure 2.7).

Figure 2.7 Nucleotide and amino acid sequence (cDNA) of murine COX-1

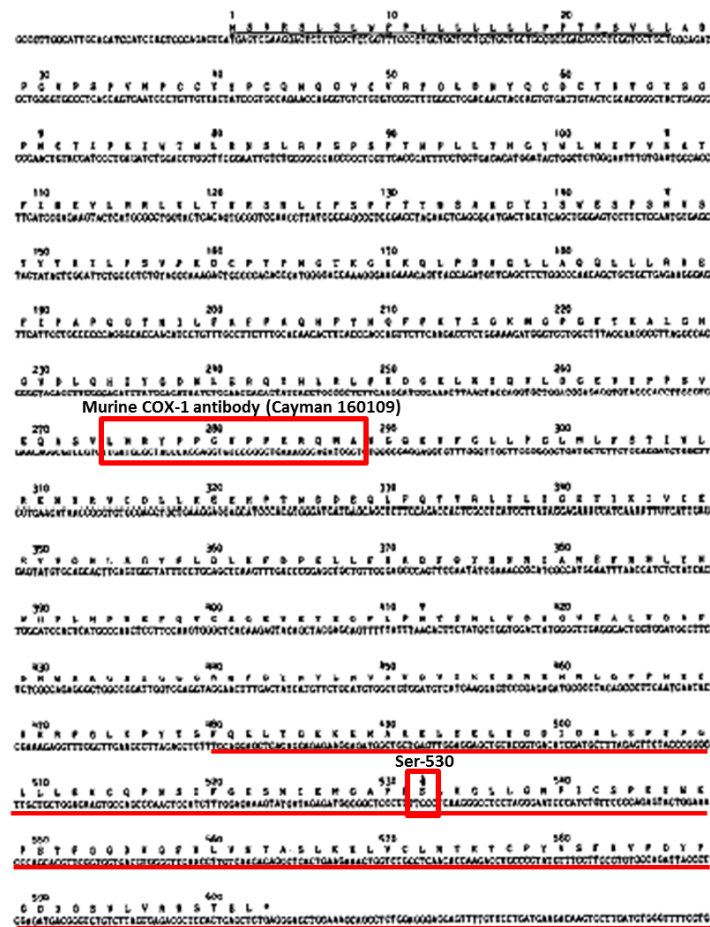


Figure 2.7 Nucleotide and amino acid sequence (cDNA) of murine COX-1. cDNA coding from a mouse 3T3 cell line (DeWitt *et al.*, 1990). Standard one letter symbols above the nucleotides indicate the corresponding amino acids and numbers above refers to the amino acid position. The large red boxes mark out the position of the antigen recognised by the Cayman murine COX-1 antibody (160109) (amino acid sequence LMRYPGPVPPERQMA). The small red box indicates the location of the Serine 530 (Ser-530), where aspirin irreversibly acetylates COX-1 by adding a bulky side chain which interferes with arachidonic acid (substrate) binding and COX-1 enzymatic activity. This was the target site for disrupting COX-1. The red line indicates the carboxy-terminal 120 amino acids that would be missing if a COX-1 protein was made in a mouse. Figure modified from (DeWitt *et al.*, 1990).

It is important to note that the murine COX-1 antibody used in this thesis (Cayman 160109) recognises a COX-1 peptide-antigen constructed to a sequence before the targeted COX-1 disruption and could therefore hypothetically pick up a truncated version of a COX-1 protein expressed in COX-1^{-/-} mice (Figure 2.7). Although a truncated version is most likely to be inactive since it would lack the carboxy-terminal end (including Ser-530), its presence could theoretically be detected in western blots and/or immunohistochemical experiments presented in this thesis.

In the original paper describing the COX-1^{-/-} mice, the authors analysed gene and protein expression in kidneys and colons to see if a truncated version of COX-1 could be detected by northern and western blotting. There was a detectable COX-1 messenger RNA (mRNA) message in the northern blot of 2.8kb in the WT (COX-1^{+/+}) mice, this signal was reduced to about half in the COX-1^{+/-} and absent in the COX-1^{-/-} mice (Langenbach *et al.*, 1995). Moreover, an antibody raised to a very similar antigen sequence (LMRYSPPGVPPERQMAC residues 274-289) as the polyclonal COX-1 antibody used in this thesis (LMRYSPPGVPPERQMA residues 274-288) did not detect measurable truncated protein in western blots (Langenbach *et al.*, 1995; Morita *et al.*, 1995).

Strategic generation of COX-2^{-/-} mice

The targeting strategy when creating the COX-2 deficient (COX-2^{-/-}) mice was to disrupt the nucleotides encoding amino acids crucial for COX-2 activity (Tyr-371 and His-374) on exon 8 with a Neo gene insert, a 104bp deletion and a truncated exon 10 (Figure 2.8) (Morham *et al.*, 1995). Mouse COX-2, or prostaglandin-endoperoxidase synthase 2 (*Ptgs2*), was isolated from the mouse strain 129 derived ES cell line E14TG2a. A probe consisting of 408 bp specific to exon 10 on *Ptgs2* (not present in the corresponding region of *Ptgs1*), was used to locate *Ptgs2* as a λ bacteriophage clone containing a 16kb fragment of BamHI digested genomic DNA (Morham *et al.*, 1995). To confirm the identity of the λ clone and to discriminate it from *Ptgs1*, a *Ptgs2* cDNA (O'Banion *et al.*, 1992) probe was used as well as specific primers to exon 1, 4, 7, 8 and 10 in *Ptgs2* (Morham *et al.*, 1995). This λ clone targeting construct, (containing a neo gene inserted between EcoRI (E) and BstXI (Bx) in exon 8, a 104 bp deletion in exon 8 (of the regions encoding for Tyr-371 and His-374) and a truncated version of exon 10), was used to disrupt the *Ptgs2* gene in the ES cell line E14TG2a (Figure 2.8A-C).

11 out of 192 clones passed the positive/negative selection process (by G418 and GANC), and was thereby assumed to have undergone successful homologous recombination. These 11 clones carrying the desired gene target were digested with SacI for Southern blot analysis, which confirmed the presence of the 3.8kb targeted allele fragment (Figure 2.8D).

Two of these successfully targeted E14TG2a ES cell lines were injected into C57BL/6 blastocysts, which resulted in the birth of male chimeric mice that were then mated with C57BL/6 females. The targeted *Ptgs2* allele was passed on to the 129/C57BL/6 offspring and these COX-2 heterozygote mice were subsequently inbred to give rise to WT (COX-2^{+/+}), heterozygote (COX-2^{+/-}) and homozygote (COX-2^{-/-}) mutant mice (see breeding setup schematic for the COX-1^{-/-} mice in Figure 2.5A-D).

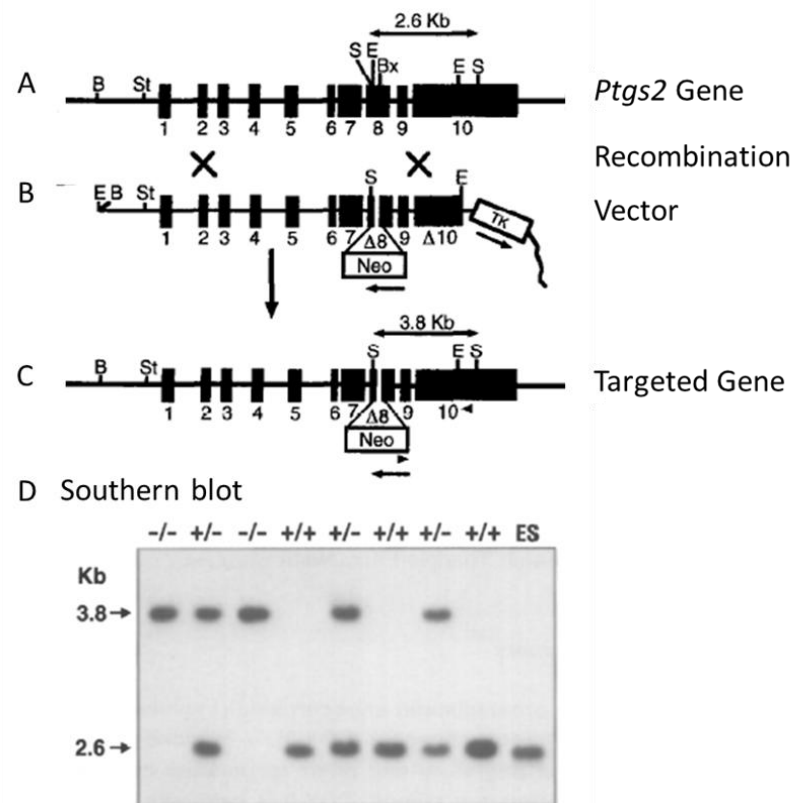
Figure 2.8 Disruption of the *Ptgs2* gene by recombination with the vector construct

Figure 2.8 Disruption of the *Ptgs2* gene by recombination with the vector construct. (A) The *Ptgs2* gene locus. Black boxes represent exons 1-10. Restriction site associated DNA markers: Bg (BglIII), St (StyI), S (SacI), E (EcoRI), Bx (BstXI). Double ended arrow indicates the 2.6kb Sacl fragment (between S and S) characteristic of the WT *Ptgs2* gene. X indicates homologous recombination. (B) Linearised λ clone vector construct; $\Delta 8$ represents neomycin resistance gene (Neo) inserted at exon 8 along with a 104bp deletion in the E to Bx region, eliminating nucleotides encoding the amino acid residues Tyr-371 and His-374, both essential for COX-2 activity. $\Delta 10$ represents a truncated version of exon 10. A herpes simplex thymidine kinase (Tk) gene was inserted for the negative selection process of correctly targeted genes. Wavy line indicates the continued plasmid sequence. (C) Successful homologous recombination of the vector construct into the targeted gene with a Neo insert and 104bp deletion in exon 8 and truncated exon 10. The correctly inserted gene is Neo positive and Tk negative, and would therefore pass through both positive and negative selection processes. Double ended arrow indicate the 3.8kb Sacl fragment (between S and S) characteristic of the targeted *Ptgs2* gene. (D) Southern blot of mouse genomic DNA digested with Sacl illustrating that DNA from WT mice ($^{+/+}$) has a single band at 2.6kb on Southern blot. Heterozygote COX-2 mice ($^{+/-}$) has one allele with the targeted gene (band at 3.8kb) and one allele with the WT gene (at 2.6kb). Homozygote COX-2 mice ($^{-/-}$) has a single band at 2.6kb (both allele carries the targeted gene). ES denotes DNA from the parental ES cell line (E14TG2a). Figure modified from (Morham et al., 1995).

Details about the COX-2 antibody used in this thesis

The COX-2 antibody that has been used throughout this thesis is a polyclonal antibody from Cayman antibodies (160126), raised in rabbit against a peptide corresponding to the amino acids 584-598 in murine COX-2 (Figure 2.9). This COX-2 antibody also cross reacts with human, rat and bovine COX-2, but has no reported cross reactivity with COX-1 in any of these species.

Figure 2.9 Murine COX-2 antibody and the amino acid sequence of *Ptgs2*

A

Murine COX-2 antibody (Cayman 160126)

Antigen: The COX-2 polyclonal antibody was raised against a peptide corresponding to amino acids 584-598 of murine COX-2.
Cross-reactivity: (+) murine, rat, human, and ovine COX-2; expected to be reactive with bovine and canine COX-2; other species not tested; (-) COX-1 from all species.

B Amino acid sequence of *Ptgs2*

Translation (604 amino acids):

MLFRAVLLCAALGLSQAANPCCSNPCQNRGECMSTGFDQYKCDCTRTGFYGENCTTPEFLTRIKLLLKPT
 PNTVHYILTHFKGVWNIVNNIPFLRSLIMKYVLTSSYLIDSPPTYNVHYGYKSWEAFSNLSYYTRALPP
 VADDCTPMGVKGNKELPDSKEVLEKVLLRREFIPDPQGSNMMFAFFAQHFTHQFFKTDHKGPGFTRGL
 GHGVDLNHIYGETLDRQHKRLRFKDGKLYQVIGGEVYPPTVKDTQVEMIYPPHIPENLQFAVGQEVFGL
 VPGLLMYATIWLREHNRVCDILKQEHPEWGDEQLFQTSRLILGETIKIVIEDYVQHLSGYHFKLKDPE
 LLFNQQFQYQNRIASEFNTLHWHPLLPDTFNIEDQEYSFKQFLYNNSILLEHGLTQFVESFTRQIAGR
 VAGGRNVPIAVQAVAKASIDQSREMKYQSLNEYRKRFSKPYTSFEELTGEKEMAAELKALYSIDVME
 LYPALLVEKPRPDAIFGETMVELGAPFSKGLMGNPICSPQYWKPSTFGGEVGFKIINTASIQSLICNNVKG
 CPFTSFNVQDPQPTKTATINASASHSRLDDINPTVLIKRRSTEL

Figure 2.9 Murine COX-2 antibody and the amino acid sequence of *Ptgs2*. (A) The details about the antigen and cross-reactivity of the murine COX-2 antibody 160126 from Cayman. (B) One letter coded amino acid sequence for *Ptgs2*. Dark grey indicates exon 8 which was the target for the insertion to disrupt *Ptgs2*. The targeting strategy also included a 104 bp deletion in exon 8 to eliminate nucleotides coding for the two amino acids crucial for COX-2 activity (Tyr-371 and His-374 in exon 8, marked in red). The amino acid sequence (SHSRLDDINPTVLIK, position 584-598,) coding for the antigen recognised by the polyclonal COX-2 antibody (Cayman 160126) used in this thesis is indicated by underlined bold black letters. Gly-469 - Leu 604 in exon 10 (highlighted in light grey) was truncated in the targeted gene which would affect the sequence recognised by the COX-2 antibody.

Truncated COX-2 protein in COX-2^{-/-} mice

A truncated version of the disrupted COX-2 protein would have a missing section at exon 10, (including the amino acid sequence for the antigen recognised by the COX-2 antibody used in this thesis, Figure 2.9). Furthermore, earlier reports from these COX-2^{-/-} mice indicate that these mice completely lack normal induction of *Ptgs2* mRNA, protein and PGE₂ synthesis in macrophages upon stimulation with LPS, measured by Northern blot, Western blot and competitive radioimmunoassay (RIA) (Morham *et al.*, 1995).

COX-1 and COX-2 protein phenotypes in WT, COX-1^{-/-} and COX-2^{-/-} mice

Information described above from the literature would suggest that the mice used in my PhD have confirmed phenotypes. However, members of our group (Mr William Wright and Ms Ivana Vojnovic) have performed western blotting validation experiments using fibroblasts isolated from lungs of the WT, COX-1^{-/-}, COX-2^{-/-} mice from our colony (Figure 2.10).

Figure 2.10 Western blots of COX-1 and COX-2 protein from lung fibroblasts isolated from our colony WT, COX-1^{-/-} and COX-2^{-/-} mice

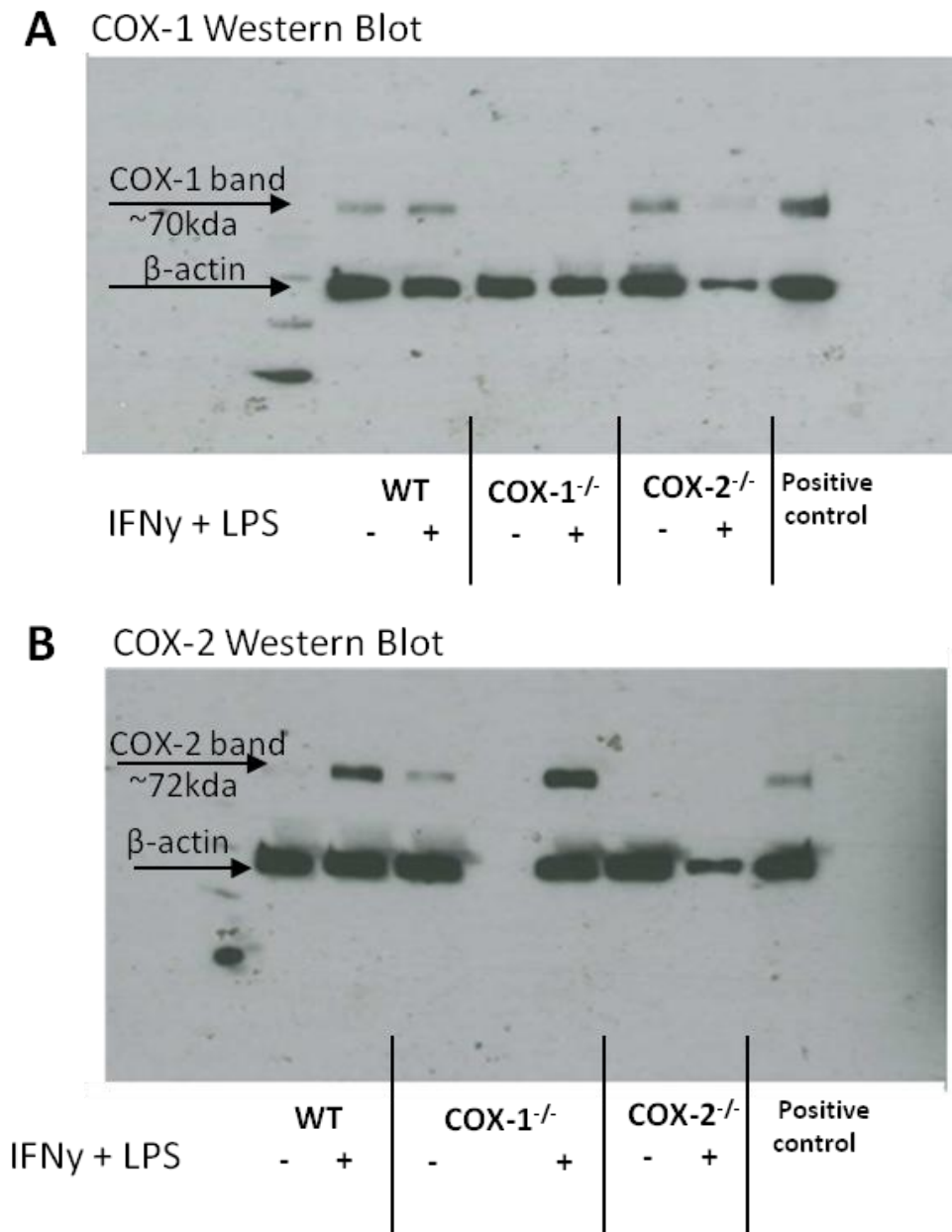


Figure 2.10 Western blots of COX-1 and COX-2 protein from lung fibroblasts isolated from our colony WT, COX-1^{-/-} and COX-2^{-/-} mice. Western blots showing (A) COX-1 and (B) COX-2 protein in lung fibroblasts from WT, COX-1^{-/-} and COX-2^{-/-} mice. Lung fibroblasts grown out from pieces of lung incubated in vitro were isolated and either left untreated (-) or treated with interferon γ (IFN γ , 10ng/ml) and LPS (serotype 0111:B4, 1 μ g/ml) to induce COX-2. The positive control was J774 macrophages treated with LPS and the strong bands underneath sample bands is β -actin. The weak band of COX-1 in the COX-2^{-/-} mice treated with IFN γ and LPS may be due to an error in protein loading since the β -actin band underneath is also greatly reduced.

Fibroblasts from WT mice and COX-2^{-/-} mice, treated with or without interferon γ (IFN γ)+ LPS, gave clear bands on the Western blot corresponding to the expected size of COX-1 protein (~70kda in size) (Figure 2.10A). These bands were absent in fibroblasts from COX-1^{-/-} mice (Figure 2.10A). In the COX-2 Western blot, there were clear bands at the expected size for COX-2 protein (~72kda), in fibroblasts from WT and COX-1^{-/-} mice treated with IFN γ + LPS, but this COX-2 band was absent in fibroblasts from COX-2^{-/-} mice (Figure 2.10B). The faint band of COX-2 in the untreated COX-1^{-/-} mice is likely to represent COX-2 induced during fibroblast proliferation, consistent with COX-2 being induced by growth factors present during proliferation (Figure 2.10B).

Reported phenotype of the COX-1^{-/-} mice

The COX-1^{-/-} females were reported to have normal fertility and implantation but to have delayed parturition leading to a prolonged gestation (by 2-3 days compared to WT mice), which negatively influenced the pups survival rate (Gross *et al.*, 1998). Langenbach *et al* reported that despite a 99% reduction in constitutive prostaglandins, the COX-1^{-/-} mice developed normally, appeared healthy and had no significant pathology when they performed necropsy and microscopic examinations of heart, lung, liver, spleen and gastrointestinal and reproductive tracts (n=3 COX-1^{-/-} male and n=3 COX-1^{-/-} female mice).

However, after closer examination of the kidneys they discovered that 3 out of 6 kidneys from the COX-1^{-/-} mice had small foci of basophilic, immature tubules, which did not occur in the WT mice (Langenbach *et al.*, 1995).

In addition, in a standard ear swelling assay, where swelling of the ear was measured in response to topical application of inflammatory agents, the COX-1^{-/-} mice had a 30% reduced inflammatory response to arachidonic acid compared to the WT mice, whereas there was no difference following exposure to tetradecanoyl phorbol acetate (TPA) (Morham *et al.*, 1995). This was thought to show that constitutive COX-1 contributes to oedema and inflammation induced by arachidonic acid, whereas TPA induced inflammation was mediated by induction of COX-2 (Morham *et al.*, 1995).

Furthermore, as expected, platelets from COX-1^{-/-} mice aggregated more slowly and were less responsive to arachidonic acid *in vitro* than platelets from WT mice (Morham *et al.*, 1995). Another phenotypic characteristic reported of these COX-1^{-/-} mice is that they had no spontaneous stomach ulcers and their gastric mucosa appeared normal (although gastric pH, which is regulated by COX-1, was reduced) (Loftin *et al.*, 2002).

Reported phenotype of the COX-2^{-/-} mice

The COX-2^{-/-} female mice were reported to be infertile due to defects in ovulation and implantation (Lim *et al.*, 1997). Meanwhile, the COX-2^{+/-} female mice had normal fertility. This was the reason why we needed to use heterozygote females in our breeding colonies. The most striking phenotypic characteristic reported about the COX-2^{-/-} mice is their renal abnormalities, with severe developmental renal defects (Morham *et al.*, 1995). In mice, the development of the kidneys continues postnatal, and COX-2 seems to play a pivotal role during this critical time (Figure 2.11) (Kömhoff *et al.*, 2000). Adult COX-2^{-/-} mice also have disrupted salt sensing which has consequences on renin-angiotensin system activation (Yang *et al.*, 2000). COX-2 is normally induced in the macula densa and thick ascending limb in mice, by reductions in salt levels. COX-2 therefore appears to be essential for renal homeostasis including salt retention, volume and blood pressure regulation (Yang *et al.*, 2000).

Figure 2.11 Histology of kidneys from 21 days old WT versus COX-2^{-/-} mice

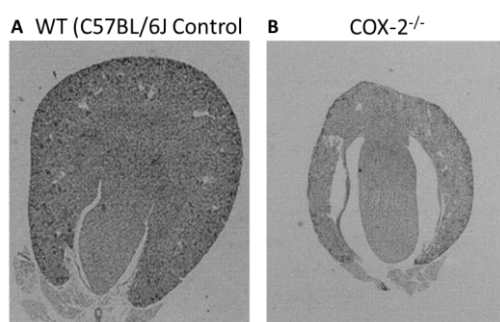


Figure 2.11 Histology of kidneys from 21 days old WT versus COX-2^{-/-} mice. Section of a kidney from a 21 days old **(A)** WT (C57BL/6J) mouse and **(B)** COX-2^{-/-} mouse, stained with hematoxylin. Figure modified from (Kömhoff *et al.*, 2000).

A summary of the main phenotypic characteristics reported in the literature for the COX-1^{-/-} and the COX-2^{-/-} mice originally used to generate our colonies is provided in table 2.12.

Table 2.12 Phenotypic characteristics of COX-1^{-/-} and COX-2^{-/-} mice

Physiology/pathology	COX-1 ^{-/-} mice	COX-2 ^{-/-} mice	References
Constitutive prostaglandin synthesis	Decreased	Normal	(Langenbach <i>et al.</i> , 1995; Morham <i>et al.</i> , 1995)
Inducible prostaglandin synthesis	Normal	Decreased	(Langenbach <i>et al.</i> , 1995; Morham <i>et al.</i> , 1995)
Ovulation	Normal	Impaired	(Gross <i>et al.</i> , 1998; Lim <i>et al.</i> , 1997)
Implantation	Normal	Impaired	
Parturition	Delayed	Unknown	
Neonatal mortality	Normal	Increased	(Dinchuk <i>et al.</i> , 1995; Langenbach <i>et al.</i> , 1995; Loftin <i>et al.</i> , 2001; Morham <i>et al.</i> , 1995)
Adult mortality	Normal	Increased	(Morham <i>et al.</i> , 1995)
Patent ductus arteriosus	Normal	Increased	(Loftin <i>et al.</i> , 2001)
Postnatal kidney development	Normal	Impaired	(Dinchuk <i>et al.</i> , 1995; Kömhoff <i>et al.</i> , 2000; Langenbach <i>et al.</i> , 1995; Loftin <i>et al.</i> , 2001; Morham <i>et al.</i> , 1995)
Spontaneous gastric ulceration	Normal	Normal	(Langenbach <i>et al.</i> , 1995; Morham <i>et al.</i> , 1995)
Peritonitis incidence	Normal	Increased	(Langenbach <i>et al.</i> , 1995; Morham <i>et al.</i> , 1995)
Induced acute colitis	Increased	Increased	(Morteau <i>et al.</i> , 2000)
Platelet aggregation	Impaired	Normal	(Langenbach <i>et al.</i> , 1995; Morham <i>et al.</i> , 1995)
Wound Healing	Unknown	Impaired	(Langenbach <i>et al.</i> , 1999)
Febrile response	Normal	Decreased	(Li <i>et al.</i> , 2001; Li <i>et al.</i> , 1999)
Influenza A viral infection	Increased	Decreased	(Carey <i>et al.</i> , 2005)
Inflammation (AA induced Ear inflammation model, air pouch model and allergen induced pulmonary inflammation model)	Altered	Altered	(Gavett <i>et al.</i> , 1999; Langenbach <i>et al.</i> , 1999; Langenbach <i>et al.</i> , 1995)
Collagen Induced Arthritis	Normal	Decreased	(Myers <i>et al.</i> , 2000)
Abdominal Aortic Aneurysms (Chronic angiotensin II infusion model)	Unknown	Decreased	(Gitlin <i>et al.</i> , 2007)
Lipid deposition in the aorta following an atherogenic diet	Unknown	Increased	(Narasimha <i>et al.</i> , 2007)

Table 2.12 Phenotypic characteristics of COX-1^{-/-} and COX-2^{-/-} mice. Table modified from (Loftin *et al.*, 2002).

In summary, despite greatly decreased constitutive prostaglandin synthesis, the COX-1^{-/-} mice were generally healthy without signs of spontaneous stomach ulcers (but with a more acidic gastric environment and heightened responsiveness to acutely induced colitis), had altered inflammatory response to influenza or bacterial infection, had normal fertility but delayed parturition (leading to smaller litter sizes) and impaired platelet aggregation responses (Langenbach *et al.*, 1995).

The COX-2^{-/-} mice had normal levels of constitutive prostaglandins, but altered induced levels. Furthermore, the female homozygote knockout mice were infertile, many pups have premature closure of the patent ductus arteriosus and post natal renal abnormalities that deteriorated with age and led to an increased neonatal and adult mortality rate. The COX-2^{-/-} mice neither had any spontaneous gastric lesions but had increased incidence of peritonitis and acutely induced colitis. Moreover, wound healing was impaired, the febrile, influenza and inflammatory response was decreased and the immune response to collagen induced arthritis was reduced. Upon challenge with an atherogenic diet, there was more lipid deposition in the aorta and increased systemic markers of inflammation in the COX-2^{-/-} mice.

Mice lacking both COX-1 and COX-2 (double knockout COX-1^{-/-} COX-2^{-/-} mice), die from a patent ductus arteriosus within 12 hours of birth (Loftin *et al.*, 2001), hence, this prevent study of the consequences of a lack of both COX isoforms.

Breeding and phenotypic data from our WT, COX-1^{-/-} and COX-2^{-/-} colonies

The average litter sizes for mice used in this thesis were: WT (6.4 ± 0.3), COX-1^{-/-} (4.7 ± 0.4) and COX-2^{-/-} (7.3 ± 0.3); the COX-1^{-/-} litter size was significantly smaller than the WT litter size (One-Way ANOVA with Dunnett's post-test, $p < 0.05$ was considered significant). This is consistent with what was reported by Langenbach et al 1995 where the delayed parturition in the COX-1^{-/-} mice, is likely to lead to smaller litter sizes. The genotypic ratio from the heterozygote COX-2 breeding was 39% COX-2^{-/-} versus 61% COX-2^{+/-} in the last 678 mice bred. Genotyping protocols were thoroughly adhered to and for some experiments; sample tissue was saved in order to double-check the genotype of the animals in a particular study.

Blood chemistry

Whole blood cell counts and serum chemistries were analysed by a commercial veterinary diagnostics service (IDEXX Laboratories, UK) for the following parameters: number of red blood cells, white cells, neutrophils, lymphocytes, monocytes, eosinophils and platelets and haemoglobin content, haematocrit, mean corpuscular size of the red blood cells, concentration of haemoglobin, albumin, globulin, urea, creatinine, alanine transferase, alkaline phosphatase, aspartate transaminase and creatine phosphokinase.

The most prominent result were that the COX-2^{-/-} mice had microcytic anaemia (smaller red blood cells based on their mean corpuscular volume). This type of anaemia in patients is normally associated either with iron deficiency caused by poor absorption of iron from the diet or loss of iron via for instance intestinal blood loss from ulcers. Another example of this type of anaemia can occur in patients on chronic dialysis with chronic renal insufficiencies.

Elevated plasma urea was a further significant difference in the COX-2^{-/-} mice. Urea is made in the liver as a breakdown product of protein and is filtered and to some extent reabsorbed by the kidneys before the remainder is excreted in the urine. Elevated plasma urea concentration can be a marker of impaired kidney function but can also occur due to increased protein consumption and/or metabolism or due to dehydration.

There was also a trend for the COX-2^{-/-} mice to have an increased number of platelets in their blood (WT; $1551 \pm 107 \times 10^9/L$ versus COX-2^{-/-}; $2045 \pm 335 \times 10^9/L$, n=6-7) although this did not reach statistical significance (One-Way ANOVA with Dunnett's post-test, $p < 0.05$ was considered significant).

Resin casts of the arterial tree in WT, COX-1^{-/-} and COX-2^{-/-} mice

It is known that COX-2 immunoreactivity is expressed in highly specialised cells of the renal vasculature and as outlined earlier; COX-2 gene deletion results in profound renal remodeling and loss of kidney functions (Kömhoff *et al.*, 2000; Morham *et al.*, 1995; Yang *et al.*, 2000). In pilot studies, performed in collaboration with Dr Antony Hunt at the Bioengineering department at Imperial College, I made resin casts of the arterial tree in WT, COX-1^{-/-} and COX-2^{-/-} mice (Figure 2.13A). The kidneys in the casts from the WT mice appeared enriched in small interlobular arteries, whereas the kidneys from the COX-2^{-/-} mice (but also to a lesser extent in the COX-1^{-/-} mice) appeared pruned in comparison (Figure 2.13B). This observation is in agreement with the general phenotype of the COX-2^{-/-} mice with their reported renal abnormalities (Kömhoff *et al.*, 2000; Morham *et al.*, 1995; Yang *et al.*, 2000).

Figure 2.13 Resin casts of the arterial tree from WT versus COX-1^{-/-} and COX-2^{-/-} mice

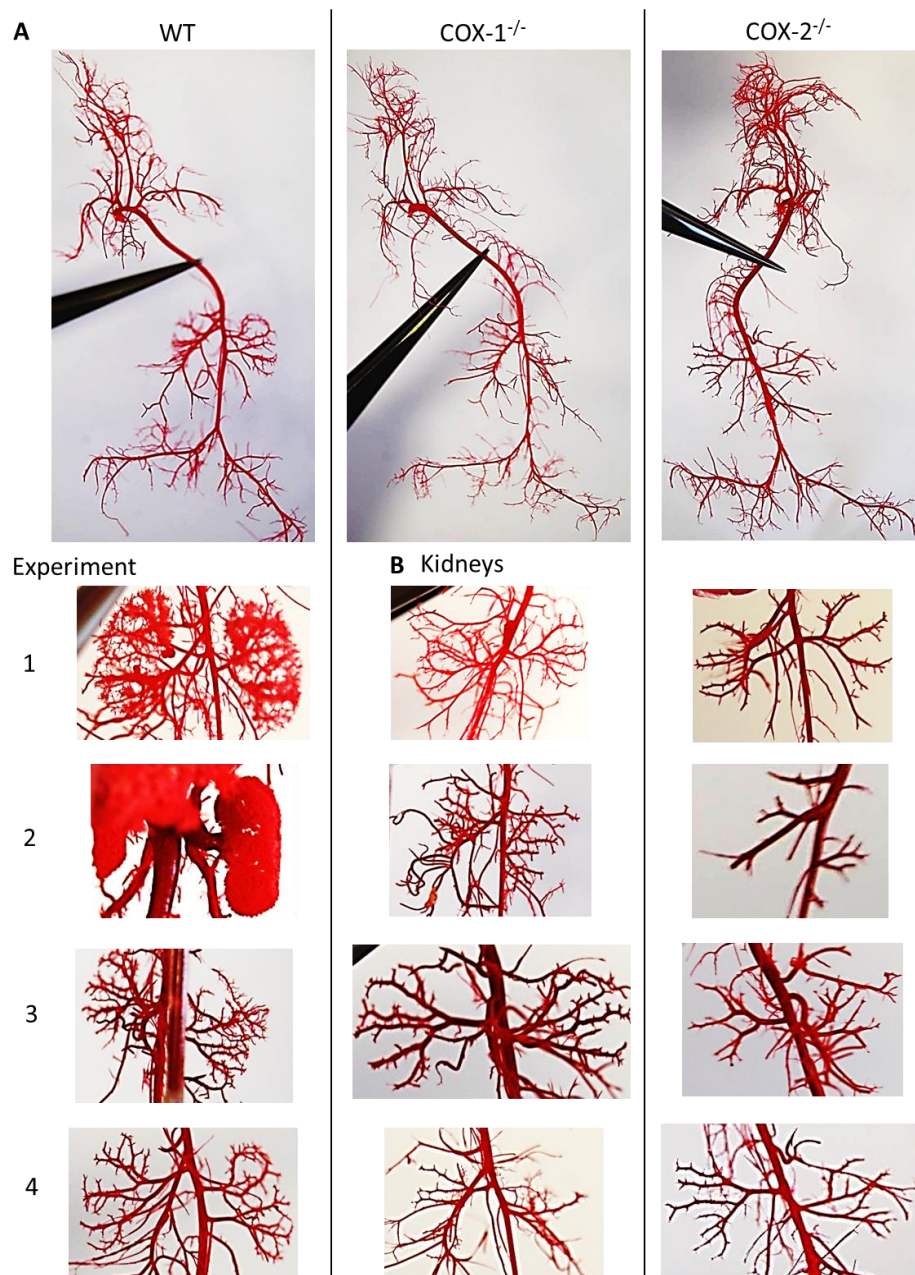


Figure 2.13 Whole mouse resin casts of the arterial tree from WT versus COX-1^{-/-} and COX-2^{-/-} mice. Representative images from resin casts of the arterial tree in n=4 male, young adult (3 month old) WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice. **(A)** General outline of the arterial tree in WT, COX-1^{-/-} and COX-2^{-/-} mice. **(B)** Photos of kidneys in the resin casts from the WT, COX-1^{-/-} and COX-2^{-/-} mice. In brief, mice were injected with 1000units/ml heparin (i.v. through the tail vein) followed by immediate CO₂ asphyxiation. The mice were then injected with a red resin mixture through the left ventricle of the heart at a pressure of 95-100mgHg, which was then allowed to set and left overnight. The resin cast of the arterial tree was then separated from the rest of the carcass by maceration/corrosion using potassium hydroxide (30% KOH). After 3 days maceration, the arterial cast was washed gently and allowed to dry.

ApoE^{-/-} mice

In my thesis I have used tissue from a mouse model of atherosclerosis. These models are not ideal since their lipid profile differs from that in man. Another short coming with mouse models of atherosclerosis is that despite the clear formation of atherosclerotic plaques, spontaneous plaque rupture (with the clinical manifestations of heart attack and stroke) have not been demonstrated (Daugherty, 2002). Nevertheless, these models are useful and have been used by countless groups to help understand human disease. The model I used in this thesis was the Apo lipoprotein deficient (ApoE^{-/-}) mouse model of atherosclerosis and these mice were a gift from Professor Shu Ye at the William Harvey Research Institute. They were originally purchased from Jackson labs (ApoE^{tm1Unc} strain, from a C57BL/6J background). The ApoE^{-/-} mice colony was managed by one of my colleagues Dr Nicholas Kirkby.

These mice have increased total plasma cholesterol levels (hypercholesterolemia), due to their deficiency in ApoE, which is essential for normal cholesterol metabolism. ApoE is normally present at the surface of high density lipoprotein (HDL) and very low density lipoprotein (VLDL) particles where it modulates interactions with low density lipoprotein (LDL) receptors (Daugherty, 2002). Consequently, ApoE^{-/-} mice develop fatty streaks in the aortic arch at around 3 month age which progress with age. In these mice, development of fatty streaks is accelerated when they are fed an atherogenic high cholesterol diet. The lesions found in these mice are enriched with so called foam cells which are lipid laden macrophages, and with time the lesions progress to contain necrotic cores and fibrous caps (Daugherty, 2002). In addition to the ApoE disruption, other genetically dependent factors such as strain background influence the disease progression and ApoE^{-/-} mice on a C57BL/6J background are particularly susceptible to atherosclerosis (Daugherty, 2002).

In this thesis I have performed experiments in both normal chow fed ApoE^{-/-} mice as well as ApoE^{-/-} mice that have been fed an atherogenic diet, in order to consider a model of early atherogenesis as well as more advanced and established atherosclerosis respectively.

Normal chow fed ApoE^{-/-} mice as a model of early atherogenesis

The normal chow fed ApoE^{-/-} mice are still reported to have markedly elevated plasma cholesterol (hypercholesterolemia) and have pre-atherosclerotic lesions or fatty streaks in their proximal aorta from 3 month of age and these lesions progress with age (even without an atherogenic diet) (Zhang *et al.*, 1992). These animals also have increased markers of vascular inflammation with monocyte adhesion to intact endothelium in 2 month old ApoE^{-/-} mice on a normal diet (Daugherty, 2002).

High cholesterol chow fed ApoE^{-/-} mice as a model of accelerated atherosclerosis

At 12 weeks of age some of the ApoE^{-/-} mice were put on an atherogenic high fat and high cholesterol diet (21% fat, 1.25% cholesterol, cholate-free; Research Diets, D12108) for 10 weeks in order to accelerate the rate of atherosclerotic plaque formation. This diet dramatically increased serum cholesterol. Aortas were then stained with Sudan staining to confirm characteristic distribution of atherosclerotic plaques in branch points and curvatures in aortas from ApoE^{-/-} mice fed an atherogenic diet (Figure 2.14).

Figure 2.14 Distribution of atherosclerotic plaques in ApoE^{-/-} mice

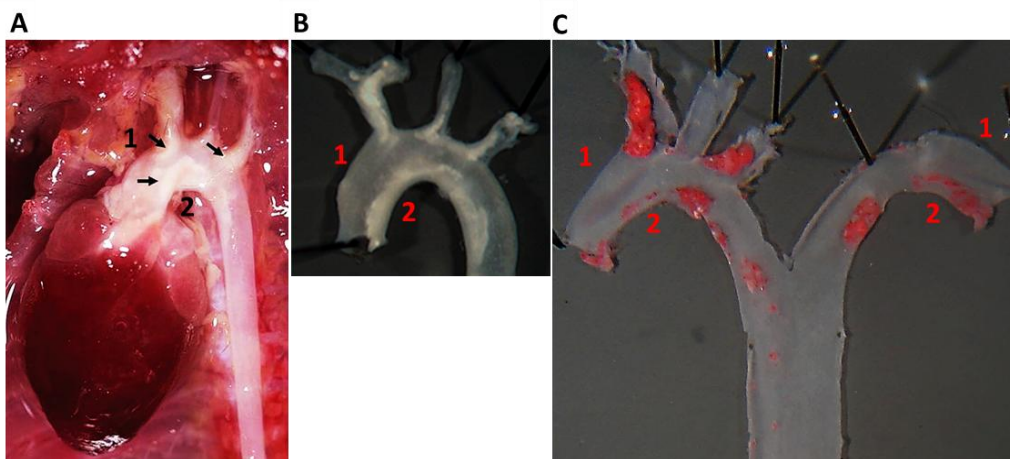


Figure 2.14 Distribution of atherosclerotic plaques in ApoE^{-/-} mice. (A) Atherosclerotic lesions (in white, indicated by black arrows) in an 8 month old ApoE^{-/-} mouse fed a chow diet, showing that ApoE^{-/-} mice spontaneously develop plaques with time (even on a normal chow diet). 1 = greater curvature, 2 = lesser curvature of the mouse aortic arch. Picture from (Catanozi *et al.*, 2009). (B) Atherosclerotic lesions in an ApoE^{-/-} mouse fed an atherogenic diet. (C) Sudan stained plaques (red) in an opened aortic arch from an ApoE^{-/-} mouse fed an atherogenic diet. 1 = greater curvature, 2 = lesser curvature of the mouse aortic arch. B + C representative pictures taken by my colleague Dr Nickolas Kirkby, of the aorta and aortic arch of one of the ApoE^{-/-} mice fed an atherogenic diet used in my PhD.

Rats

In collaboration with Dr Alexander Lyon (Cardiologist, Imperial College), I was able to use tissue from male Sprague Dawley rats surgically treated to model post-infarction chronic heart failure (Lyon *et al.*, 2011; Lyon *et al.*, 2009). Our collaborator Dr Alexander Lyon performed the surgery, maintenance and husbandry of the rats. All animal surgical procedures and perioperative management were carried out in accordance with the United Kingdom Home Office Guide on the Operation of the Animals (Scientific Procedures) Act 1986.

Rat post-infarction chronic heart failure model

In brief, male Sprague Dawley rats (250-300g, 2-3 month old) were anaesthetized and the left proximal coronary artery was ligated 1mm below the left atrial appendage, encompassing both the proximal left anterior descending artery and first diagonal coronary arteries, giving a large infarct size (~30% left ventricular myocardium) to induce chronic myocardial infarction. Animals were allowed to recover and were followed for approximately 4 months after the procedure, where markers of heart failure manifest.

- The transmural infarct covered more than 30% of the left ventricular circumference (compared to rats with sham ligation) when examined after 4 months (Figure 2.15A) (Lyon *et al.*, 2009).
- Pressure-volume (PV) loop analysis in the left ventricle was performed after 4 months using a microconductance catheter via an apical approach under anesthesia (1.5% isoflurane) and steady state data was recorded after 15 minutes stabilisation. PV loops from rats with chronic heart failure demonstrated increased ventricular volumes and increased diastolic blood pressure compared to sham controls (Figure 2.15B) (Lyon *et al.*, 2009).
- Heart hypertrophy (as a marker of heart failure) was estimated by correcting heart weight for tibia length (heart weight (g) / Tibia length (mm)), rather than body weight given the variety of complex and opposing changes that influence body weight in chronic heart failure. The heart weight/ tibia length ratio was significantly increased in the rats with chronic heart failure used for the experiments in this thesis, which validates that these rats had the characteristic heart failure hypertrophy of the viable left ventricle (Figure 2.15C) (Lyon *et al.*, 2009).

- Contractile function was also significantly impaired in the rats with the chronic infarction, consistent with the chronic heart failure phenotype (Lyon *et al.*, 2009).

Figure 2.15 Markers of post-myocardial infarction induced chronic heart failure in rats

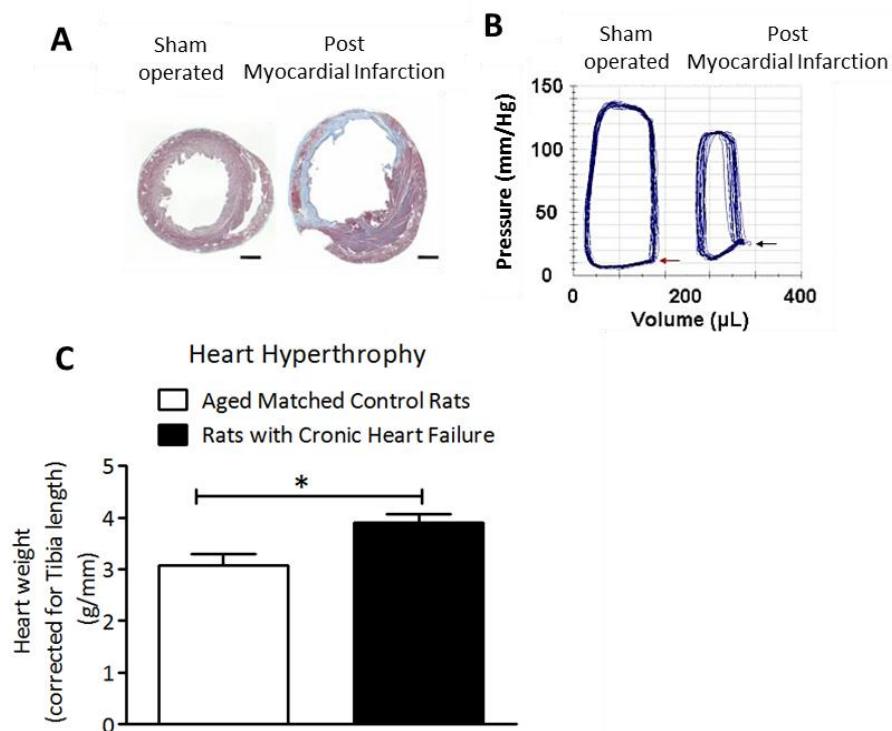


Figure 2.15 Markers of post-myocardial infarction induced chronic heart failure in rats. After 4 month post infarction markers of heart failure manifest: **(A)** Midventricular sections of sham operated and a chronically infarcted rat heart stained with Masson's trichrome stain. Black scale bar represent 2mm. **(B)** Representative steady state pressure-volume (PV) loops from in vivo measurements from sham operated and chronically infarcted rats. Arrows indicate end-diastolic blood pressure. **(C)** Heart hypertrophy (as a marker of chronic heart failure) was estimated by correcting heart weight for tibia length (heart weight (g) / Tibia length (mm)) from aged matched control versus rats with chronic heart failure used in the experiments presented in this thesis. Data (supplied by Dr Lyon) is the mean $\pm$ S.E.M. from $n=5$ rats (6-7 months old) and was analysed using paired Student's t-test and $*p<0.05$ taken as significant.

Aged matched controls versus sham operated controls

In initial experiments performed by Dr Lyon, when the heart failure model was set up, sham operated animals were used as control, but after confirming that there were no significant differences between sham ligated and aged matched control rats (personal communication, Dr Lyon), the latter were used as controls in the experiments presented in this thesis. Considering that the time of sacrifice is so remote in time (at least 4 months after) from the infarct surgery, it is less likely for spillover of 'operation effects' per se on the vasculature.

Human pulmonary artery

During the course of my PhD I had the opportunity to image COX-1 and COX-2 in a human blood vessel (pulmonary artery). Whilst this could only be done as a pilot study, I have included the results in my thesis in order to serve as validation for my data using rodent vascular tissue. The human pulmonary artery was from lungs obtained from an American tissue bank (International Institute for Advancement of Medicine), with confirmed consent from the donor) and used under appropriate ethical approval held by Professor Belvisi (Imperial College) which our group is also named on (ethics number 09/H0708/72). The donor was 67 year old Caucasian female with 49 pack year smoking history (1 pack a day for 49 years) and a known medical history of chronic obstructive pulmonary disease (COPD), essential hypertension and diabetes mellitus. Cause of death was spontaneous intracerebral haemorrhage. Brain stem death was declared at 6pm 15th March 2011 (US Mountain Time Zone, MT). Patient was maintained on artificial ventilation with her heart beating spontaneously until cross-clamp and lung removal the next evening (11pm, 16th March 2011). Time from cross-clamp to dissection in our lab (cold ischemia time) was approximately 36 hours.

After transport to the UK, the lungs arrived in our lab on the 18th of March. The aortic arch and the pulmonary artery was dissected and placed in 2% formaldehyde for 20 minutes (in room temperature) before it was moved to a vial with phosphate buffered saline (PBS). The aortic arch was cut open and pinned out on wax in order to take photos of the intima (Figure 6.1 in the General Discussion Chapter 6). Whereas the pulmonary artery was cleared of fat and connective tissue and cut into small ~1-2mm rings and placed in a well with 200µl of PBS in a 96-well plate for incubations with reagents and antibodies (Figure 2.18).

Experimental models

En face confocal imaging of the endothelium in the mouse aortic arch model

En face confocal imaging of the mouse aortic arch was adapted from previously published techniques (Hajra *et al.*, 2000; Iiyama *et al.*, 1999; Zakkar *et al.*, 2008; Zakkar *et al.*, 2009). This model was developed following the observation that atherosclerotic plaques formed in defined regions of the aortic arch in LDL receptor knockout mice (LDLR^{-/-} mice) fed a high cholesterol diet. It was shown, that the aortic arch provided a robust model encompassing both a low probability region for lesion development (greater curvature) and a high probability region for atherosclerotic plaque formation (lesser curvature) (Figure 2.16) (Iiyama *et al.*, 1999).

Figure 2.16 Mapping the mouse aortic arch for high and low probability regions of atherosclerosis development

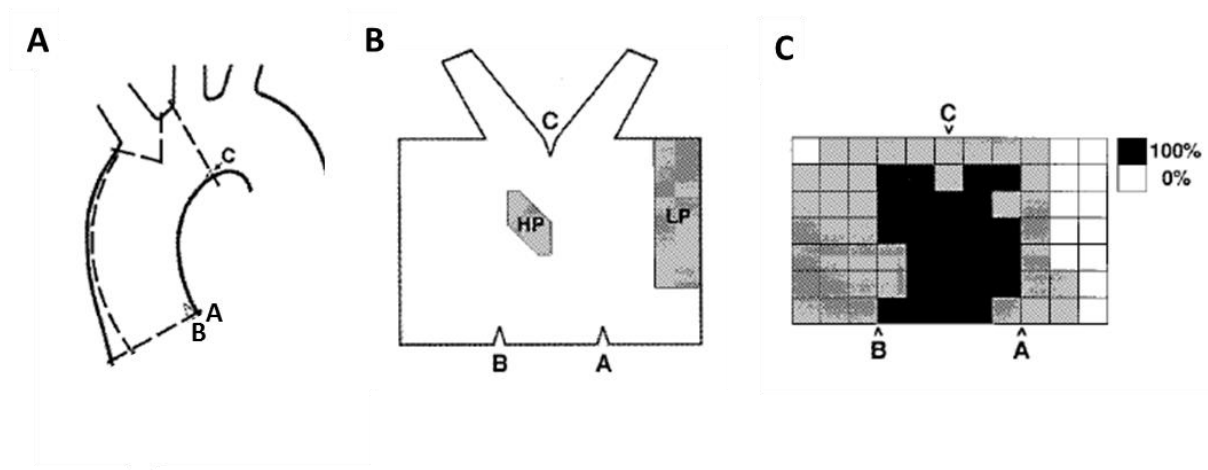


Figure 2.16 Mapping the mouse aortic arch for high and low probability regions of atherosclerosis development. (A) A schematic of the proximal ascending aortic arch with marked dissection points A+B indicate aortic valves C is the cut of point after brachiocephalic artery branch. (B) The mouse aortic arch shown opened (flattened) with A+B+C as anatomical landmarks. LP = the Low Probability region, where lipid content (visualised using oil red O stain) was absent (0%) indicating that this area was clear of atherosclerotic plaques, in n=8 LDLR^{-/-} mice fed a semi purified high cholesterol diet for 4 weeks (the same was true after 20 weeks). HP = the High Probability region, where oil red O stain was abundant (100%) indicating that this area had atherosclerotic plaques, in n=8 LDLR^{-/-} mice fed a semi purified high cholesterol diet for 4 weeks. (C) Map % of mice with oil red o stain in the aortic arch. HP is shown in black where 100% (8 out of 8) LDLR^{-/-} mice had oil red o stain and LP shown in white where 0% (0 out of 8) LDLR^{-/-} mice had oil red o stain. Figure modified from (Iiyama *et al.*, 1999).

Hajra et al also reported that the endothelium lining the lesser curvature of the aortic arch was ‘primed for activation’ with elevated levels of nuclear NF- κ B and cell adhesion molecules (Hajra *et al.*, 2000). As introduced in Chapter 1, hemodynamic shear stress profiles affect the endothelial cell morphology and susceptibility to inflammation and atherosclerosis in curvatures and branch points of the vasculature. In general terms, the laminar, unidirectional and high shear stress experienced by the endothelium in the greater curvature typically corresponds to a ‘resting’ endothelial phenotype which is similar to other straight segments of the vasculature such as the descending aorta and/or the thoracic aorta.

The two regions of high versus low susceptibility of atherosclerosis in the aortic arch have a somewhat confusing and inconsistently used, but still interchangeable nomenclature. Therefore, in order to help clarify the terminology used to refer to different regions of the aortic arch throughout the thesis; please see table 2.17. I will refer to the two regions as the greater and the lesser curvature of the aortic arch.

Table 2.17 Interchangeable terms to describe the greater and the lesser curvature of the mouse aortic arch

Commonly used descriptors for the ‘greater curvature’ of the aortic arch	Commonly used descriptors for the ‘lesser curvature’ of the arch	References
• Greater curvature • Outer curvature • Low probability region (LP) • Athero-resistant region • Athero-protected region • Protected region	• Lesser curvature • Inner curvature • High probability region (HP) • Athero-prone region • Athero-susceptible region • Susceptible region	• (Iiyama <i>et al.</i>, 1999) • (Davies, 2009) • (Hajra <i>et al.</i>, 2000) • (Kinderlerer <i>et al.</i>, 2008) • (Warboys <i>et al.</i>, 2011) • (Zakkar <i>et al.</i>, 2009)

Figure 2.17 Interchangeable terms to describe the greater and the lesser curvature of the mouse aortic arch.

General outline of procedure for the mouse aortic arch

In this thesis, mice were either left untreated or injected i.p. with 4mg/kg LPS for 4 hours, (serotype 0111:B4, L4391, Sigma) or 4mg/kg or 10mg/kg LPS for 30 minutes or 4.5 hours (serotype, 055:B5, L16529, Sigma), see individual figure legends, before CO₂ asphyxiation. The thoracic cavity was opened up and the aortic arch was then immediately subject to either systemic transcatheter perfusion fixation or *ex vivo* semi perfusion fixation as described below.

Systemic transcardial perfusion fixation procedure

Following CO₂ asphyxiation, the thoracic cavity was immediately opened up and a needle (connected to a 20 ml syringe) was inserted through the left ventricle of the heart and the mouse was systemically perfused with 20ml of phosphate buffered saline (PBS, Sigma D8537) (at room temperature) to flush out all the blood from the aortic arch. Subsequently, the perfusion procedure was repeated with 20ml of 2% formaldehyde (histological fixative from 10% buffered formalin solution that naturally contain ~4% formaldehyde, diluted 1:2 with PBS to create a ~2% solution, Sigma HT501320) to cross link (fix) the proteins in the tissue and thereby stop decay and autolysis. Formaldehyde fixation is based on crosslinking the aldehydes and the proteins creating a gel that should retain the cellular constituents and *in vivo* appearance for accurate histopathologic evaluation.

The manual systemic perfusion was always performed using the same syringe and needle size and at a 'manually sustained' constant pressure, for around 20 seconds per syringe (20 seconds PBS perfusion and 20 seconds formaldehyde perfusion). After ~ 5 minutes of fixation (at room temperature), the heart (including the aortic arch and the thymus protecting the arch), was dissected off in one piece, by holding the trachea and cutting along the back bone of the chest, avoiding stretching of the aortic arch and put into a vial with PBS. The proximal aortic arch was then carefully dissected off the heart/thymus complex at the start of the aortic root and carefully cleared off connective tissue, before transferring the arch as an intact tube to a well with 200µl PBS in a 96-well plate for incubations with reagents and antibodies (Table 2.18-2.20).

Ex vivo semi-perfusion fixation procedure

When the experiment was part of an animal share that did not allow for systemic transcardial perfusion fixation, the *ex vivo semi-perfusion fixation procedure* was performed as outlined. Following CO₂ asphyxiation, and shortly after death, the heart (including aortic arch and thymus) was quickly dissected out and flushed through with 2% formaldehyde using a needle inserted through the heart and placed in a vial with 2% formaldehyde for 10 minutes, then put into a vial with PBS (at room temperature). The proximal aortic arch was then cut from the heart (by the start of the aortic root) and carefully cleared off connective

tissue, before transferring the arch as an intact tube to a well with 200µl PBS in a 96-well plate for incubations with reagents and antibodies (Table 2.18-2.20).

It is important to note that no discernible difference was seen in endothelial or other cell morphology in the aortic arch of mice prepared by either fixation procedure. Nevertheless the nature of the fixation procedure used (systemic transcardial perfusion versus ex vivo semi-perfusion) in each data set is given in the methods section of individual results chapters as well as indicated in the figure legends.

General outline of staining protocol

1. Joint permeabilisation and blocking step (0.1-0.5% Triton X and 20% normal goat serum in PBS) in room temperature (RT) for a few hours or overnight (O/N).
2. 2-3, 5 minutes washes with 200µl of PBS. (Carefully avoiding contact of the tissue with the pipette tip, which can damage the tissue)
3. Primary antibody incubation step (shorter incubations were made in PBS at RT and longer incubations were made in the fridge (4°C) O/N or for ~24 hours).
4. 2-3, 5 minutes washes with 200µl of PBS.
5. Secondary antibody raised against the species that the primary antibody was raised in and conjugated to an Alexa Fluor 568 or 594 fluorophore (this was always a much shorter step than the primary, around 2 hours RT in PBS) During this step and onwards, the 96' well plate was kept in the dark (covered in tinfoil or kept in a cupboard) since fluorophores are light sensitive.
6. 2-3, 5 minutes washes with 200µl of PBS.
7. Primary antibody against the endothelial cell marker CD31, directly conjugated to Alexa Fluor 488. Incubated for a few hours RT or O/N or 24-48 hours in the fridge.
8. 2-3, 5 minutes washes with 200µl of PBS.
9. 4', 6'-diamidino-2-phenylindole (DAPI) nuclear stain (5 minutes incubation in RT in distilled water (dH₂O) since it falls out of solution in PBS).
10. 2-3, 5 minutes washes with 200µl of (dH₂O).

Once a staining protocol proved successful (according to positive and negative controls in the pilot study), the protocol was strictly adhered to for the remaining duration of the study.

Table 2.18 Immunohistochemistry protocols for COX immunoreactivity

<u>Protocol</u>	<u>Incubation</u>	<u>Reagents</u>
Permeabilisation and blocking: PBS with (0.5% or 0.1% Triton X and 20% normal goat serum) Mouse tissue: 0.5% Triton X Rat and human tissue: 0.1% Triton X	Mouse Tissue: (O/N in RT) Rat tissue: (2h RT) Human tissue: (O/N in RT)	PBS (Sigma, D8537x6) Triton X-100, (Sigma T8787) Normal goat serum (Santa Cruz, SC-2043 or Vector labs S-1000)
COX-1 and 2 blocking peptide Mouse tissue: 1:50 dilution in PBS Rat tissue: 1:200 dilution in PBS Human Tissue: 1:250 dilution in PBS	Incubate blocking peptide and primary antibody 1:1 for 1h RT, add mix to the designated wells for incubation as primary	Blocking peptides: from Cayman to block antibody/protein complex COX-2 murine block (360106) COX-1 murine block (360109) COX-1 ovine+ human block (360108)
Primary antibody staining: Mouse tissue: 1:50 dilution in PBS Rat tissue: 1:200 dilution in PBS Human Tissue: 1:250 dilution in PBS	Mouse tissue: (160109, 160126) 4h RT Rat Tissue: (160109, 160126) 2h RT Human Tissue: (160108 and 160126) O/N 4°C	Polyclonal antibodies: raised in rabbit from Cayman: COX-1 anti-mouse and rat (160109) COX-1 anti-ovine + human (160108) COX-2 rabbit anti-mouse, rat, and human (160126)
Secondary antibody staining: Mouse tissue: 1:100 dilution in PBS Rat tissue: 1:100 dilution in PBS Human tissue: 1:500 dilution in PBS	Mouse tissue (2h) RT Rat Tissue (1h RT) Human Tissue (2h RT)	Secondary antibody: Invitrogen Alexa Fluor 594 conjugated goat anti-rabbit (A-11012)
CD31 primary antibody staining: Mouse Tissue (1:100 in PBS) Rat tissue (no CD31 step) Human tissue (1:50 in PBS)	Mouse tissue (O/N at 4°C) Rat Tissue (no CD31 step) Human Tissue (O/N 4°C with primary step)	CD31 primary antibody: Mouse tissue: Alexa Fluor 488 conjugated anti-mouse CD31 (Biolegend, 102514) Human tissue: Alexa Fluor 488 conjugated murine anti-human CD31 (AbD Serotec MCA1738F)
DAPI nuclear stain 25µg/ml in dH ₂ O	Mouse + rat tissue: 5 min RT Human Tissue: 15 min RT	DAPI from Invitrogen (D1106)
Mounting One drop during mounting, top up with more before glass slide, mount and coat edges with nail varnish	O/N RT under ~7lb pressure (3 boxes of gloves)	Hardset mounting medium (Vector Labs, Vectashield H-1400) Cover slips, electrostatic (SC-24976) Glass slides, electrostatic (SC-24975)

Table 2.18 Immunohistochemistry protocols for COX immunoreactivity.

Table 2.19 Immunohistochemistry protocol for NF- κ B immunoreactivity in mice

<u>Protocol</u>	<u>Incubation</u>	<u>Reagents</u>
Permeabilisation and blocking: PBS with (0.5% Triton X and 20% normal goat serum)	O/N in RT	PBS (Sigma, D8537x6) Triton X-100, (Sigma T8787) Normal goat serum (Santa Cruz, SC-2043)
Primary antibody staining: 1:500 dilution in PBS	24h at 4°C	Polyclonal antibody: anti-p65/NF- κ B antibody raised in rabbit (Santa Cruz, SC-372)
Secondary antibody staining: 1:1000 dilution in PBS	2h RT	Secondary antibody: Alexa Fluor 568 conjugated goat anti-rabbit (Invitrogen, A11026)
CD31 primary antibody staining: 1:100 in PBS	Mouse tissue (O/N at 4°C)	CD31 primary antibody: Alexa Fluor 488 conjugated anti-mouse CD31 (Biolegend,102514)
DAPI nuclear stain 25 μ g/ml in dH ₂ O	5 min RT	DAPI from Invitrogen (D1106)
Mounting One drop during mounting, top up with more before glass slide, mount and coat edges with nail varnish	O/N RT under ~7lb pressure (3 boxes of gloves)	Hardset mounting medium (Vector Labs, Vectashield H-1400) Cover slips, electrostatic (SC-24976) Glass slides, electrostatic (SC-24975)

Table 2.19 Immunohistochemistry protocols for NF- κ B immunoreactivity in mice.

Table 2.20 Immunohistochemistry protocol for CD41 immunoreactivity in mice

<u>Protocol</u>	<u>Incubation</u>	<u>Reagents</u>
Permeabilisation and blocking: PBS with (0.1% Triton X and 20% normal goat serum)	2h RT	PBS (Sigma, D8537x6) Triton X-100, (Sigma T8787) Normal goat serum (Vector labs S-1000)
Primary antibody staining: 1:50 dilution in PBS	O/N at 4°C	Polyclonal antibody: rat anti-mouse (AbD serotec, MCA2245GA)
Secondary antibody staining: 1:200 dilution in PBS	2h RT	Secondary antibody: Alexa Fluor 594 conjugated goat anti-rat (Invitrogen, A-11007)
CD31 primary antibody staining: 1:100 in PBS	2h RT	CD31 primary antibody: Alexa Fluor 488 conjugated rat anti-mouse CD31 (Biolegend,102514)
DAPI nuclear stain 25µg/ml in dH ₂ O	5 min RT	DAPI from Invitrogen (D1106)
Mounting One drop during mounting, top up with more before glass slide, mount and coat edges with nail varnish	2h RT under ~7lb pressure (3 boxes of gloves)	Aortic arches: Hardset mounting medium (Vector Labs, H-1400) Blood smears: Aqueous mounting medium (Vector labs, Vectashield H-1000) Cover slips, electrostatic (SC-24976) Glass slides, electrostatic (SC-24975)

Table 2.20 Immunohistochemistry protocols for CD41 immunoreactivity in mice.

Mounting of aortic tissue

After the designated incubation time with antibodies (Table 2.18, 2.19 and 2.20) the mouse, rat or human tissue was mounted on a coverslip slide with a drop of hard set mounting medium and was subject to the following mounting procedure. The slide was visualised under a light dissection microscope and the mouse aortic arch was cut just before the brachiocephalic artery and cut open along the greater curvature, then carefully flipped over with the luminal surface facing down against the coverslip glass (Figure 2.21). The flipping and flattening procedure can cause a lot of damage to the endothelium, but trial and error showed that cutting the arch at an angle from the lesser curvature to the greater curvature, gives a more stable, butterfly-like shape that is less likely to curl up once mounted (Figure 2.21).

More mounting medium was then added to ensure even spread without air bubbles and a cover glass slide with clear nail varnish coated sides was slowly folded on top of the coverslip with the tissue, allowing the mounting medium to spread evenly. The labelled slide was then placed under 3 boxes of gloves (~7lb) and left at RT O/N to ensure that the tissues were sufficiently flattened.

Figure 2.21 Photos and schematics illustrating enface mounting of the mouse aortic arch

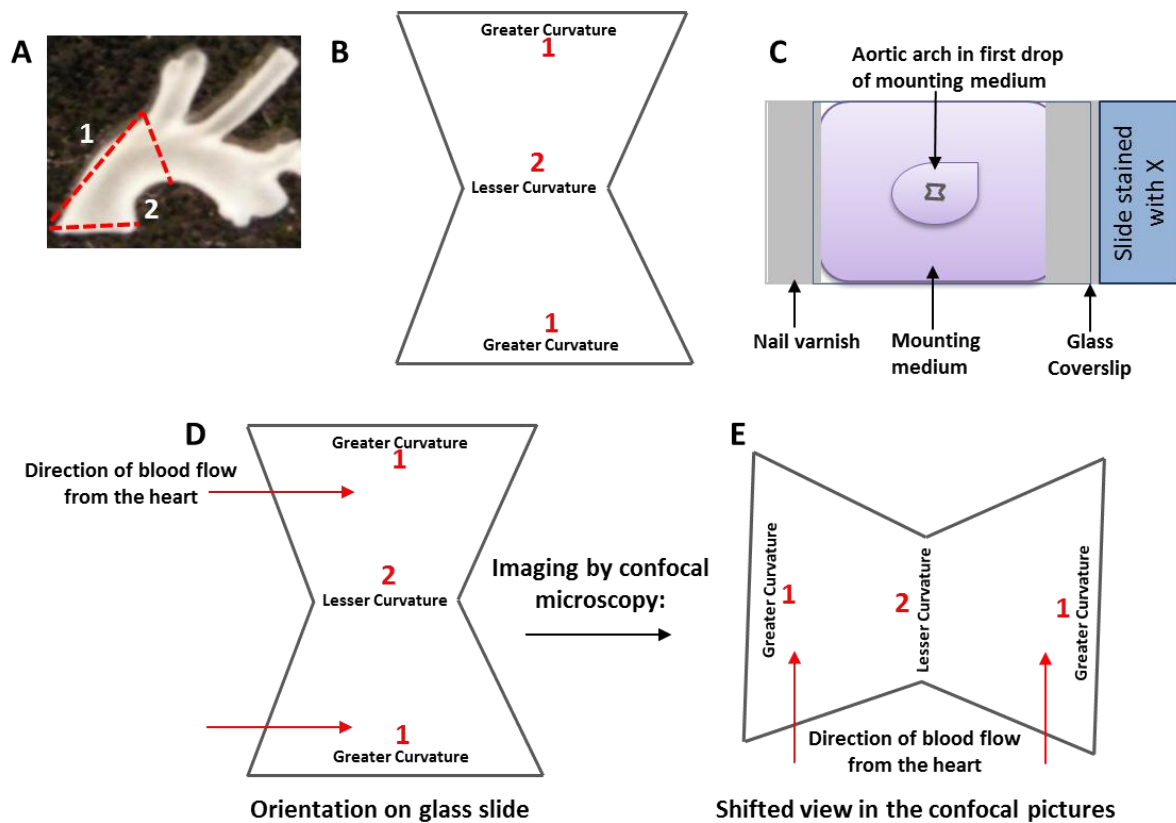


Figure 2.21 Photos and schematics illustrating enface mounting of the mouse aortic arch. (A) Picture of the proximal ascending aortic arch from a wild type (C57BL/6J) mouse. Red dotted line indicates cutting points along the greater curvature (1) and at an angle from the lesser curvature (2) to the greater curvature (1) of the aortic arch. (B) Drawing showing the butterfly shaped aortic arch once cut open and flattened. (C) Schematic of a flattened aortic arch on a glass slide, where the luminal surface (aortic arch endothelium) is towards the lower glass slide. The coverslip was always mounted (with clear nail varnish) close to the title of the slide to ensure that the slide would lay flat on top of the inverted Leica confocal microscope stage. In the confocal imaging, there is a 90° shift to the right of the actual orientation of the aortic arch on the glass slide (D) to (E).

Confocal microscopy

Confocal microscopy; defined as ‘having the same focus’, means that the final image has the same focus as the point of focus in the specimen. The main advantage of confocal microscopy (developed by Marvin Minsky in the 1950’s), compared to conventional wide-field fluorescence microscopy is that out-of-focus signal can be eliminated by the use of point illumination and a confocal pinhole in front of the microscope photo-detector or camera.

A conventional microscope is limited by how deep into the tissue that the light source can penetrate, whereas confocal microscopy has an increased optical resolution, which permits imaging one layer of tissue (depth) at a time. Due to the confocal pinhole (which only allows entry of in-focus signal), the overall brightness (amount of light) coming from the specimen is greatly reduced. To compensate for this, powerful lasers are used (instead of conventional mercury lamps) to produce very bright light at a particular wavelength to excite the fluorophore stained specimen. The fluorescent signal emitted from the specimen is then detected by a highly sensitive photo-multiplier detector (PMT) (set at a specified wavelength range) to detect the reduced (but in-focus) signal through the pinhole. Another feature of the confocal microscope is images showing co-localisation of different fluorophores (collected from different PMT channels).

In my thesis, I have used a Leica SP5 inverted confocal microscope, located at the Imperial Facility for Imaging by Light Microscopy (FILM) facility in Sir Alexander Fleming Building at the South Kensington campus. This microscope is equipped with a diode laser for excitation at 405nm (and a UV light for visualisation in the microscope eye piece), an argon laser for excitation at 488nm and a helium neon laser for excitation at 543 nm.

Confocal settings

Once the confocal settings had been optimised for the fluorescently labelled primary antibody of interest (during the pilot run of each study), they were kept identical and strictly adhered to for the remainder of the study so that fluorescence levels could be compared between experimental runs. This is imperative since, as shown in Figure 2.23, changes in settings such as the gain can greatly alter fluorescence values.

Since relative COX-1 and COX-2 immunoreactivity was compared head to head, in the same experiments, on the same experimental runs, their experimental procedure (including staining protocols) and confocal settings were kept the same, so that levels of immunoreactivity from the two isoforms could be compared relative to one another.

All the confocal images presented in this thesis were taken using an oil based 40x1.25 objective (giving a field of view of 387.5x387.5 μ m) and these following settings were also kept the same: argon laser was set at 15% under the configurations tab, pinhole was set at 1 (airy unit, AU equivalent of 67.9 μ m) for all channels and the line frequency was kept at 400 Hz (higher value means higher time resolution). Invitrogen spectra viewer was used to decide on suitable spectra wavelengths to detect nuclear stain DAPI, endothelial marker CD31 (directly conjugated to Alexa Fluor 488) and Alexa Fluor 594 or 568 conjugated protein of interest (Figure 2.22).

Figure 2.22 Invitrogen spectra viewer showing fluorophore emission wavelengths for DAPI, Alexa Fluor 488 and Alexa Fluor 594/568

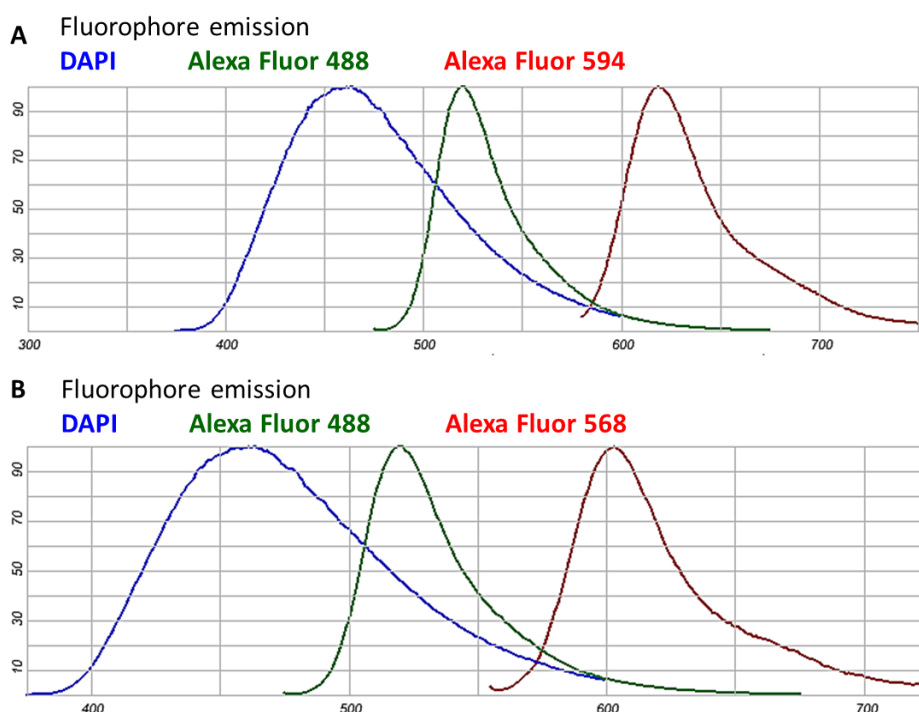


Figure 2.22 Invitrogen spectra viewer showing fluorophore emission wavelengths for DAPI, Alexa Fluor 488 and Alexa Fluor 594/568. Fluorophore emission for DAPI (blue), CD31 as Alexa Fluor 488 (green) and Alexa Fluor conjugated to primary antibody of interest (red) for (A) Alexa Fluor 594 and (B) Alexa Fluor 568.

The gain (brightness enhancement) setting was optimised for each fluorophore channel with the aim to avoid saturation, but to be far above the 'zero' level. The zero level was adjusted by the offset (amount of dark background) to ensure that all labeled tissue was detectable. The positive control (the sample expected to be the brightest) was imaged first to estimate a gain level with a clear positive signal, yet below saturation. Then the secondary control (background, expected to be the darkest) slide was imaged to see how the gain setting chosen for the positive control fits with the background level of signal, with the overall aim to choose a gain setting which covers a wide range between 0 and 100% signal (Figure 2.23).

Figure 2.23 Effects on the different gain settings on the mean fluorescence (signal)

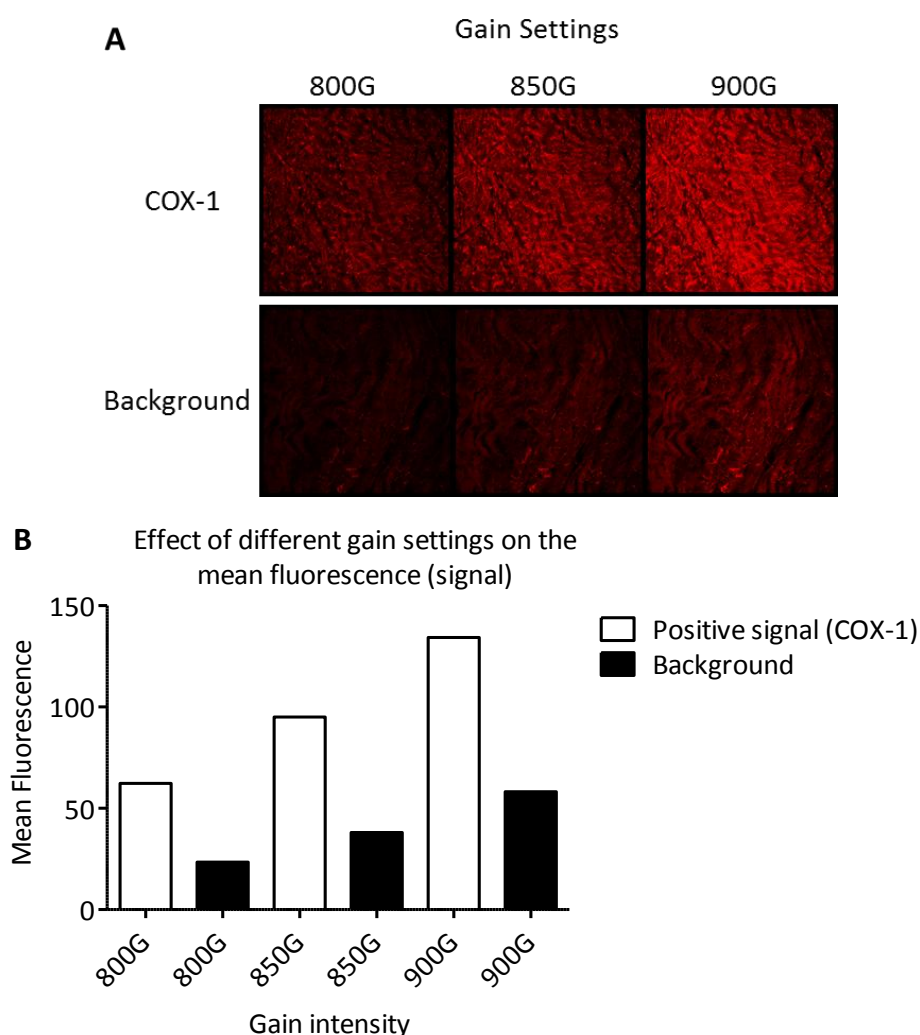


Figure 2.23 Effects on the different gain settings on the mean fluorescence (signal). (A) Representative images of COX-1 immunoreactivity and background (secondary control) below, at 800, 850 and 900 Gain (G). (B) Quantified mean fluorescence at 800, 850 and 900Gain for COX-1 (positive signal) and background n=1.

Specific confocal settings for each experiment are indicated in table 2.24.

Table 2.24 Confocal settings

Study	Lasers	Format
Chapter 3 (3month old) Male WT mice Endothelial morphology study	DAPI: 50% UV 405 laser Detection (420-480nm) CD31: 30% Argon 488 laser Detection: (500-550nm)	Image Size: 203.95x203.95µm 2048x2048 pixels 1.9 zoom, Line average 2 DAPI (PMT1) channel: Gain: 550 Offset: -2% CD31 (PMT2) channel: Gain: 880 Offset: -2%
Chapter 3 (3month old) Male WT mice Chapter 4 (3month old) Female mice, Chapter 4 (12month old) Male WT mice COX-1/COX-2 immunoreactivity studies	DAPI: 15% UV 405 laser Detection (405-458nm) CD31: 15% Argon 488 laser Detection: (493-544nm) COX-1/COX-2 (Alexa Fluor 594): 50% Helium Neon 543 laser Detection: (590-700nm)	Size: 387.5x387.5µm 2048x2048 pixels No zoom, Line accumulation 4 COX-1/COX-2 (PMT4) channel: Gain: 780 Offset: -0.6% DAPI/CD31 (PMT1 and2) channels: Gain and offset settings varied since these channels were not quantified and could therefore be adjusted if needed
Chapter 3 (3month old) mice WT, COX-1 ^{-/-} , COX-2 ^{-/-} mice Chapter 4 (12month old) mice WT, COX-1 ^{-/-} , COX-2 ^{-/-} mice COX-1/COX-2 Immunoreactivity and blocking peptides studies	DAPI: 50% UV 405 laser Detection (420-480nm) CD31: 30% Argon 488 laser Detection: (500-550nm) COX-1/COX-2 (Alexa Fluor 594): 50% Helium Neon 543 laser Detection: (590-700nm)	Size: 387.5x387.5µm 1024x1024 pixels No zoom, Frame average 2 or 3 COX-1/COX-2 (PMT4) channel: Chapter 4: Gain: 700 Offset: -1.1% Chapter 3: Gain: 850 Offset: -1.1% Frame average 3 DAPI/CD31 (PMT1 and2) channels: Gain and offset settings varied
Chapter 3 Human tissue COX-1/COX-2 immunoreactivity study	DAPI: 50% UV 405 laser Detection (420-480nm) CD31: 30% Argon 488 laser Detection: (500-550nm) COX-1/COX-2 (Alexa Fluor 594): 50% Helium Neon 543 laser Detection: (590-700nm)	Size: 387.5x387.5µm 1024x1024 pixels No zoom, Line average 2 COX-1/COX-2 (PMT4) channel: Gain: 850 Offset: -1.0% DAPI/CD31 (PMT1 and2) channels: Gain and offset settings varied

Table 2.24 Confocal settings (continues on the next page).

Table 2.24 (continued) Confocal settings

Study	Lasers	Format
Chapter 4 Rat tissue Heart failure study in rat COX-1/COX-2 immunoreactivity study	DAPI: 50% UV 405 laser Detection (420-480nm) COX-1/COX-2 (Alexa Fluor 594): 50% Helium Neon 543 laser Detection: (590-700nm)	Image size: 387.5x387.5µm 1024x1024 pixels No zoom, Frame average 2 COX-1/COX-2 (PMT4) channel: Gain: 900, Offset: -1.0% DAPI (PMT1) channel: Gain and offset settings varied
Chapter 4 ApoE ^{-/-} mice Lipid fed and normal chow fed COX-1/COX-2 immunoreactivity study	DAPI: 50% UV 405 laser Detection (420-480nm) COX-1/COX-2 (Alexa Fluor 594): 50% Helium Neon 543 laser Detection: (590-700nm)	Size: 387.5x387.5µm 1024x1024 pixels No zoom, Frame average 2 COX-1/COX-2 (PMT4) channel: Gain: 850, Offset: -1.0% DAPI (PMT1) channel: Gain and offset settings varied
Chapter 5 (3month old) mice WT, COX-1 ^{-/-} , COX-2 ^{-/-} mice NF-κB immunoreactivity study	DAPI: 15% UV 405 laser Detection (405-458nm) CD31: 15% Argon 488 laser Detection: (493-544nm) NF-κB/p65 (Alexa Fluor 568): 50% Helium Neon 543 laser Detection: (585-680nm)	Size: 387.5x387.5µm 1024x1024 pixels No zoom, Line accumulation 8 NF-κB/p65 (PMT4) channel: Gain: 900 Offset: -0.6% DAPI/CD31 (PMT1 and2) channels: Gain and offset settings varied
Chapter 5 (3month old) mice WT, COX-1 ^{-/-} , COX-2 ^{-/-} and normal chow fed ApoE ^{-/-} mice CD41 positive platelets study Endothelial morphology study (DAPI/CD31 settings constant)	DAPI: 50% UV 405 laser Detection (420-480nm) CD31: 30% Argon 488 laser Detection: (500-550nm) CD41 (Alexa Fluor 594): 50% Helium Neon 543 laser Detection: (585-680nm)	Size: 387.5x387.5µm 2048x2048 pixels No zoom, Line average 2 CD41 (PMT4) channel: Gain: 700 Offset: -1.0% DAPI (PMT1) channel: Gain: 550 Offset: -2% CD31 (PMT2) channel: Gain: 880 Offset: -2%

Table 2.24 Confocal settings.

Confocal settings during z-profiling

Z-profiles were created by changing the confocal mode from XYZ to XZY setting to enable z-profiling through the entire depth of the tissue (at 7.8 zoom), 50x50µm image size and with a line average 8 to (sharpen the image to make up for a more blurry optical resolution in the XZY than in XYZ mode).

Confocal settings during 'tiling'

Tile composite of 4x4 pictures was created using the automated tile function in the Leica application suite (LAS) confocal program with the same confocal settings as the study the tile was made in (i.e. study of COX-1/COX-2 immunoreactivity in 3 month old and 12 month old mice, Chapter 3 and 4), but with a lower pixel quality (512x512 pixels).

Image selection

As described below, images were selected blinded from target protein by observing only DAPI and CD31 staining down the eyepiece of the confocal microscope, with the red channel showing (COX-1, COX-2, DC41 or NF-κB) immunoreactivity switched off. 2-3 images were taken from separate areas from each region (greater and lesser curvature) of each slide/aortic arch. The area was selected based on anatomy (greater curvature on the edge and lesser curvature running along the middle section of the tissue) and using the characteristic differences in endothelial cell morphology, with nuclear density being the most striking one, as a guild.

As described fully in Chapter 3, the endothelial nuclei in the greater curvature are more evenly spaced out compared to the more crammed in and randomly orientated and often overlapping endothelial nuclei in the lesser curvature. Clear differences in endothelial cell alignment (best visible by CD31 immunoreactivity) can also easily be distinguished between the two regions. CD31 was also important as a marker of intact endothelium as damage (inflicted during the mounting process). Therefore, when selecting areas to image, areas with clear (experimental handling inflicted) damage were avoided and an area with flat, intact endothelium was imaged (where possible). Thus, the captured image was selected based on endothelial integrity visualised under the blue (DAPI) and green (CD31) channels on the microscope (not the red channel showing protein of interest). The red channel with

the relevant red staining (i.e. COX, NF- κ B or CD41) was not seen until the area to image had been chosen (based on endothelial integrity/morphology as defined above) and the picture was captured, for the first time showing the red channel on the screen.

The aim with this procedure was to avoid bias in image selection. Furthermore, the tiling procedure also ensured that when a large area was captured (by an automated tiling function on the mobile microscope stage) it was confirmed that the individual images displayed a fair representation of results in the whole tissue.

General quantification procedure

Since the aim was to image the aortic arch (which is a curved tube structure that has been forced flat), the endothelium was not always flat in the full view of the image (387.5x387.5 μ m), resulting in some images with roughly half of the area in focus with the other half out of focus. In order to avoid out of focus artefacts during the quantification process, a Region Of Interest (ROI) box of ~ half the total image area was manually moved to the area in focus using Leica Application Suite Advanced Fluorescence Lite software program (LAS AF, no licence, Leica Microsystems). During this image handling process, the red PMT4 channel with the protein of interest (i.e. COX-1 or COX-2 or NF- κ B or CD41) was switched off from view to avoid bias and to allow the ROI to be selected only based on the area being in focus with clear DAPI in blue and CD31 endothelial outline in green, and the mean fluorescence immunoreactivity from this particular area was noted down (Figure 2.25). The mean fluorescence was quantified for each image, which represents the total fluorescence divided by the area (either the total area or the area selected for analysis by an ROI box).

Figure 2.25 Examples of selecting an area in focus for quantification

A Example of ~full image in focus: **B** Example of ~half an image in focus:

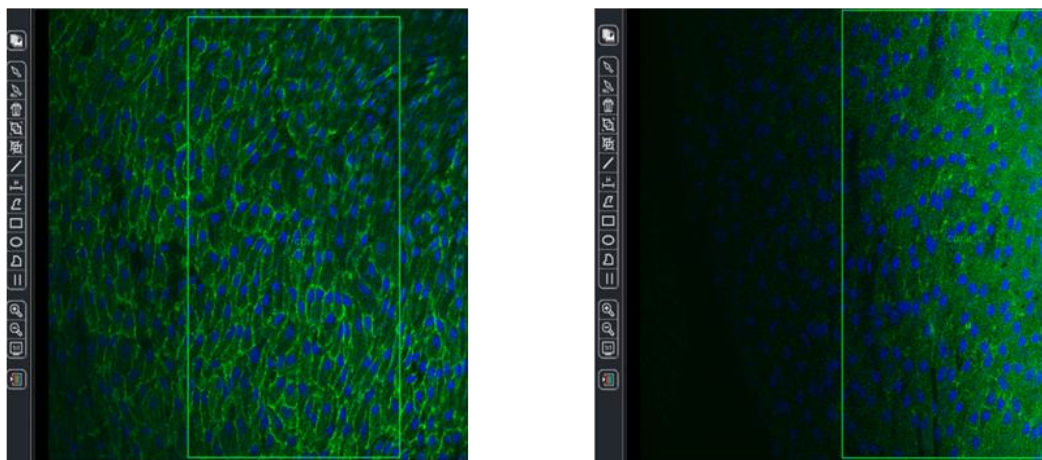


Figure 2.25 Examples of selecting an area in focus for quantification. Half the area of the picture was selected for quantification (of red) based on blue and green in focus, using a region of interest (ROI) box (in green). Original picture size: 2048x2048 pixels (4194304 pixels) (387.5x387.5 μ m) Green region of interest (ROI) box picture size: 2068892 pixels (193 μ m by 378 μ m). The red immunoreactivity in this selected area was quantified as mean fluorescence. (A) Representative image where almost the full image is in focus. The green box was placed in an area with intact endothelium in focus. (B) One of the worst examples of an image where almost half of the image is completely out of focus (which would negatively and inaccurately affect the mean fluorescence). The green ROI box was moved to the endothelial area in focus for quantification.

Subtraction of secondary control (background) mean fluorescence

The average background mean fluorescence from each area of the 'secondary control slide' (tissue stained only with secondary control antibodies) was subtracted from each image before the data was presented in graphs. It was established early in my PhD that there was no discernable difference in secondary control fluorescence values obtained from either region of the arch or indeed from adjacent tissue of the descending aorta. Thus, as my studies progressed I used aortic arch or descending aorta interchangeably as tissue for secondary control baseline data. The secondary control background immunoreactivity was in general very low and diffuse/ non-specific looking.

Data analysis- definition of n-values for the aortic arch imaging studies

Within the mouse aortic arch definable morphology for the lesser and greater curvature regions allows for multiple sampling from the same animal. Thus, n-values were defined (in the first instance) as separate sections/images of a particular region of the arch with n=2-3 data points being gathered from arches of each mouse and with tissue from at least 4 separate animals used for each study. In the pilot study using human tissue, 3 separate images were taken from segments of pulmonary artery from 1 human. In the imaging studies of the rat tissue, the endothelial cell morphology was more homogenous (due to the straighter and more uniform sections) and therefore, n-values were defined as data from individual animals where values of 2-3 images per animal were averaged.

Quantification of NF- κ B immunoreactivity in endothelium of aortic arches from WT, COX-1^{-/-} and COX-2^{-/-} mice

In order to calculate 'non-specific binding' of the secondary antibody and resultant non-NF- κ B associated immunofluorescence, control incubations were performed, using descending aorta from WT mice. Non-specific binding was found to be very low and did not vary much between experiments, nor did it differ significantly in tissue from WT and COX-1^{-/-} or COX-2^{-/-} mice. Specific NF- κ B immunoreactivity was calculated after the values for non-specific binding were subtracted. It should be noted that, due to a technical error, accurate secondary antibody staining data could not be achieved for one of my experimental runs in this study.

For this experiment, background values were assumed from the mean of the full data set (the other experimental runs). Importantly, inclusion of this data set was not critical to the conclusions of the study.

Endothelial NF- κ B immunoreactivity was objectively analysed using Velocity 5 (licensed imaging software) in the following way:

- 1) Total levels of NF- κ B immunoreactivity; total NF- κ B fluorescence minus the total fluorescence in the secondary control (non-specific binding) of the cropped areas.
- 2) Nuclear NF- κ B immunoreactivity; nuclear bodies, imaged as 'independent' DAPI positive objects were quantified in the same cropped images (225x225 μ m). At least 50 separate nuclei were analyzed from each image. Using Velocity 5 software the amount of nuclear NF- κ B immunoreactivity, captured as 'red' co-localized with 'blue' within the selected nuclei, was calculated. An average amount of NF- κ B immunoreactivity per nuclei imaged was then calculated for each image and referred to as 'Nuclear NF- κ B immunoreactivity (average nuclear fluorescence)'.

Quantification of non-nucleated CD41 positive platelet-like bodies in the mouse aortic arch- manual blind scoring procedure

CD41 was used as a platelet marker to stain platelets adhered to the endothelium in aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and ApoE^{-/-} mice. 2 images (387.5x387.5 μ m) were taken from each area (greater and lesser curvature), from each aortic arch. Unlike CD31, CD41 is only expressed on platelets, (not the endothelium or other nucleated cells), hence clear and distinct platelet-like bodies could be identified using CD41 as a marker and were easily quantified by blind manual scoring. In order to fully validate the blind scoring approach, all images were scored by me and my colleague, Ms Claire Potter, and the two sets of results from our manual blind scoring were averaged.

Images were manipulated using the LAS AF software program to zoom in the full sized (387.5x387.5 μ m) images (with all of the channels zoomed in simultaneously), to identify DAPI negative, CD31 positive, CD41 positive distinct platelet-like bodies.

Quantification of non-nucleated CD41 positive platelet-like bodies in the mouse aortic arch- automated quantification using Image J

The quantification of the CD41 positive platelets were then repeated by a more automated method using Image J software. Before analysis, the scale was set to 5.283 pixels/ μm . Full sized images (387.5x387.5 μm) were thresholded using the '*intermodes function*' and adjusted to around 70 (out of 255) on the thresholding scale of brightness and saturation. This was manually adjusted for each image (blinded to the identity of the animal), to a threshold level that included the full area of any CD41 positive signal present in the image, but excluded unspecific background noise. At this setting: '*intermodes function*' (at 70 out of 255), the secondary control images were zero for all measurements. The total number of CD41 positive platelets present in each image was then quantified based on the sum of these thresholded CD41 positive objects that exceeded a size of $1\mu\text{m}^2$. The average size in diameter of these more or less round objects was estimated based on the formula for the area of a circle = $\pi \times \text{radius}^2$, where the average area (μm^2) was divided with π ($\pi=3.14$), and the square root of this result was multiplied with 2 to get an estimate of the diameter of the circle like (roundish) CD41 positive platelets in the aortic arches and in the blood smears.

Quantification of non-nucleated CD41 positive platelet-like bodies in blood smears

The total number and total area taken up by CD41 positive platelets in blood smears from WT, COX-1^{-/-}, COX-2^{-/-} and ApoE^{-/-} mice was estimated from images taken from a consistent location on the blood smear slides. These images were thresholded using the default criteria (Image J) and the selected CD41 positive platelets (1 μm in size to infinity) were included in the analysis.

Quantification of nuclear density and CD31 immunoreactivity in the greater and lesser curvature of the mouse aortic arch

Nuclear area and CD31 immunoreactivity was quantified from two different data sets, in two different studies presented in Chapter 3 and in Chapter 5 of this thesis.

Nuclear density and CD31 immunoreactivity in aortic arches from WT mice (Chapter 3)

This study was performed using images that had been taken with a 1.9 zoom on the confocal microscope to ensure a full area of intact endothelium in focus. In order to estimate the nuclear density in the endothelial layer and in the smooth muscle layer of these images, they were processed using Image J Software. Before analysis, the system was calibrated to the known distance in pixels (physical length divided by the pixel size) to set the scale to how many pixels there are in 1 μ m of the image (i.e. 5.015 pixels/ μ m). Images were then thresholded using the automated Huang criteria function (in Image J), to allow consistent and non-biased determination of a suitable threshold level for each image. The analysis was defined as 25 to infinity and the total area (of DAPI positive, thresholded nuclei, larger than 25 pixels) was analysed.

Nuclear density and CD31 immunoreactivity in aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and chow fed ApoE^{-/-} mice (Chapter 5)

The images from this study came from the CD41 study and were taken without any zoom on the confocal microscope (full size: 387.5x387.5 μ m). The images were processed using Image J software. Before analysis, the scale was set to 5.283 pixels/ μ m. CD31 immunoreactivity was quantified as mean fluorescence in images that were cropped using Image J software (from 387.5x387.5 μ m in size to 200x200 μ m) using a saved ROI box (200x200 μ m, 1059x1059 pixels) that was dragged to an area in focus selected based on endothelial integrity (in an area devoid of damage from handling). This area was then earmarked for cropping and quantification of the DAPI stained nuclei. The DAPI stained nuclei in the cropped ROI image were thresholded using the 'default' criteria in the thresholding function (thresholded images show the thresholded DAPI positive objects in red) and the DAPI positive nuclear density (or total area size in μ m), was calculated based on the total sum of the thresholded objects (exceeding a size of 2 μ m²). The average number of DAPI positive nuclei and average size of these nuclei was estimated based on the same data as the total sum was calculated from and any object with an area less than 2 μ m² was excluded from the analysis.

Measurement of COX activity in vascular tissue (bioassay protocols)

The 'equilibrated study' protocol of COX bioactivity, measured by prostacyclin release detected as the breakdown product 6-keto PGF_{1α} using radioimmunoassay (RIA)

Aortic arches or thoracic aortic segments from WT, COX-1^{-/-} or COX-2^{-/-} mice were dissected out in PBS, cleaned of connective tissues and fat, cut into rings (~2mm length) and placed in plastic wells of a 96-well plate. They were then left to equilibrate in 200μl of Dulbecco's Modified Eagle Medium (DMEM) for 1 hour at 37°C. The medium was then replaced with fresh pre-warmed DMEM or DMEM plus 100μM of the COX-1/COX-2 inhibitor NSAID diclofenac (100μM) for 30 minutes at 37°C. After equilibration, 50μl of sample was removed from each well as baseline measure of prostacyclin release. The calcium ionophore, (A23187, 50μM) was then added to activate PLA₂ in order to facilitate maximal prostacyclin release and the incubations continued for a further 30 minutes. At the end of this time, samples were collected. All samples were stored frozen and -80°C until measurements of the stable prostacyclin breakdown product 6-ketoPGF_{1α} could be made (all the samples were analysed together on completion of the study).

The 'Acute study' protocol of COX bioactivity measured by prostacyclin release using Enzyme Immunoassay (EIA)

Aortic arches or thoracic aortic segments from WT, COX-1^{-/-} or COX-2^{-/-} mice were dissected out, quickly cleaned of connective tissues and fat and cut into rings (~2mm length). These rings were immediately (<5 minutes from time out of the animal) added to wells containing calcium ionophore, (A23187, 50μM in 200μl of DMEM for 30 minutes at 37°C) to activate PLA₂ in order to facilitate maximal prostacyclin release. At the end of this time, samples were collected and stored in -80°C until measurements of the stable prostacyclin breakdown product 6-ketoPGF_{1α}.

In early experiments 6-ketoPGF_{1α} release was measured using the group's in-house radio immunoassay (RIA; see individual figure legends) by Ms Ivana Vojnovic at William Harvey Research Institute. However, for subsequent experiments 6-ketoPGF_{1α} was measured using commercial Enzyme Immunoassay (EIA) kits. In some experiments thromboxane A₂ was also measured (as the breakdown product thromboxane B₂) again using EIA.

Enzyme Immunoassay (EIA) measurement of prostacyclin release detected as the breakdown product 6-keto PGF_{1α} and thromboxane release detected as TXB₂

The EIA kits from Cayman Chemical to detect 6-keto PGF_{1α} (Cayman 515211) and TXB₂ (Cayman 159031) are competitive EIAs. In brief, the basic principle of the assay is based on competition for a limited number of 6-keto PGF_{1α} specific rabbit antibody binding sites, between free 6-keto PGF_{1α} or TXB₂ (sample) and Acetylcholinesterase (AChE) conjugated 6-keto PGF_{1α} or TXB₂ (provided in the kits as tracers). The applied concentration of tracer will be constant whereas the free 6-keto PGF_{1α} or TXB₂ will vary between samples, which means that the higher concentration of free 6-keto PGF_{1α} or TXB₂ in the sample, the less tracer will be able to bind. This is measured by the extent of colour change upon development with Ellman's Reagent (which is the substrate for the AChE) and this enzymatic reaction results in a product that with a distinct yellow colour (which absorbs strongly at 412 nm) that can be determined spectrophotometrically by a plate reader. The more yellow (at 412nm) the samples are, the less free 6-keto PGF_{1α} or TXB₂ do they contain and vice versa. The IC₅₀ (50%B/B₀) of the 6-keto PGF_{1α} EIA is 40pg/ml and the detection limit is 6pg/ml (80%B/B₀) (Figure 2.26A). The IC₅₀ (50%B/B₀) of the TXB₂ EIA is 57pg/ml and the detection limit is 11pg/ml (80%B/B₀) (Figure 2.26B).

Figure 2.26 Representative standard curves from the 6-ketoPGF_{1α} and TXB₂ EIA assays

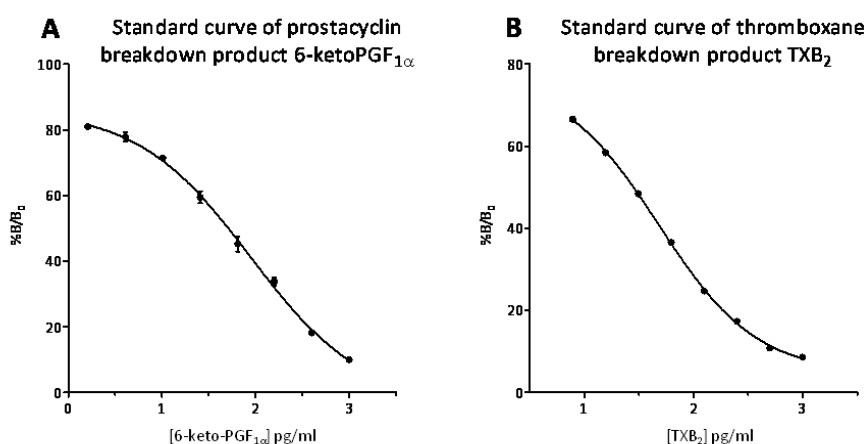


Figure 2.26 Representative standard curves from the 6-ketoPGF_{1α} and TXB₂ EIA assays. %B/B₀ (%Bound/Maximum Bound) is the ratio of the absorbance of a standard (or sample) well (%B) to the maximum amount of tracer that the antibody can bind in the absence of standard (or sample). **(A)** Standard curve for prostacyclin breakdown product 6-ketoPGF_{1α} in the EIA assay (Cayman Chemical, 515211). **(B)** Standard curve for thromboxane breakdown product TXB₂ in the EIA assay (Cayman Chemical, 159031).

PGE₂ measured by Homogeneous Time-Resolved Fluorescence (HTRF) assay

Supernatants from the 'equilibrated' protocol from the rat heart failure study were also assayed for PGE₂ (as a measure of COX-2 induction after the overnight incubation with LPS). These measurements were done by Homogeneous Time-Resolved Fluorescence (HTRF) assay (Cisbo) and performed with the help of my colleague Dr Nicholas Kirkby at the William Harvey Research Institute (see standard curve in Figure 2.27). HTRF is a combination of Fluorescence Resonance Energy Transfer (FRET) and Time-Resolved Fluorescence (TRF). FRET is based on a donor and an acceptor fluorophore and upon excitation, the donor transfers energy to the acceptor, (provided that the acceptor is within a given proximity to the donor), and once this energy reach the donor, light of a certain wavelength is emitted. HTRF combines FRET with TRF in order to reduce background fluorescence by the use of fluorophores with long half-lives (compared to the short lived background fluorescence). The excitation was set to 320nm and emission at 620nm (control, non-bound PGE₂) and 665nm (increased signal at this wavelength if energy is transferred). The long-lived and stable donor fluorophore was Europium cryptate and the acceptor fluorophore was d2. High levels of PGE₂ correspond to a low signal and the results are normalised to a control and are presented as arbitrary units (Figure 2.27).

Figure 2.27 A representative standard curve from the PGE₂ HTRF assay

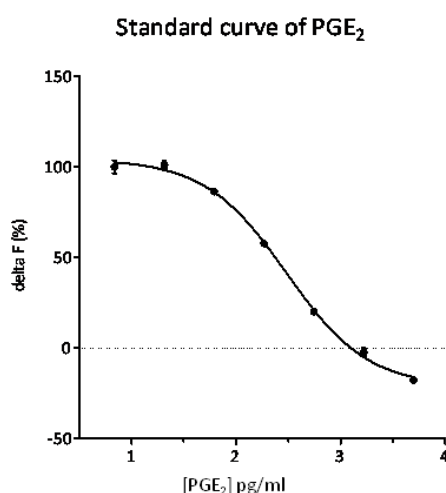


Figure 2.27 A representative standard curve from the PGE₂ HTRF assay. Delta F (%) reflects the signal to background for the assay calculated using HTRF ratios. Due to less energy transfer, higher levels of PGE₂ correspond to lower levels of Delta F (%). Delta F (%) corresponds to arbitrary units and negative values can occur due to normalisation.

Statistics

Results are expressed as the mean $\pm$ Standard error of the mean (S.E.M). Statistical analysis was performed with Graph Pad Prism 5 statistical software, using statistical tests as indicated in individual figure legends. Differences at a probability value of <0.05 was taken as significant and denoted by * and $p>0.05$ was considered non-significant and denoted by ns. For within group analysis of difference in immunoreactivity between the two regions (images from the greater versus lesser curvature of the aortic arch); a paired two-tailed t-test was employed according to the statistical test reported in publications using the same model (Hajra *et al.*, 2000; Iiyama *et al.*, 1999).

For between group analyses an unpaired two tailed t-test was employed if there were only two groups. When there were more than two groups, (i.e. WT versus COX-1^{-/-} or COX-2^{-/-} mice) a One-Way ANalysis Of Variance (ANOVA) was performed with post-tests where relevant (either Bonferroni or Dunnett's post test).

Chapter 3

*Endothelial morphology in the aortic arch:
Examination of the constitutive expression and activity
of COX-1 and COX-2 in healthy endothelium*

Rationale behind Chapter 3

Endothelial cells line the luminal surface of all blood vessels. Their morphology is regulated by, and associated with, different types of physical factors including shear stress and cyclic strain. In areas of unidirectional laminar flow endothelial cells appear aligned whereas in areas of complex or turbulent flow they appear to have a cobblestone appearance (Hajra *et al.*, 2000; Potter *et al.*, 2011). The aortic arch is thought to experience both types of shear stress patterns; with the endothelium in the greater curvature exposed to unidirectional shear stress, whereas the endothelium lining the lesser curvature is exposed to multidirectional, oscillatory shear stress (Hajra *et al.*, 2000; Suo *et al.*, 2007). Importantly, endothelial cells lining the lesser curvature are particularly susceptible to activation and the underlying wall is prone to low grade chronic inflammation and atherosclerosis, whilst the endothelium of the greater curvature is protected (Iiyama *et al.*, 1999; Jongstra-Bilen *et al.*, 2006; Zakkar *et al.*, 2009). This phenomenon has given rise to the hypothesis that the nature of shear stress that endothelial cells are exposed to predefines their physiological and pathophysiological phenotype. However, a detailed analysis of the morphology of the endothelium across these two regions and including the interface portion of the aortic arch and the underlying smooth muscle layer has not previously been conducted.

It is well established that vascular endothelium is enriched in COX and makes prostacyclin as a consequence of COX and prostacyclin synthase co-expression. However, COX isoform expression has routinely been studied in cultured endothelial cells grown under static conditions *in vitro*. These cells may not be representative of endothelial phenotypes present *in vivo*. Furthermore, COX-2 expression has been shown to be regulated by shear stress applied to cells *in vitro* (Topper *et al.*, 1996). Thus, it would seem that the best way to understand COX expression in endothelial cells would be to look at vascular endothelium on vessels *in situ*. However, the relative distribution of COX-1 versus COX-2 immunoreactivity in endothelial cells of different flow-derived phenotypes *in situ* is not known. Moreover, the relative contribution of each isoform to prostacyclin production by different blood vessels has not been addressed.

Thus, in this chapter I have used *en face* confocal microscopy to image the endothelial intracellular junction marker CD31 as well as COX-1 and COX-2 in the endothelium and underlying smooth muscle across the greater and the lesser curvature of the aortic arch from mice or in endothelium of the human pulmonary artery. For some experiments animals were treated with LPS *in vivo* to intentionally increase COX-2 levels in the vasculature. Finally, I have taken aortic arch and thoracic aortic tissue from WT, COX-1^{-/-} and COX-2^{-/-} to measure prostacyclin release under different experimental conditions. The research questions and aims of this chapter can be summarised as follows:

How is the morphology of the endothelium and the underlying smooth muscle defined in the gross area of the mouse aortic arch?

- Study CD31 positive intracellular junctions and DAPI positive nuclear density in both endothelial and smooth muscle layers in different regions of the aortic arch and examine the depth of the endothelial layer through z-profiling.

Which COX isoform predominates in vascular endothelium and how does this relate to endothelial cell morphology?

- Investigate the normal distribution of COX-1 and COX-2 in endothelial cells lining the atherosclerosis-protected greater curvature and the atherosclerosis-prone lesser curvature of the mouse aortic arch from young (3 month old) healthy male WT mice, using immunohistochemistry and *en face* confocal microscopy.
- Repeat protocol in a pilot study using human pulmonary artery.
- Perform validation experiments:
 - Positive control (in vivo injection i.p. with endotoxin/LPS to upregulate COX-2).
 - Negative control (background immunofluorescence from secondary control).
 - Specificity of COX antibodies by the use of specific blocking peptides.
 - Examine COX-1 and COX-2 immunoreactivity in COX-1 and COX-2^{-/-} mice.

Which COX isoform drives endothelial prostacyclin production in healthy blood vessels?

- Establish the functional relevance of COX-1 versus COX-2 in the aorta in terms of prostacyclin releasing capacity by using tissue from young (3 month old) healthy WT versus COX-1^{-/-} and COX-2^{-/-} mice.

Methods

Animals

WT, COX-1^{-/-} and COX-2^{-/-} mice were bred, maintained and genotyped as described in the General Methods Chapter 2. For this chapter, animals were male and female (but predominantly male) and used at 10-12 weeks (3 month) of age (considered as young adult mice). In some experiments mice were injected with LPS (E.coli serotype 0111:B4, 4mg/kg i.p. for 4 hours) or (E.coli serotype 055:B5, 10mg/kg i.p. for 4.5 hours) see individual figure legends. All WT mice were from the C57BL/6J strain; some WT were purchased from Harlan, UK, whilst colony WT were bred in-house with a shared background to match the COX-1^{-/-} and COX-2^{-/-} mice (backcrossed to C57BL/6J, Harlan, UK, more than 7 times) see General Methods Chapter 2. Purchased WT mice will be referred to as 'C57BL/6J Harlan, UK' and the other COX colony WT controls will be referred to as 'C57BL/6J bred in-house'. Animals were killed by CO₂ asphyxiation and aortic arches were dissected out and fixed with 2% formaldehyde according to the '*ex vivo semi-perfusion fixation procedure*' or the '*transcardial perfusion fixation procedure*' and staining protocols described in the General Methods Chapter 2 and as specified in the figure legends.

Human tissue

This chapter includes data from a pilot study of COX immunoreactivity in human pulmonary artery endothelium (from one donor). The pulmonary artery was dissected out from lungs from an American tissue bank (International Institute for Advancement of Medicine) with confirmed consent from the donor and under the Royal Brompton hospital ethics regulations (ethics number 09/H0708/72). The donor was a 67 year old female Caucasian who died from a cerebrovascular accident (intracerebral haemorrhage). For more details about the past medical history of this donor and staining protocols for this tissue, see General Methods Chapter 2.

En Face imaging of arch lumen

In this chapter I have imaged CD31, COX-1 and COX-2 using antibodies and the stain DAPI to visualise nuclei as described in the General Methods Chapter 2 (Table 2.18). In order to investigate the relative COX-1 and COX-2 isoform expression in the endothelium, tissues were subject to the same staining protocol, with or without the addition of blocking peptides to the antigen that the COX primary antibodies were raised against and imaged head to head by confocal microscopy (Leica SP5 inverted confocal microscope, 40x oil objective), with the same confocal settings maintained throughout the study. Immunoreactivity was quantified as mean fluorescence using LAS AF software and Image J software was used to calculate nuclei area as described in General Methods Chapter 2.

Measurement of COX activity

COX activity was measured in two protocols; the first '*equilibrated study*' included a lengthy equilibration step. For validation purposes, the study was repeated without the equilibration step, referred to as the '*acute study*'. In both cases, aortic arch or thoracic aortic rings were removed from animals and incubated *in vitro* with the calcium ionophore, A23187 (50 μ M) with or without diclofenac (100 μ M), for 30 minutes. Prostacyclin release from the tissue was measured as the stable breakdown product 6-keto PGF_{1 α} by RIA or EIA as described in the General Methods Chapter 2.

Analysis and statistics

Data is presented as the mean $\pm$ S.E.M and was analysed using Graph Pad Prism 5 using statistical tests described in the figure legends. A p value of <0.05 was taken as significant and denoted by * and p>0.05 was considered non-significant and denoted by ns.

Results

Morphology of the luminal surface of the mouse aortic arch

The morphology of endothelial cells lining the luminal surface of the greater and the lesser curvature of the aortic arch were found to be clearly different, with ‘aligned’ versus ‘cobblestone’ morphology enriched in the greater curvature and lesser curvature respectively (Figure 3.1C+D). The greater curvature was enriched in endothelial cells with elongated nuclei that were evenly spread out and appeared aligned with the unidirectional laminar shear stress (Figure 3.1E). In contrast, the nuclei of the endothelial cells lining the lesser curvature had a polygonal shape and were randomly orientated with cobblestone morphology (Figure 3.1F). Figure 3.1D show that endothelial cells in the ‘interface region’ are of mixed morphology. Endothelial cells from all regions of the aortic arch stained positive for the endothelial marker CD31 (Figure 3.1C+D+E+F, shown in green).

DAPI positive nuclear density

A key and striking observation of this tiled image of the luminal surface of the arch is how ‘nuclear dense’ the cells of the lesser curvature appear (shown on the tile as blue DAPI staining, Figure 3.1C+G). Indeed, quantification of these images showed a graded increase in the total area of DAPI positive nuclei from the greater curvature to the lesser curvature (Figure 3.1 D+E). The increased nuclear density in the lesser curvature was also evident in images from a separate study (Figure 3.2A+B I, III and IV) and in the quantified pooled data (Figure 3.3A).

CD31 immunoreactivity

CD31 immunoreactivity however, declined proportionally (in comparison to DAPI staining) across the tile from the greater to the lesser curvature region (Figure 3.1D+H). This observation was again evident in images from a separate study (Figure 3.2A+B II) and in the quantified pooled data (Figure 3.4A). The expression pattern of the CD31 immunoreactivity appeared more abundantly expressed throughout the cytoplasm of the endothelial cells in the greater curvature and more localised to intracellular junctions in the lesser curvature (Figure 3.1C+D+E+F and 3.2A+B).

Images of the endothelial cell layer z-plane and the directly underlying vascular smooth muscle layer z-plane in the greater and lesser curvature of the mouse aortic arch

In separate experiments, images were taken from defined Z-planes (confocal microscopy movement through the tissue) in the greater and lesser curvature of the aortic arch (Figure 3.2). The first (manually adjusted) z-plane captured images and fluorescence data from the superficial endothelial layer (visually confirmed by characteristic endothelial like CD31 immunoreactivity and DAPI stained nuclei) (Figure 3.2). The second z-plane (deeper down through the tissue) captured images and fluorescence data from the directly underlying smooth muscle layer (visually confirmed by the absent CD31 immunoreactivity and by smooth muscle like DAPI stained nuclei) (Figure 3.2).

As seen in the tiled image (Figure 3.1), the endothelial layer showed positive CD31 staining and the typical aligned versus cobblestone morphology in the greater versus lesser curvature regions (Figure 3.2A+B I+II+III). CD31 immunoreactivity was to a large extent lost in the Z-plane of the vascular smooth muscle (Figure 3.2A+B and Figure 3.4B), but DAPI nuclear staining of the smooth muscle cells was clearly visible (Figure 3.2A+B). DAPI stained smooth muscle nuclei in the greater curvature appeared aligned, whereas DAPI stained smooth muscle nuclei in the lesser curvature displayed multi directional alignment similar to the overlying endothelial layer (Figure 3.2A+B I, III and IV). However, in contrast to the endothelial layer, the total area of nuclear staining (nuclear density) in the smooth muscle layer was not significantly different in the lesser curvature versus the greater curvature (Figure 3.3B).

Measurement of endothelial cell layer depth

Z-plane images were taken to measure the depth/thickness of the endothelial layer at the lesser and greater curvature regions of the aortic arch (Figure 3.5). There was a trend for the CD31 positive endothelial layer to be deeper in the greater curvature than in the lesser curvature region of the aortic arch but this did not reach statistical significance (Figure 3.5C).

COX-1 and COX-2 immunoreactivity in the endothelium of the aortic arch in untreated and LPS treated conditions

COX-1 immunoreactivity in the endothelial layer was readily detectable, obvious in every cell and expressed in similar levels in the greater and lesser curvature regions (Figure 3.6). The intracellular staining pattern of the COX-1 immunoreactivity appeared diffused cytoplasmic and intense perinuclear (Figure 3.6E). By contrast, COX-2 immunoreactivity in the endothelial layer was comparatively very low (Figure 3.7). Where it was detected, COX-2 immunoreactivity appeared as punctate staining (Figure 3.7E). Pooled immunoreactivity data confirmed that COX-1 immunoreactivity predominates over COX-2 immunoreactivity in both regions of the aortic arch (Figure 3.8A+B+C).

In tissue of untreated mice, the COX-2 immunoreactivity was very low, but modestly enriched in the lesser curvature of the aortic arch (Figure 3.9A+C+D). As expected, COX-2 expression was increased by LPS treatment *in vivo* (4mg/kg i.p. for 4 hours), which reached statistical significance in endothelial cells of the lesser curvature region of the arch (Figure 3.9B+C+D). The LPS induced COX-2 immunoreactivity was also significantly higher in the lesser curvature of the arch (Figure 3.9B+C+D). COX-1 immunoreactivity was highly abundant in every area of the endothelium of the aortic arch and there was no significant difference between the greater and the lesser curvature in untreated animals (Figure 3.10A+C+D). However, even though COX-1 is not an 'inducible' gene in the same way that COX-2 is, it was surprising to note that COX-1 immunoreactivity was also increased in the endothelium of the lesser curvature region of the arch from mice treated with LPS (4mg/kg i.p. for 4 hours) (Figure 3.10B+C+D). This COX-1 immunoreactivity was in contrast to untreated conditions, significantly higher in the lesser curvature.

COX-1 and COX-2 immunoreactivity in the endothelial versus smooth muscle layer

In another study, I imaged COX-1 and COX-2 again in the endothelial layer of the mid arch and descending aorta of the aortic arch in tissue from untreated animals, before changing the focus of the z-plane to the directly underlying smooth muscle layer, where I imaged COX-1 and COX-2 again (Figure 3.11). It was clear that COX immunoreactivity (in particular COX-1 immunoreactivity) was highly enriched in the endothelial layer with much less expression in the underlying vascular smooth muscle layer (Figure 3.11A-C).

Pilot study of COX-1 and COX-2 immunoreactivity in human pulmonary artery endothelium

Using the same protocol I had developed for mouse tissue I was able to fix, mount and image endothelium *in situ* from sections of a human pulmonary artery (Figure 3.12). Endothelial cells of the human vessel stained positive for CD31 and appeared aligned in their morphology (Figure 3.12A). COX-1 immunoreactivity was abundant in almost every CD31 positive endothelial cell in 3 separate images (Figure 3.12 C). COX-2 immunoreactivity was also detected, but as with the mouse tissue, it was much lower in intensity than seen for COX-1 (Figure 3.12 C+D), but was confirmed to be specific as it was reduced by the addition of COX-2 blocking peptide with only low levels of punctate immunoreactivity remaining (Figure 3.12D+E).

Overall, this pilot study showed a similar pattern in the human vessel as in the mouse vessels regarding abundant COX-1 immunoreactivity compared to sparse COX-2 immunoreactivity (Figure 3.12F). This human pulmonary artery tissue was also shown to release prostacyclin upon stimulation with the NOD-1 agonist C12-iE DAP or LPS in a bioassay performed by my colleague Dr Timothy Gatheral (basal: 15.0 ± 3.7 ng/ml, stimulated with C12-iE DAP: 127.7 ± 61.2 ng/ml or stimulated with LPS 55.1 ± 25.3 ng/ml).

Validation studies for COX-1 and COX-2 immunoreactivity; non-specific background and blocking peptides for COX-1 and COX-2

The COX-1 and COX-2 antibodies used in this thesis are well validated (General Methods, Chapter 2). However, it was important to conduct validation experiments to be able to interpret our data with confidence. Background immunofluorescence was low or undetectable, and did not differ between regions of the arch/aorta or in experiments with or without LPS (4mg/kg i.p. for 4 hours), in parallel incubations where only secondary antibody was used ('secondary controls') (Figure 3.13A+B+C).

Please note that for data shown with primary antibodies 'secondary control' immunofluorescence values obtained in parallel incubations were subtracted before the data was pooled and presented in graphs of immunoreactivity. In separate experiments, specific blocking peptides to the precise epitope (antigenic determinant) of COX-1 used to raise our antibodies, greatly reduced immunoreactivity to COX-1 in endothelium of

untreated WT mice or those treated with LPS (10mg/kg i.p. for 4.5 hours), (Figure 3.14). Similarly, blocking peptides to COX-2 inhibited COX-2 immunoreactivity in the endothelium of mice treated with LPS (Figure 3.15). For these experiments, the greater curvature was compared to the descending aorta from the same animal since these areas display similar endothelial phenotypes affected by laminar unidirectional shear stress.

COX-1 and COX-2 immunoreactivity in endothelium of the aortic arch from mice with COX-1 or COX-2 gene deletion

In my thesis I have used colonies of mice where COX-1 or COX-2 genes have been disrupted. These modifications result in mice with no function of COX-1 or COX-2 enzyme activity respectively, but may (theoretically) transcribe truncated, yet inactive, forms of the protein (Langenbach *et al.*, 1995; Morham *et al.*, 1995) (See General Methods Chapter 2).

COX-1 immunoreactivity in COX-1^{-/-} mice

The antibodies used for COX-1 were raised to a sequence in the middle of the COX-1 gene, prior to the target site for the genetic disruption (General Methods, Chapter 2), and therefore this region would potentially be preserved if a truncated COX-1 protein was synthesised and survived. This may explain why COX-1 immunoreactivity was detectable in aortic endothelium of COX1^{-/-} mice (Figure 3.16 A+B), but when quantified the levels were reduced compared to WT mice (Figure 3.16 C). This COX-1 immunoreactivity was blocked by the addition of COX-1 blocking peptide (Figure 3.17 A+B).

COX-2 immunoreactivity in COX-2^{-/-} mice

The COX-2 antibody was raised to a sequence at the end of the COX-2 gene (in exon 10), which was truncated in the targeted gene (General Methods, Chapter 2). Hence, if a truncated COX-2 protein was synthesised, the antibody used in this thesis is unlikely to recognise it. Figure 3.18 shows that the COX-2 immunoreactivity in tissue from LPS treated COX-2^{-/-} mice were significantly reduced and lacked the COX-2 staining pattern seen in the WT and the COX-1^{-/-} mice (Figure 3.18A+B+C).

COX-1 immunoreactivity in COX-2^{-/-} mice and COX-2 immunoreactivity in COX-1^{-/-} mice

I was able to establish that COX genes were not induced in compensation for each other in specific knockout animals. Thus, COX-1 immunoreactivity was not altered in endothelium of COX-2^{-/-} mice (Figure 3.16 D) and COX-2 immunoreactivity was not altered in the endothelium of COX-1^{-/-} mice (Figure 3.18 A+B+D) compared to the levels detected in WT mice.

Effect of COX-1 and COX-2 gene deletion of prostacyclin release from aortic arches and thoracic aorta

The immunohistochemical experiments presented so far suggest that COX-1 predominates over COX-2 in the endothelium of freshly fixed blood vessels. However, immunoreactivity does not necessary reflect biological activity. In order to further understand the relative role of COX-1 and COX-2 in prostacyclin release from large healthy blood vessels, bioassay experiments were performed. These experiments were performed in two ways. In the first set of experiments, vascular tissue was washed and allowed to equilibrate before being stimulated to release prostacyclin (Figure 3.19; '*equilibrated study*'). However, it has been debated that COX-2 protein may be very unstable with rapid offset kinetics from its shear-induced gene expression (Funk *et al.*, 2007; Grosser *et al.*, 2006), and considering that the blood vessels (in the equilibrated study, Figure 3.19) were equilibrated outside the animal for 1.5 hours in total before being stimulated *in vitro*, this may have affected the results. Therefore, for validation purposes, the protocol was repeated a second time where vascular tissue was immediately (<5 minutes) placed in media containing the calcium ionophore stimuli, (A23187) and the resultant prostacyclin release was measured after 30 minutes (Figure 3.20; '*acute study*').

(Figure 3.19); 'equilibrated study'

After equilibration, segments of thoracic aorta from WT mice released detectable levels of prostacyclin when stimulated with 50μM of the calcium ionophore A23187 (Figure 3.19B). This was completely inhibited by the COX-1 and COX-2 inhibitor (NSAID) diclofenac (Figure 3.19B). By contrast thoracic aorta from COX-1^{-/-} mice released minimal levels of prostacyclin in the presence or absence of A23187 (Figure 3.19B). Thoracic aorta from COX-2^{-/-} mice however, released detectable levels of prostacyclin that were not different to those

measured from tissue of WT animals (Figure 3.19B). In order to directly compare our prostacyclin release with the COX immunohistochemical data presented in this thesis, prostacyclin release experiments were repeated using aortic arch tissue in place of thoracic aorta (Figure 3.19A). As seen in aortic segments, prostacyclin was released by aortic arches stimulated with A23187 from WT and COX-2^{-/-} mice, but only very low levels were released from tissue of COX-1^{-/-} mice (Figure 3.19A).

(Figure 3.20 and 3.21); *'acute study'*

Similar results were obtained when the study was repeated and aortic arch and thoracic aortic tissue was immediately stimulated in the *'acute study'*, without the postmortem delay in the *'equilibrated study'* (Figure 3.20A+B). Using this protocol I performed a pilot study (n=2) to investigate (i) how COX deletion affected thromboxane release by blood vessels and (ii) what effect LPS treatment *in vivo* had on a prostacyclin and thromboxane release. LPS treatment had no discernable effect on the ability of aortic tissue from any genotype of mouse to release prostacyclin or thromboxane (Figure 3.21). As expected, thromboxane release was very low in aortic tissue, and again, as with prostacyclin, the levels of release were not affected by LPS. It was interesting to note that the low levels of thromboxane release detected were reduced in tissue from COX-1^{-/-}, but not COX-2^{-/-} mice (Figure 3.21).

Figures

Figure 3.1 Tile composite of 4x4 images showing the morphology of the luminal surface of the proximal aortic arch of a WT mouse

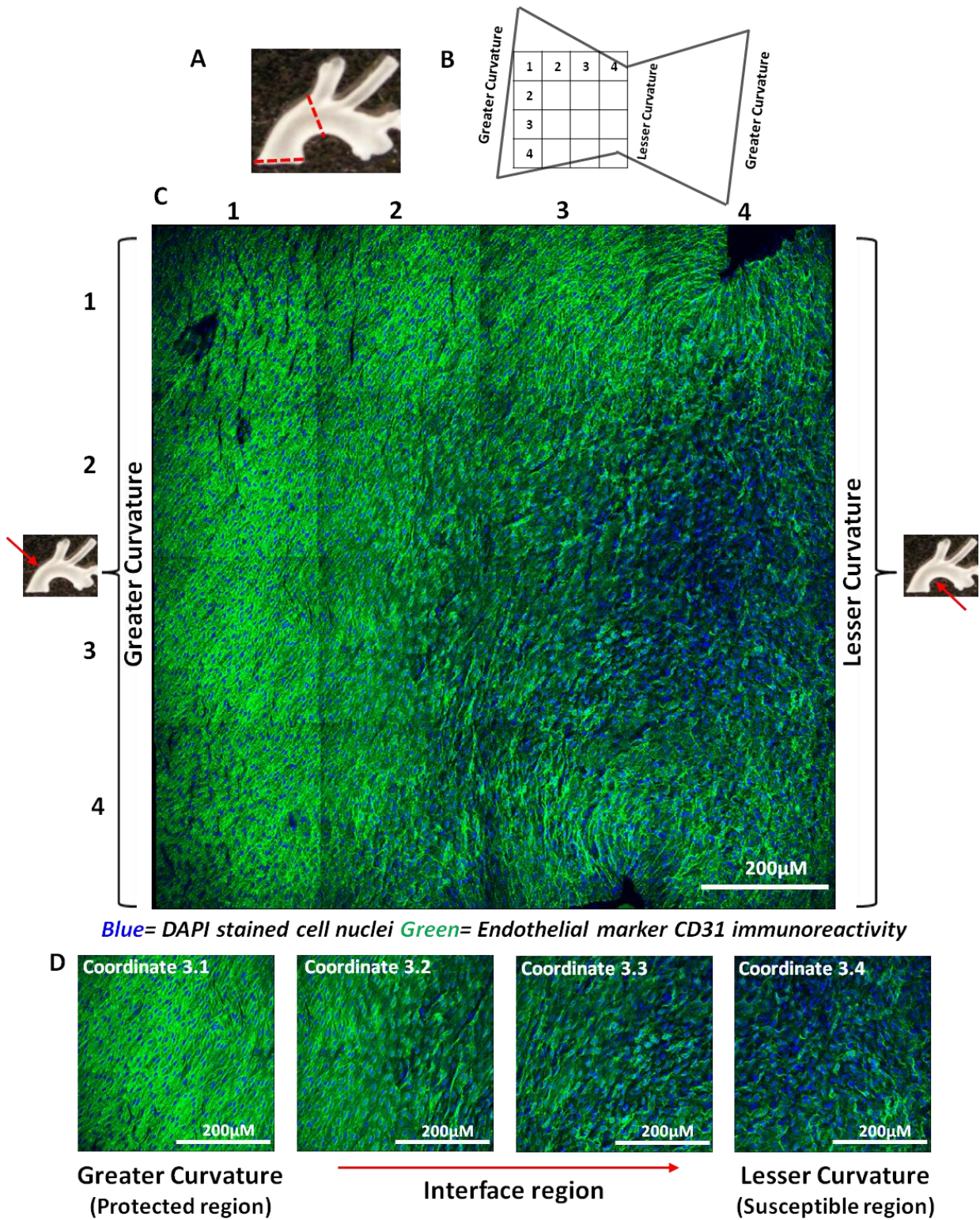


Figure 3.1 continues on the next page.

Figure 3.1 (Continued) Tile composite of 4x4 images showing the morphology of the luminal surface of the proximal aortic arch of a WT mouse

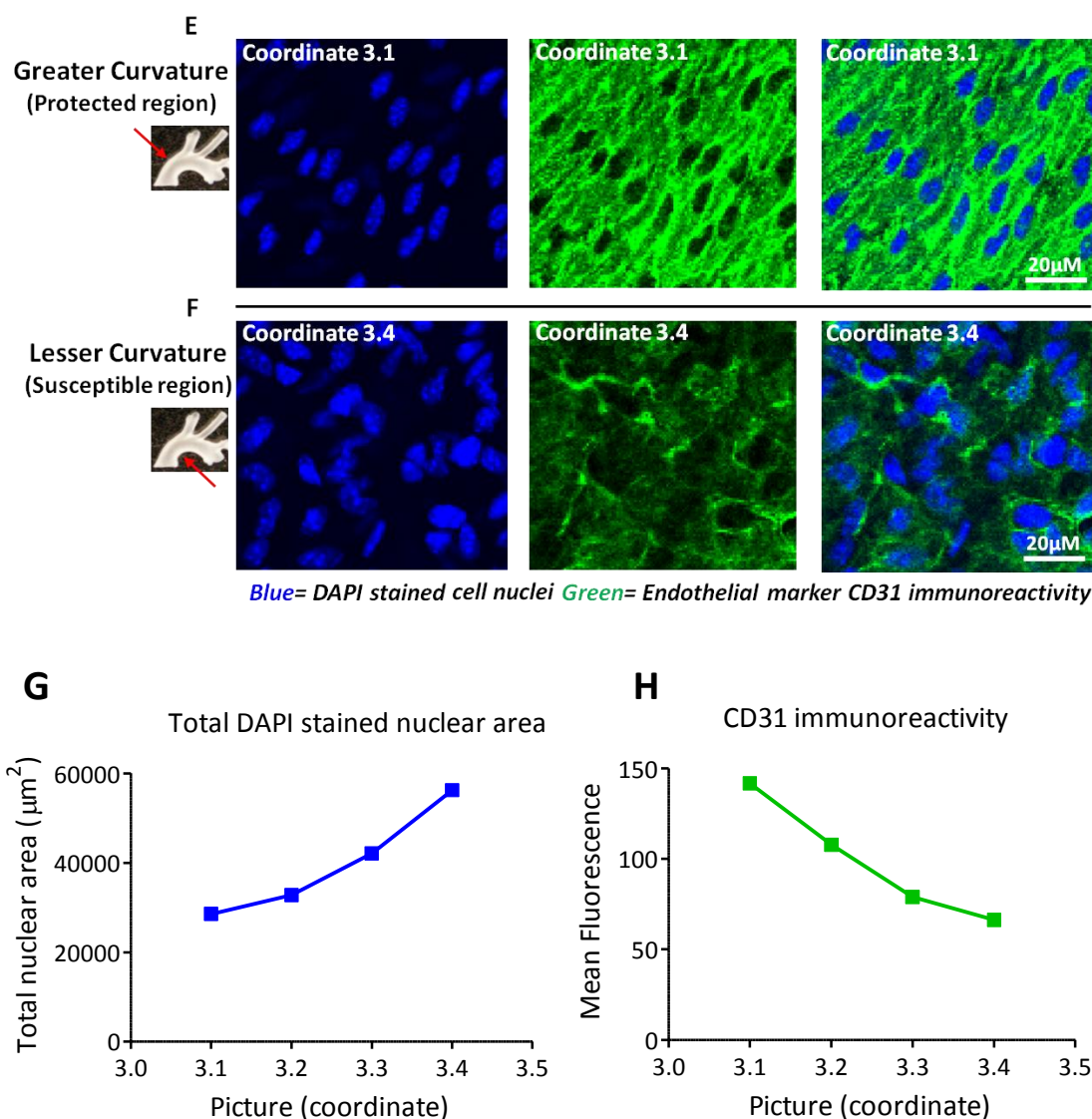


Figure 3.1 Tile composite of 4x4 images showing the morphology of the luminal surface of the aortic arch of a mouse. An aortic arch fixed according to the ex vivo semi-perfusion fixation procedure was dissected from a young (3 month old) female WT mouse (C57BL/6J, bred in-house) (A) Red dotted line, mark dissection cutting points. (B) A schematic of the mounted (opened and flattened) aortic arch, marking the location of the lesser and the greater curvature as well as the position of the tile composite (4x4 images, coordinates 1.1-4.4). (C) The entire tile of 4x4 images was captured using the automated tiling function on a Leica SP5 inverted confocal microscope. Nuclei visualised with DAPI (blue) and endothelial cells outlined with the endothelial cell marker CD31 (green). (D) Selected images from the tile, (coordinate 3.1 to 3.4) showing typical shear responsive phenotypic differences in endothelial cell morphology in the greater curvature, interface region and the lesser curvature respectively. Zoomed in images from (E) greater curvature: coordinate 3.1 and (F) lesser curvature: coordinate 3.4. (G) Total DAPI stained nuclear area (μm²) in each of the selected images in (D) (3.1-3.4). (H) CD31 immunoreactivity measured in each of the selected images in (D) (3.1-3.4) (Image size 387.5x387.5μm).

Figure 3.2 Images of the endothelial layer z-plane and the directly underlying vascular smooth muscle layer z-plane in the greater and the lesser curvature of the mouse aortic arch

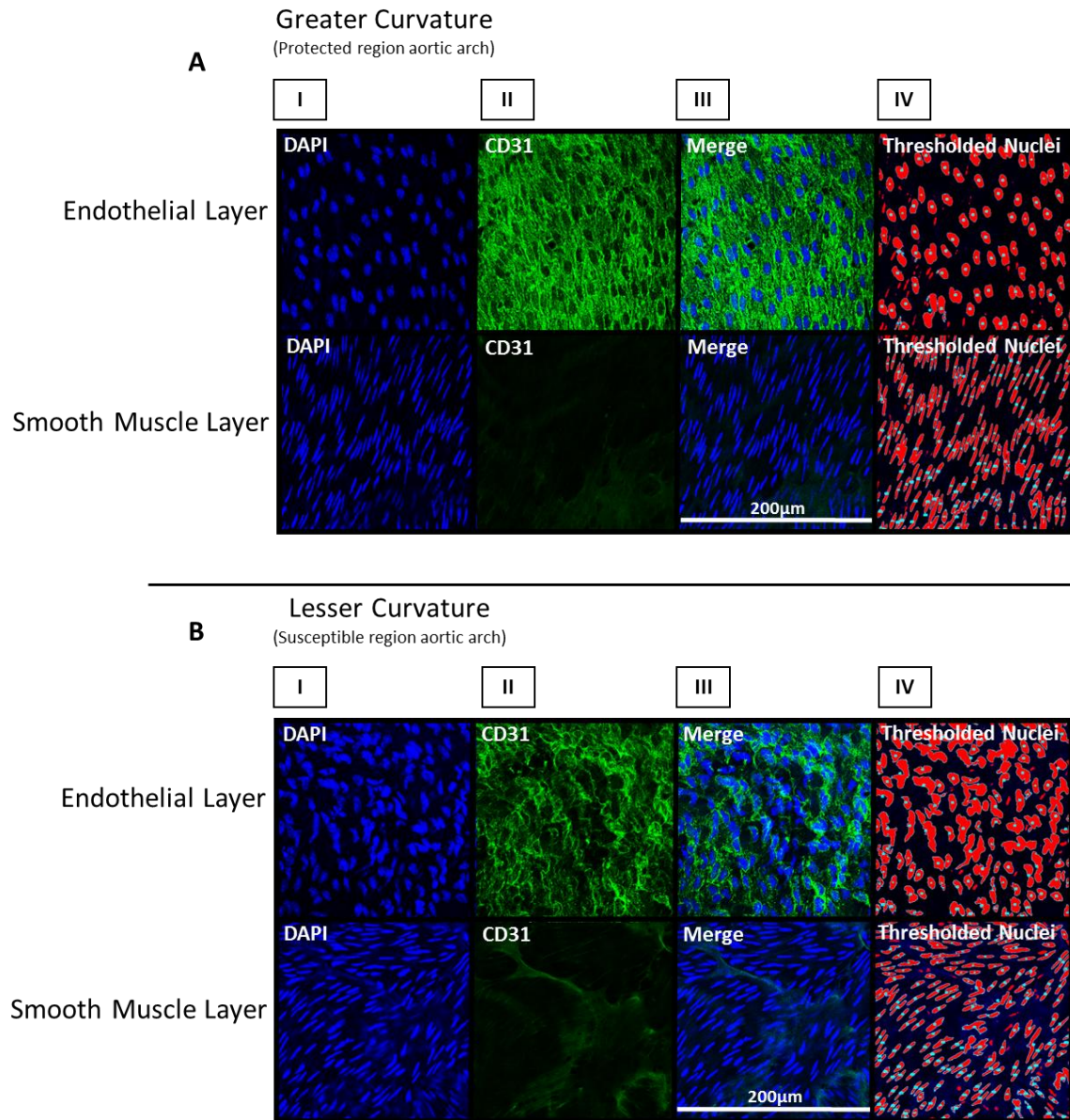


Figure 3.2 Images of the endothelial layer z-plane and the directly underlying vascular smooth muscle layer z-plane in the greater and lesser curvature of the mouse aortic arch. Morphology of the endothelial layer and directly underlying smooth muscle layer of the **(A)** greater curvature and **(B)** lesser curvature regions of the aortic arch, fixed according to the transcatheter perfusion fixation procedure. Pictures taken with a 40x objective and 1.9 zoom (image size 203.95x203.95µm). Images are representative from n=4 young (3 month old) male WT mice (C57BL/6J, bred in-house). **(I)** DAPI stained nuclei in the endothelial and underlying smooth muscle layers of both regions (**blue**). **(II)** CD31 immunoreactivity in both layers of both regions (**green**). **(III)** CD31 and DAPI images are merged with blue and green both visible. **(IV)** DAPI positive nuclei in the endothelial and the smooth muscle layers were highlighted by thresholding using the Huang criteria in Image J, and these defined DAPI positive 'objects' (endothelial and smooth muscle cell nuclei) (**red**) were selected for quantification of total nuclear area in the endothelial and smooth muscle layers of the greater and lesser curvature of the aortic arch.

Figure 3.3 Quantified total nuclear area of DAPI stained nuclei in the endothelial and smooth muscle cell layers in the greater and the lesser curvature of the mouse aortic arch

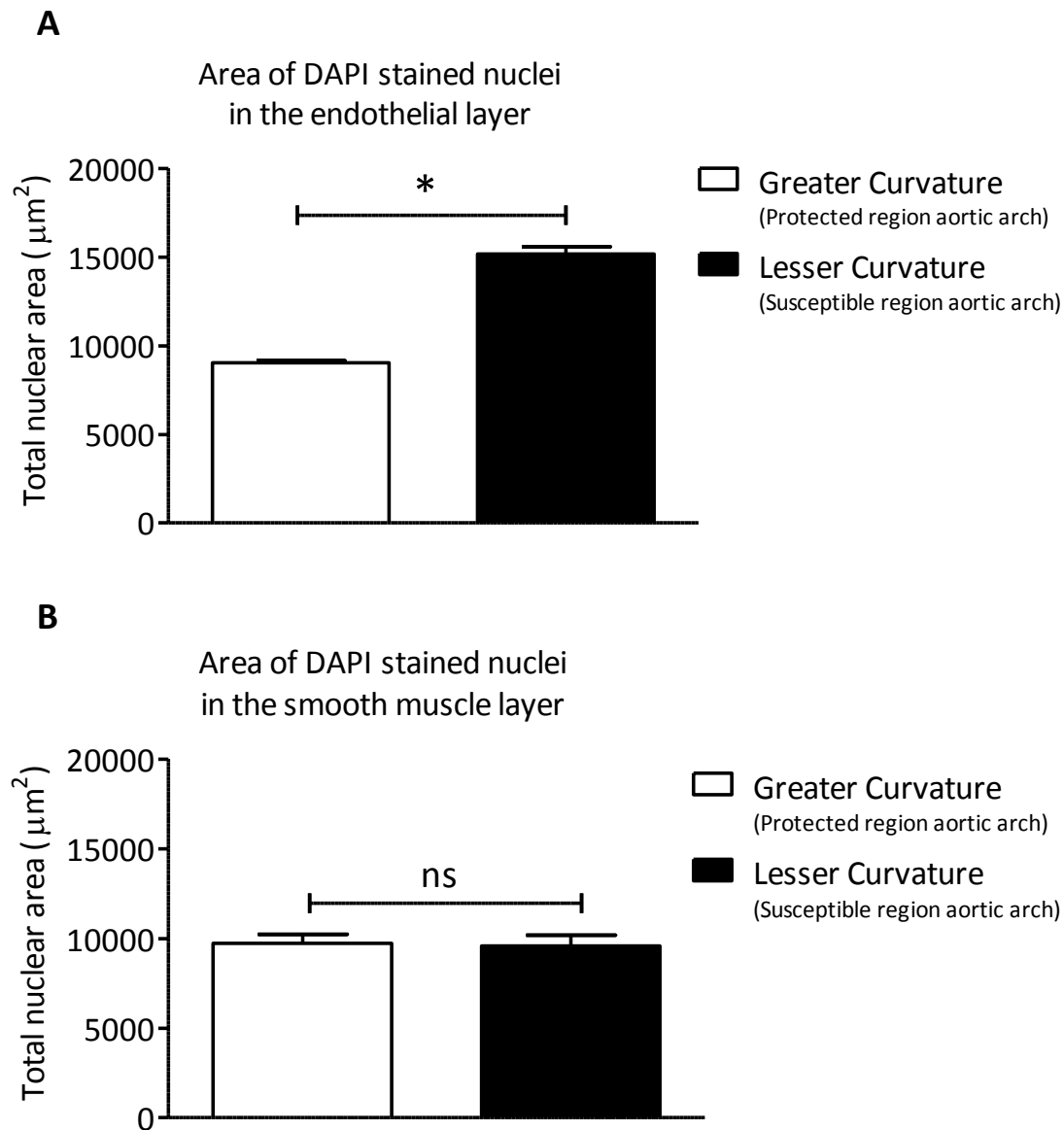


Figure 3.3 Quantified total nuclear area of DAPI stained nuclei in the endothelial and smooth muscle cell layers in the greater and the lesser curvature of the mouse aortic arch. Total Area (μm^2) of DAPI stained nuclei in the **(A)** endothelial layer and in the **(B)** smooth muscle layer of the greater curvature (white columns) and lesser curvature (black columns) regions of the mouse aortic arch fixed according to the transcatheter perfusion fixation procedure. Data was analysed from confocal pictures taken with a 40x objective and 1.9 zoom (image size 203.95x203.95 μm), see representative images (Figure 3.2). One picture was taken from each area and nuclei were selected for quantification by thresholding using the Huang criteria in Image J (Figure 3.2A+B IV). Data is presented as the mean $\pm$ S.E.M from $n=4$ young (3 month old) male WT mice (C57BL/6J, bred in-house) and was analysed using paired Student's t -test and $p<0.05$ taken as significant and denoted by * and $p>0.05$ was considered non-significant and denoted by ns.

Figure 3.4 Quantified CD31 immunoreactivity in the endothelial and smooth muscle cell layers of the greater and the lesser curvature of the mouse aortic arch

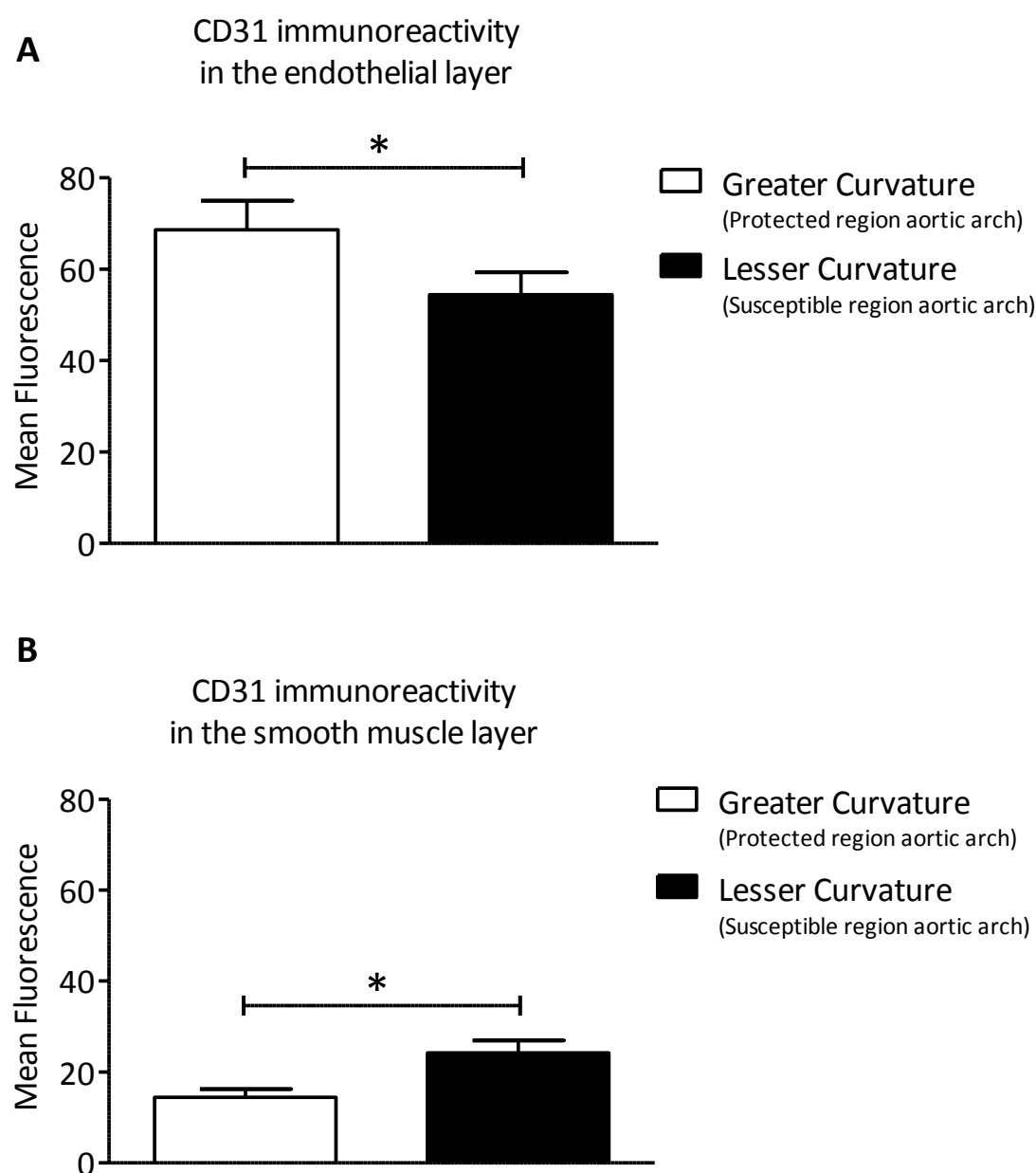


Figure 3.4 Quantified CD31 immunoreactivity in the endothelial and smooth muscle cell layers of the greater and the lesser curvature of the mouse aortic arch. CD31 immunoreactivity in the (A) endothelial or (B) smooth muscle layer of the greater curvature (white columns) and lesser curvature (black columns) regions of the mouse aortic arch fixed according to the transcardial perfusion fixation procedure. Data was analysed from confocal pictures taken with a 40x objective and 1.9 zoom (image size 203.95x203.95 μ m), see representative images (Figure 3.2A+B II). One picture was taken from each area and CD31 immunoreactivity was quantified as mean fluorescence. Data is the mean $\pm$ S.E.M from $n=4$ young (3 month old) male WT mice (C57BL/6J, bred in-house) and was analysed using paired Student's t -test and $p<0.05$ taken as significant and denoted by *.

Figure 3.5 Z-profiling of the depth of the endothelial cell layer at the greater curvature and the lesser curvature of the mouse aortic arch

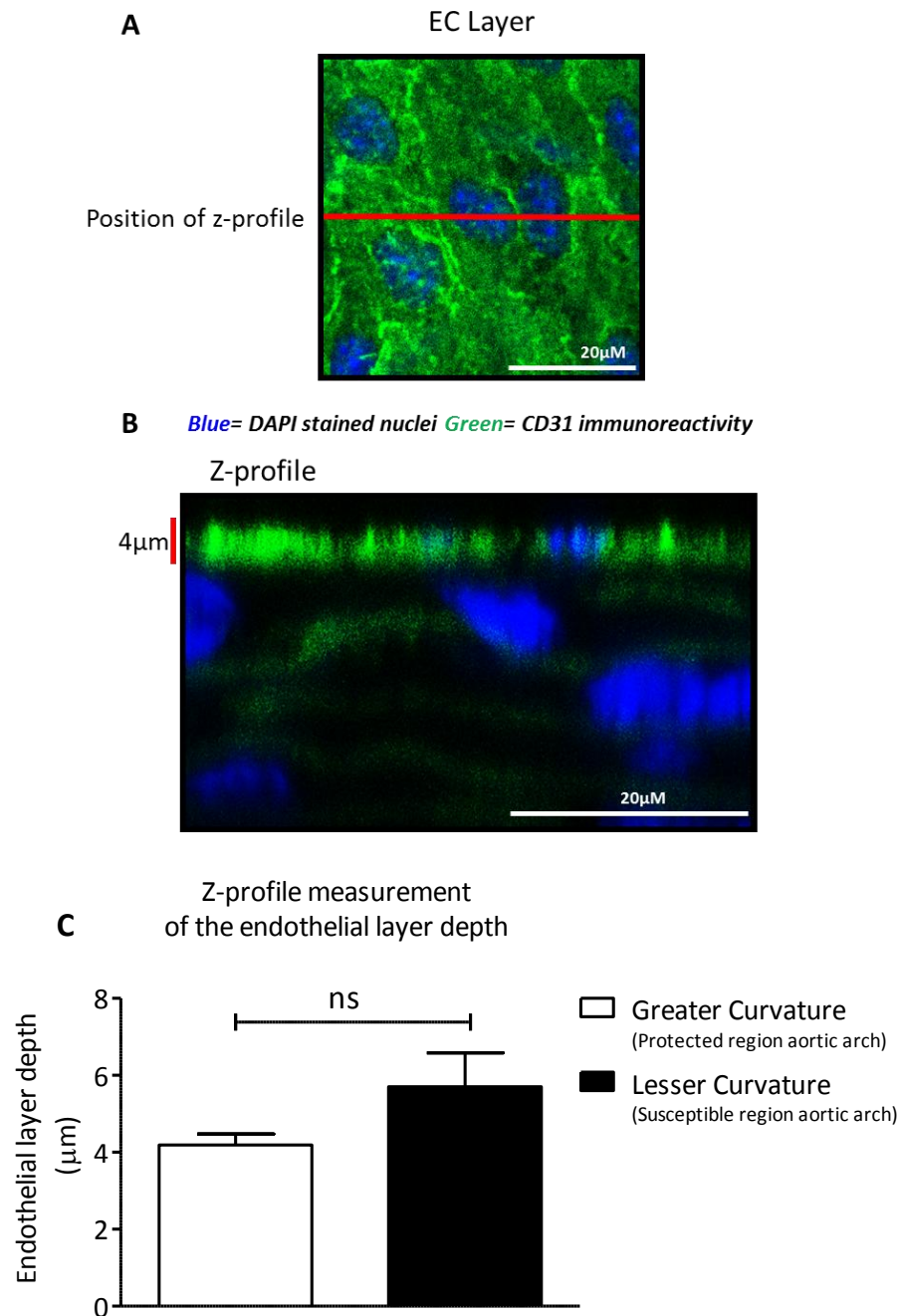


Figure 3.5 Z-profiling of the depth of the endothelial cell layer at the greater curvature and the lesser curvature of the mouse aortic arch. (A) View of luminal surface of the endothelium (40x objective at 7.75 zoom) with the position of the z-profile shown as a red line. (B) Transverse z-profile image of the endothelium (identified by CD31 immunoreactivity in green) and DAPI stained nuclei in blue. The green channel (CD31) has been enhanced for viewing purposes, to the same extent in both A and B. (C) Pooled data of the depth of the endothelial (CD31 positive) cell layer. Data is the mean $\pm$ S.E.M for $n=4$ young (3 month old) male WT mice (C57BL/6J, bred in-house) and was analysed using paired Student's *t*-test and $p>0.05$ taken as non-significant and denoted by ns.

Figure 3.6 Tile composite of 4x4 images showing COX-1 immunoreactivity in the greater and the lesser curvature of the aortic arch from a young adult male WT mouse

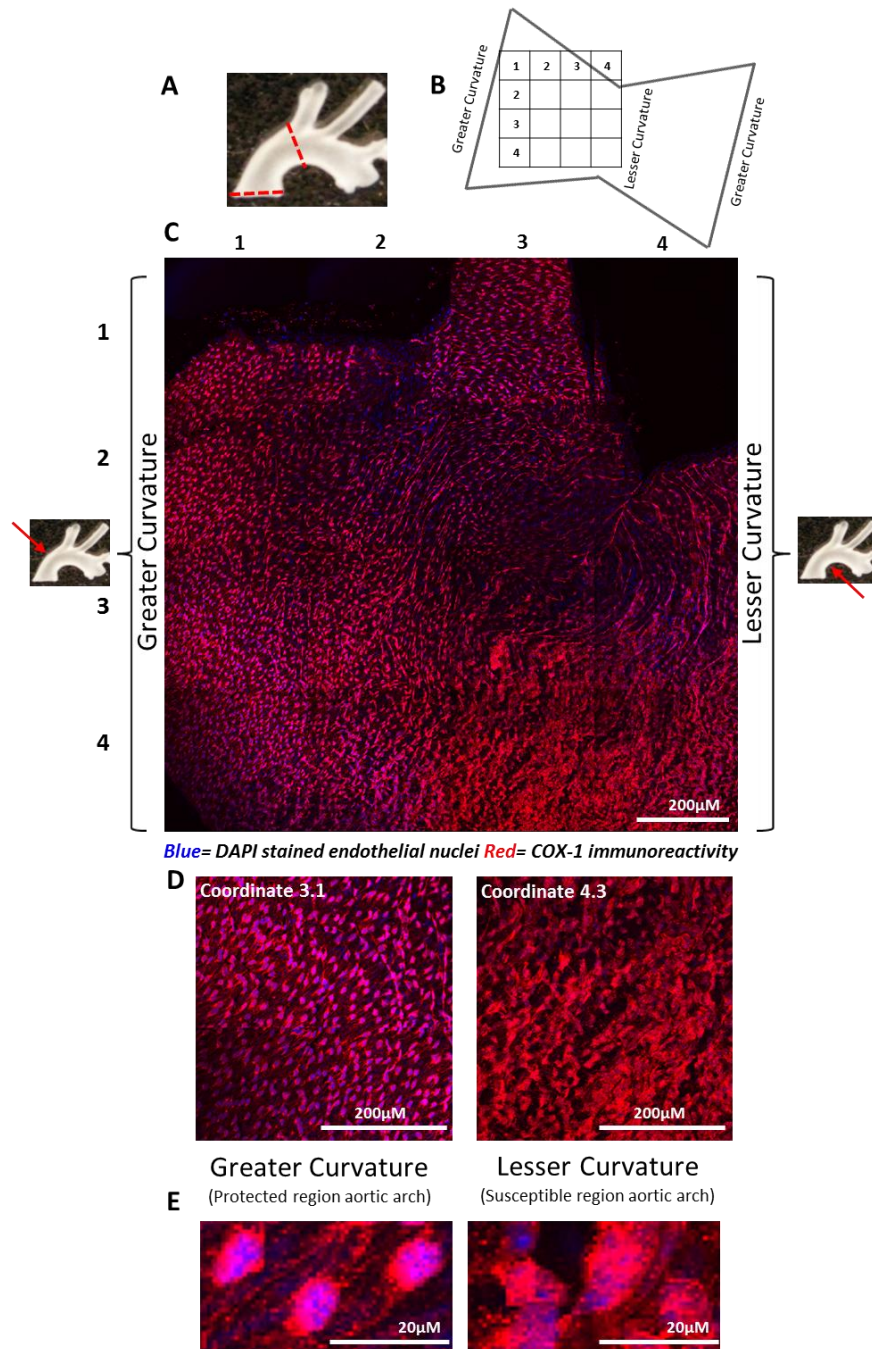


Figure 3.6 Tile composite of 4x4 images showing COX-1 immunoreactivity in the greater and the lesser curvature of the aortic arch from a young adult male WT mouse. An aortic arch fixed according to the transcatheter perfusion fixation procedure, dissected from a 3 month old (young adult) male WT mouse (C57BL/6J, Harlan, UK). (A) Red dotted line, mark dissection cutting points. (B) A schematic of the mounted (opened and flattened) aortic arch, marking the location of the greater and the lesser curvature as well as the position of the tile composite (4x4 images, coordinates 1.1-4.4). (C) The entire tile of 4x4 images. DAPI stained nuclei (blue) and COX-1 immunoreactivity (red). (D) Representative images from the lesser and the greater curvature (coordinate 3.1 and 4.3). (E) Zoomed in images showing intracellular localisation of COX-1 immunoreactivity.

Figure 3.7 Tile composite of 4x4 images showing COX-2 immunoreactivity in the greater and the lesser curvature of the aortic arch from a young adult male WT mouse

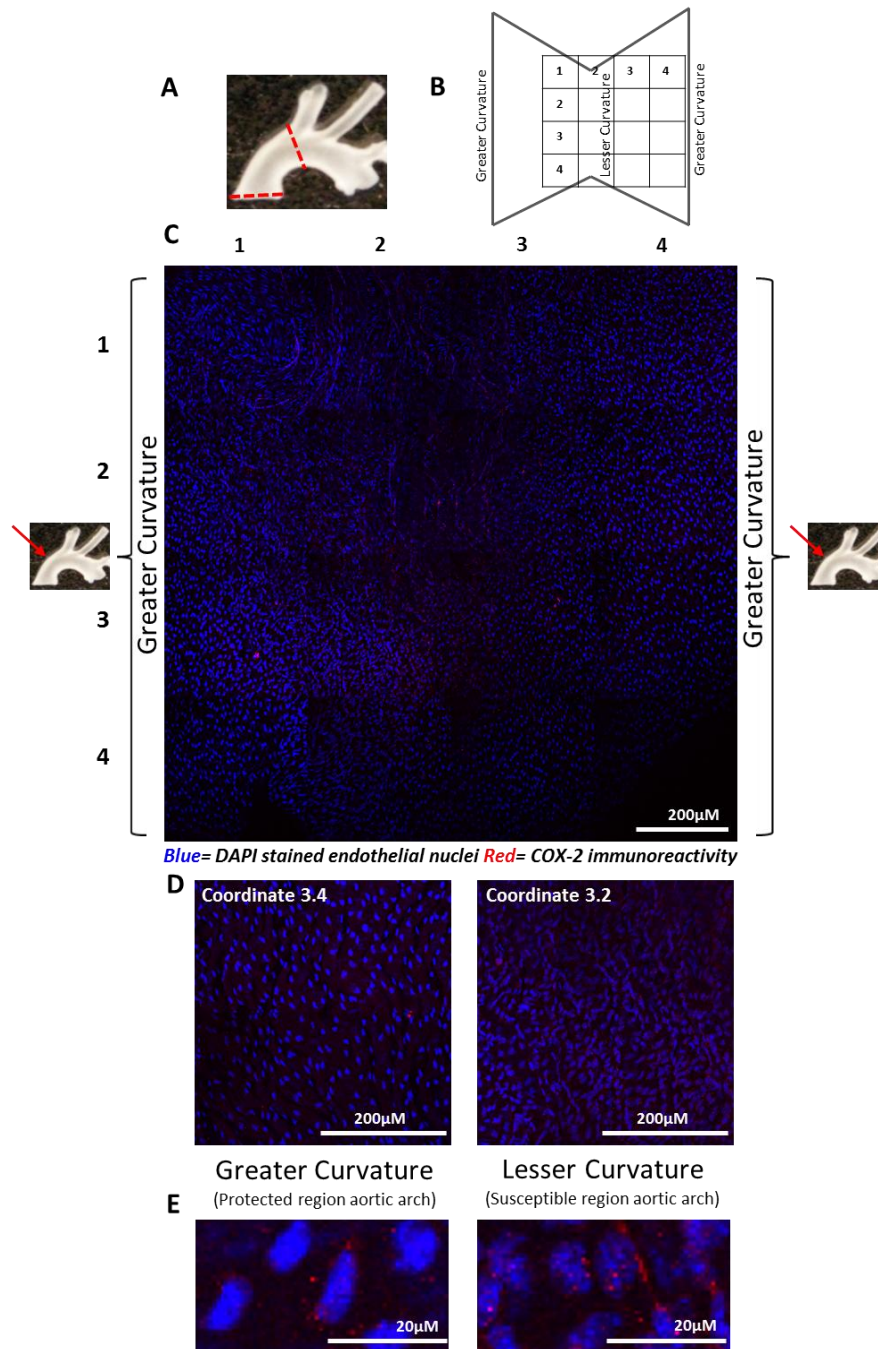


Figure 3.7 Tile composite of 4x4 images showing COX-2 immunoreactivity in the greater and the lesser curvature of the aortic arch from a young adult WT mouse An aortic arch fixed according to the transcatheter perfusion fixation procedure, dissected from a 3 month old (young adult) male WT mouse (C57BL/6J, Harlan, UK). (A) Red dotted line, mark dissection cutting points. (B) A schematic of the mounted (opened and flattened) aortic arch, marking the location of the greater and the lesser curvature as well as the position of the tile composite (4x4 images, coordinates 1.1-4.4). (C) The entire tile of 4x4 images. DAPI stained nuclei (blue) and COX-1 immunoreactivity (red). (D) Representative images from the lesser and the greater curvature (coordinate 3.4 and 3.2). (E) Zoomed in images showing intracellular localisation of COX-2 immunoreactivity.

Figure 3.8 Quantified COX-1 versus COX-2 immunoreactivity in the greater and the lesser curvature of the mouse aortic arch from young adult male WT mice

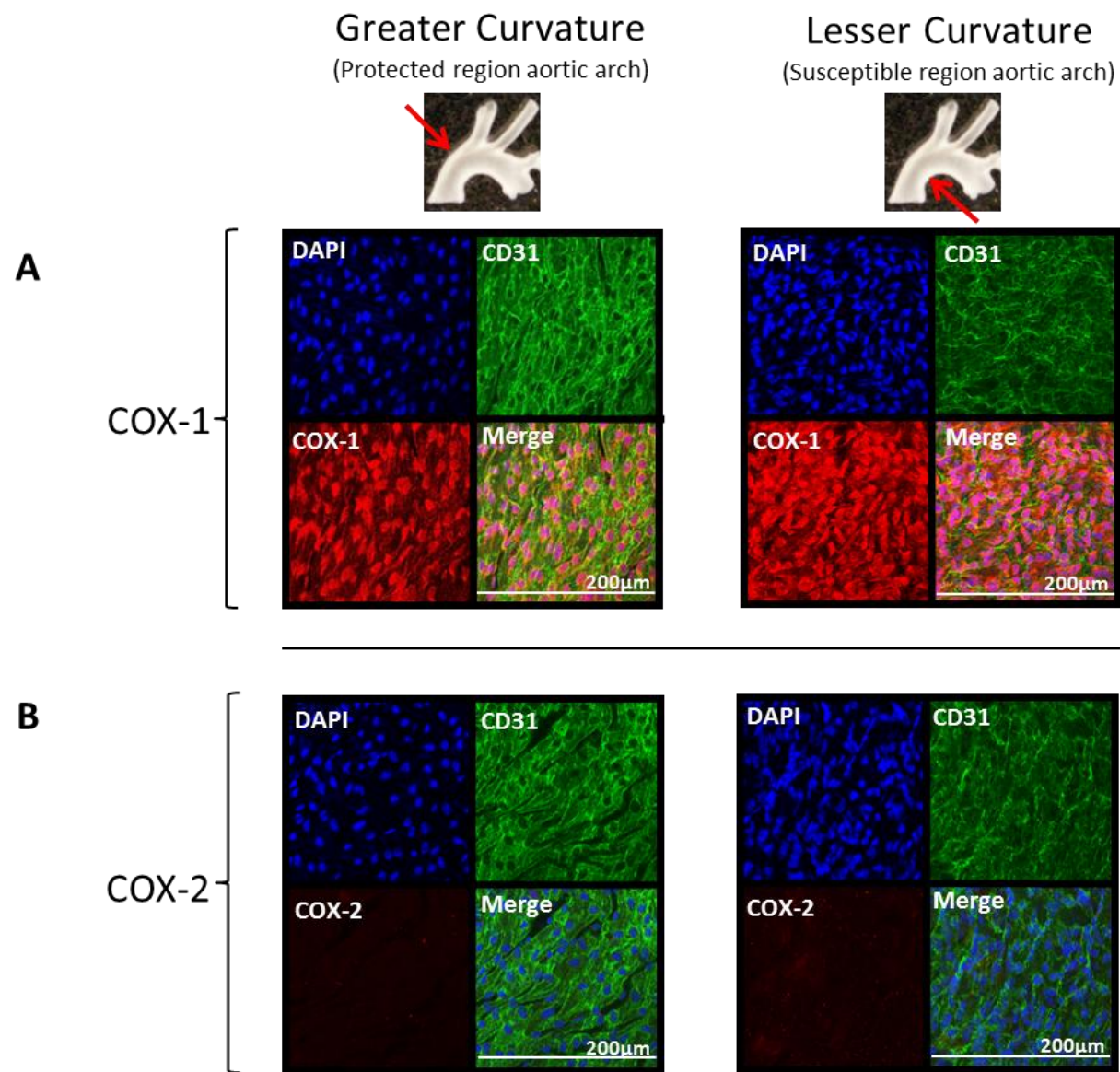


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Figure 3.8 (Continued) Quantified COX-1 versus COX-2 immunoreactivity in the greater and the lesser curvature of the mouse aortic arch from young adult male WT mice

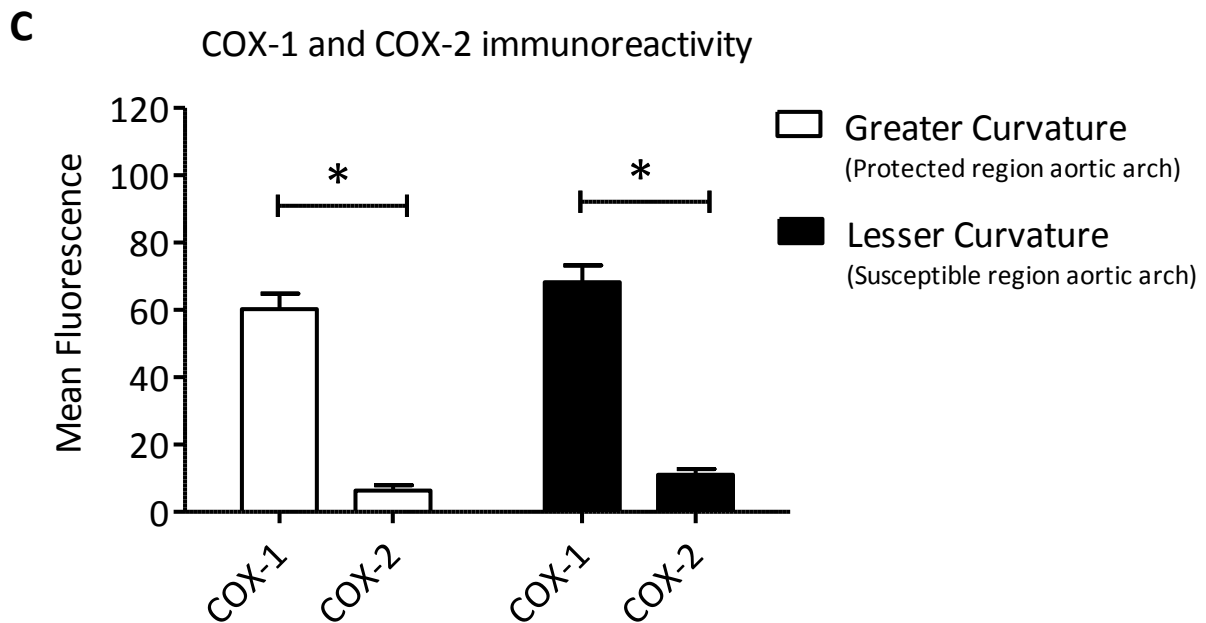


Figure 3.8 Quantified COX-1 versus COX-2 immunoreactivity in the greater and the lesser curvature of the mouse aortic arch from young adult male WT mice. Representative cropped images (200x200 μ m) of COX-1 immunoreactivity (A) and COX-2 immunoreactivity (B) (red), from the greater and the lesser curvature of the mouse aortic arch fixed according to the transcatheter perfusion fixation procedure. Nuclei visualised with DAPI (blue) and endothelial cells outlined with CD31 (green). (C) Pooled data comparing COX-1 and COX-2 immunoreactivity from 6 head to head experiments. 3 unique images were captured from the greater curvature and the lesser curvature respectively. COX immunoreactivity was quantified as mean fluorescence and secondary control background immunoreactivity from each region has been subtracted from each image. Data is the mean $\pm$ S.E.M of $n=14-18$ images from separate regions of tissue from $n=6$ young adult (3 month old) male WT mice (C57BL/6J, Harlan, UK). Data was analysed using one-way ANOVA with Bonferroni's multi comparison post-test and $*p<0.05$ was taken as significant and denoted by*.

Figure 3.9 COX-2 immunoreactivity in the greater and the lesser curvature of aortic arches from untreated versus LPS treated young adult male WT mice

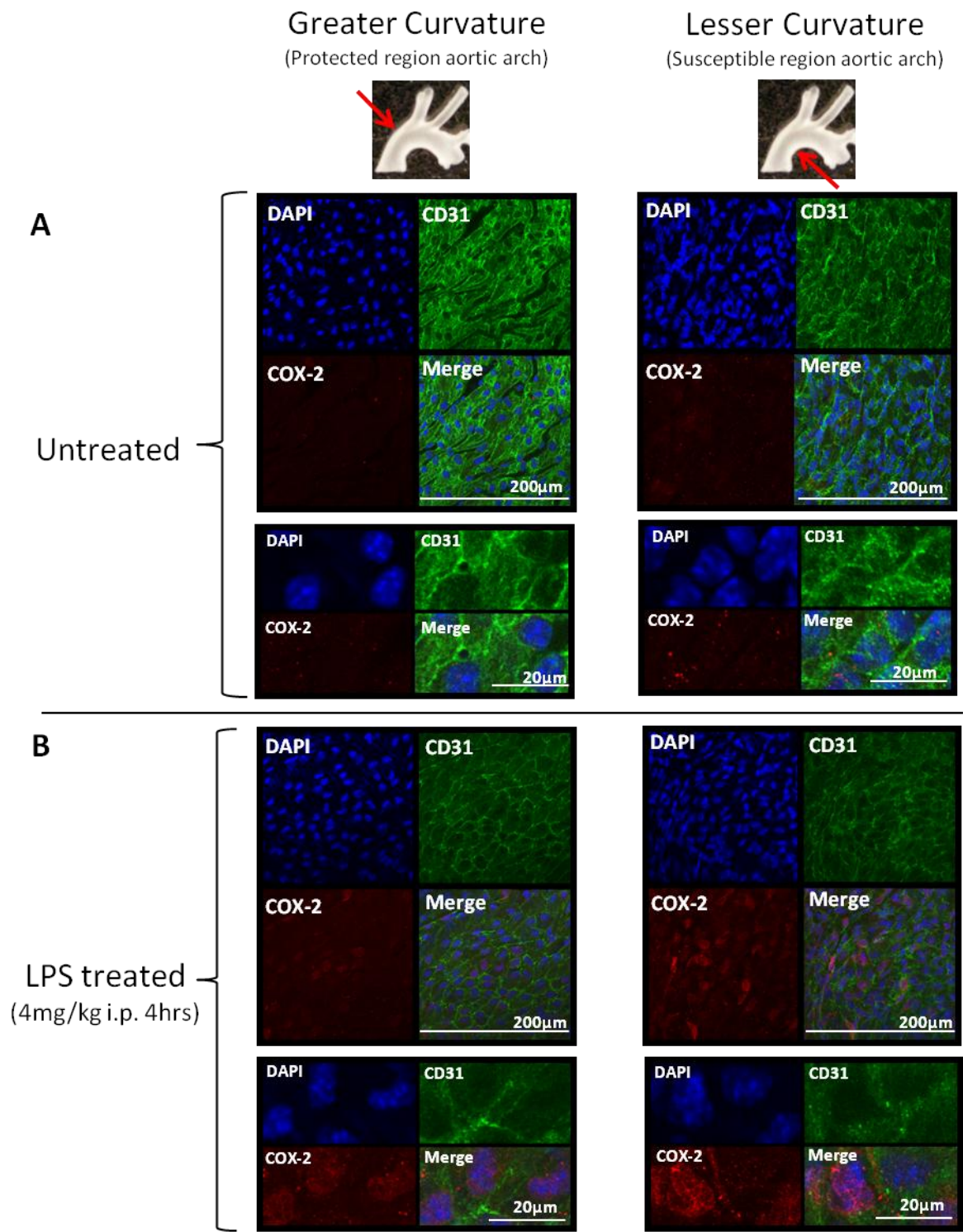


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Figure 3.9 (Continued) COX-2 immunoreactivity in the greater and the lesser curvature of aortic arches from untreated versus LPS treated young adult male WT mice

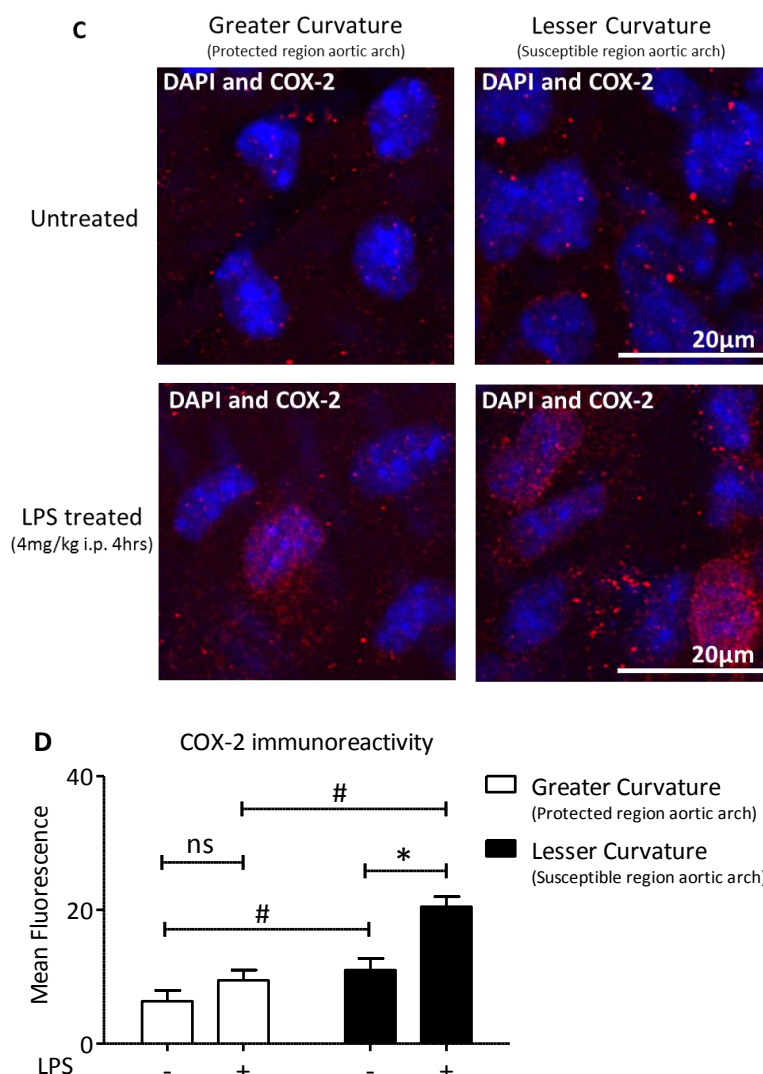


Figure 3.9 COX-2 immunoreactivity in the greater and the lesser curvature of aortic arches from untreated versus LPS treated young adult male WT mice. Representative cropped images (200x200μm) of COX-2 immunoreactivity (in red) from (A) untreated and (B) LPS injected (*E.coli* serotype 0111:B4, 4mg/kg i.p. for 4 hours) WT mice. Aortic arches were fixed according to the transcardial perfusion fixation procedure. Nuclei visualised with DAPI (blue) and endothelial cells outlined with CD31 (green). (C) Zoomed in images (40x40μm) of COX-2 staining pattern in untreated and LPS treated WT mice. (D) Pooled data of COX-2 immunoreactivity in the greater and the lesser curvature in n=6 aortic arches from untreated (-) and LPS treated (+) WT mice (secondary control background was subtracted from each image before analysis). Data is the mean ± S.E.M of n=18 (3 separate images were taken per region) from n=6 young adult (3 month old), WT mice (C57BL/6J, Harlan, UK). Within group analysis was performed using paired Student's t-test and when significant denoted by #. Between groups analysis was analysed using one-way ANOVA with Bonferroni's multi comparison post-test and when significant denoted by *. P<0.05 taken as significant and denoted by * or # and p>0.05 was taken as non-significant and denoted by ns.

Figure 3.10 COX-1 immunoreactivity in the greater and the lesser curvature of aortic arches from untreated versus LPS treated young adult male WT mice

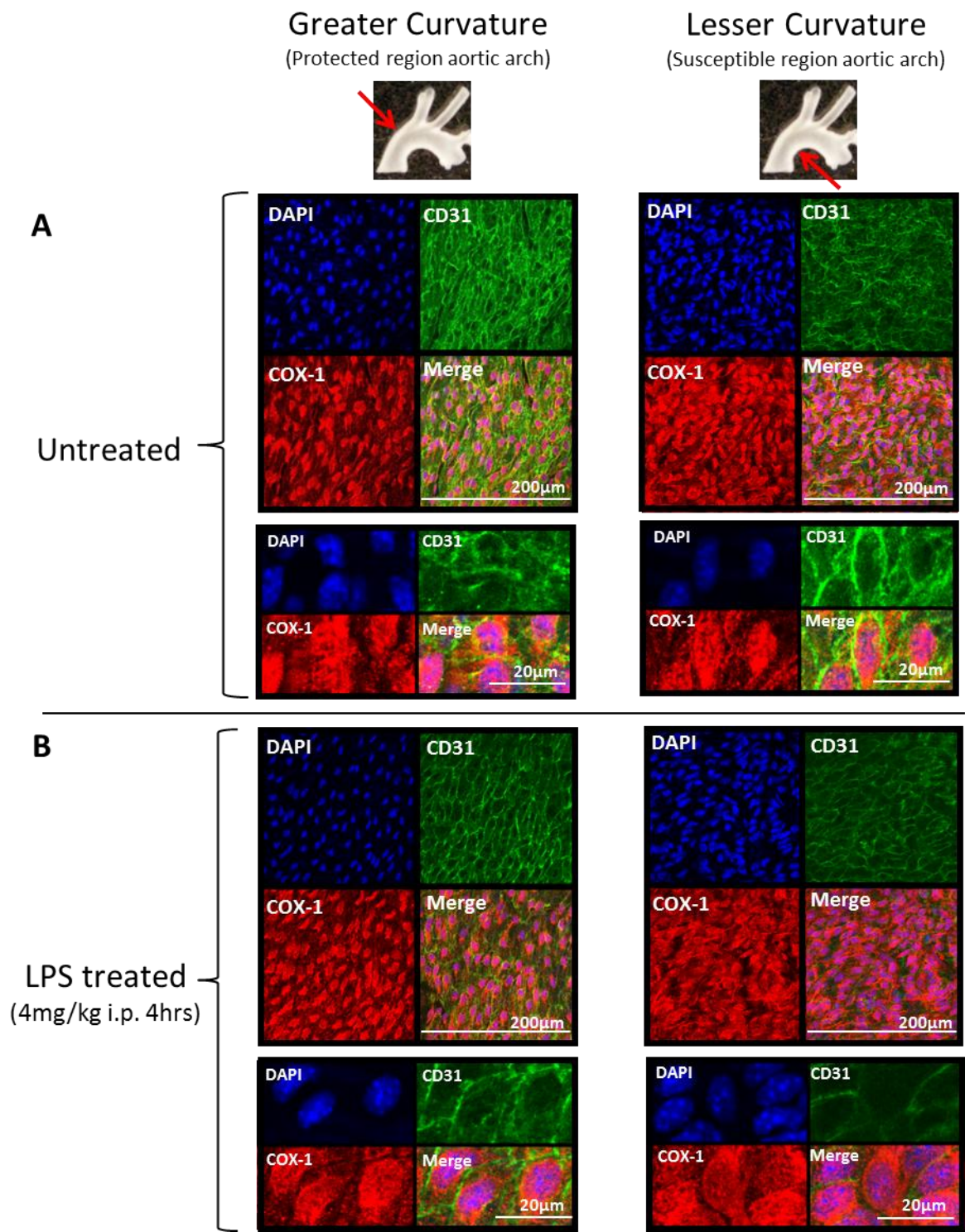


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Figure 3.10 (Continued) COX-1 immunoreactivity in the greater and the lesser curvature of aortic arches from untreated versus LPS treated young adult male WT mice

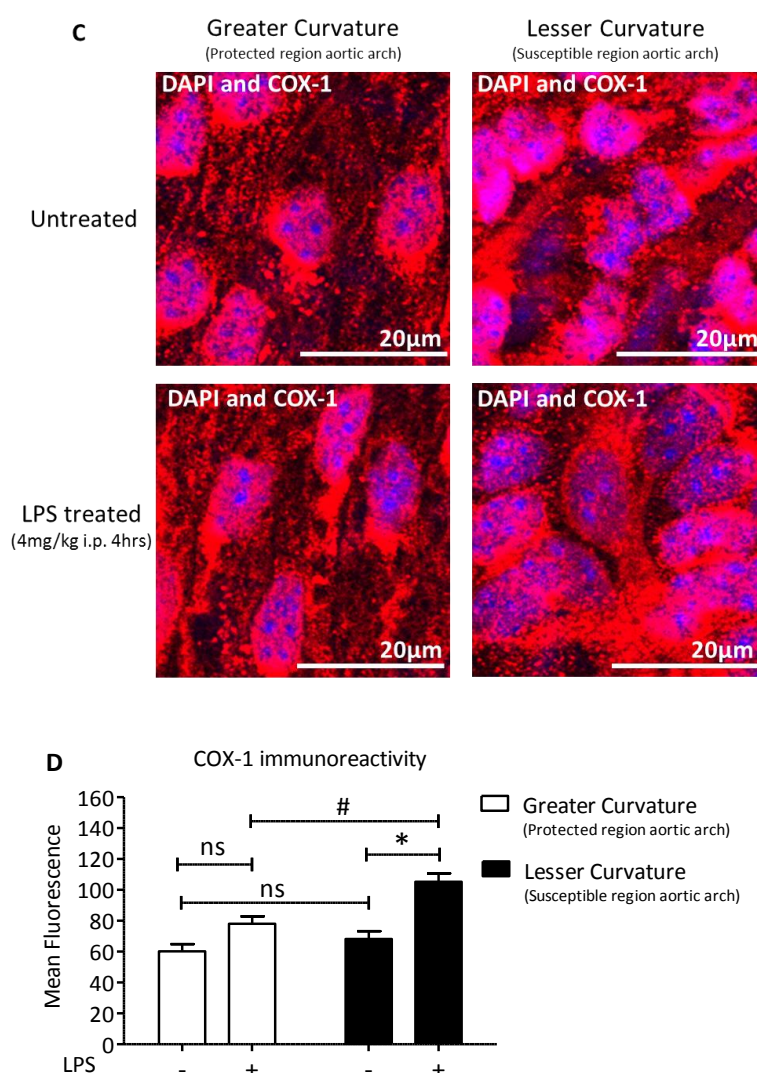


Figure 3.10 COX-1 immunoreactivity in the greater and the lesser curvature of aortic arches from untreated versus LPS treated young adult male WT mice. Representative cropped images (200x200μm) of COX-1 immunoreactivity (red) from (A) untreated and (B) LPS injected (*E.coli* serotype 0111:B4, 4mg/kg i.p. for 4 hours) WT mice. Aortic arches were fixed according to the transcatheter perfusion fixation procedure. Nuclei visualised with DAPI (blue) and endothelial cells outlined with CD31 (green). (C) Zoomed in images (40x40μm) of COX-1 staining pattern in untreated and LPS treated WT mice. (D) Pooled data of COX-1 immunoreactivity in the greater and the lesser curvature in $n=6$ aortic arches from untreated (-) and LPS treated (+) WT mice (secondary control background was subtracted from each image before analysis). Data is the mean $\pm$ S.E.M of $n=14-18$ (3 separate images were taken per region) from $n=6$ young adult (3 month old), WT mice (C57BL/6J, Harlan, UK). Within group analysis was performed using paired Student's *t*-test. Between groups analysis was analysed using one-way ANOVA with Bonferroni's multi comparison post-test. $P<0.05$ taken as significant and denoted by * and $p>0.05$ was taken as non-significant and denoted by ns.

Figure 3.11 COX-1 and COX-2 immunoreactivity in the endothelial layer versus the smooth muscle cell layer

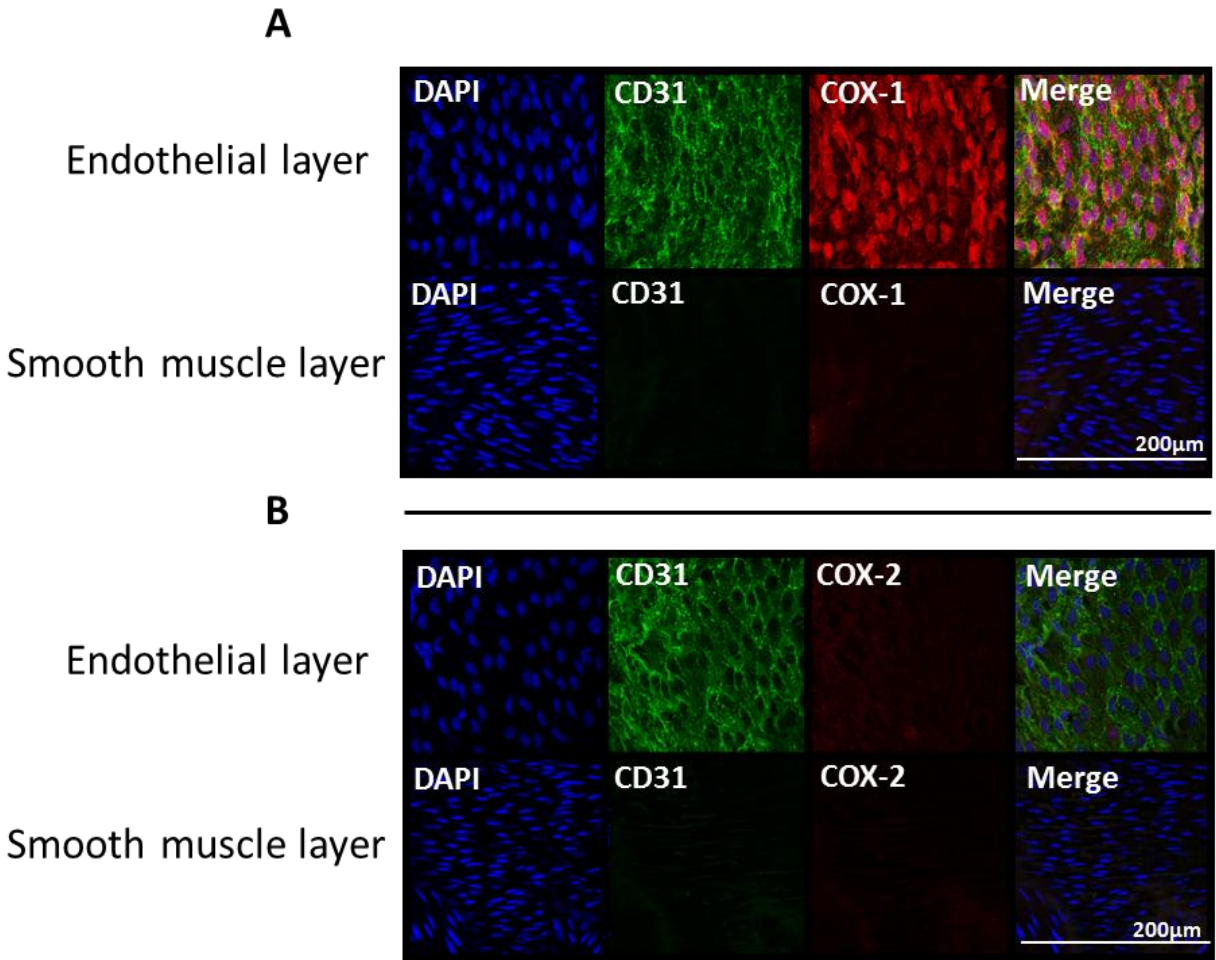


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Figure 3.11 (Continued) COX-1 and COX-2 immunoreactivity in the endothelial layer versus the smooth muscle cell layer

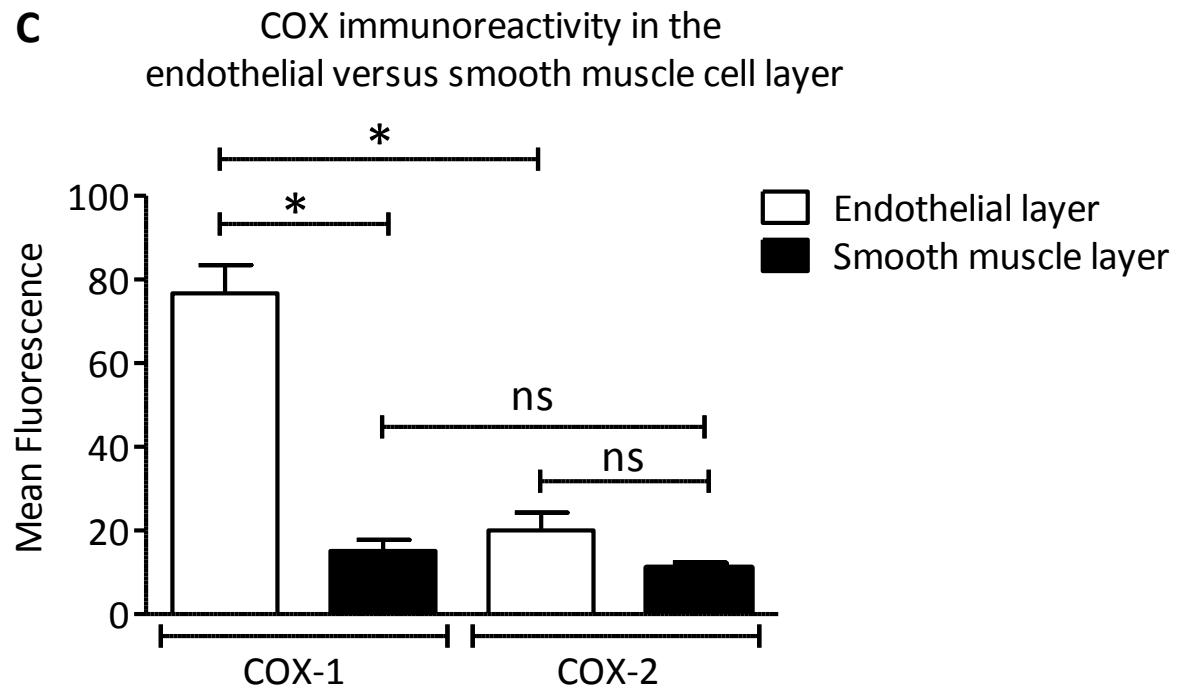


Figure 3.11 COX-1 and COX-2 immunoreactivity in the endothelial layer versus the smooth muscle cell layer. Representative cropped images (200x200 μ m) of **(A)** COX-1 immunoreactivity and **(B)** COX-2 immunoreactivity (**red**), in the endothelial layer and in the directly underlying smooth muscle layer in the mid arch (COX-2) and descending aorta (COX-1) of a mouse aortic arch fixed according to the transcardial perfusion fixation procedure. Nuclei visualised with DAPI (**blue**) and endothelial cells outlined with CD31 (**green**). **(C)** COX immunoreactivity quantified as mean fluorescence. Data is the mean $\pm$ S.E.M of $n=4$ images from $n=4$ WT mice (2 male and 2 female, 3 month old, young adult, C57BL/6J mice, bred in-house). Data was analysed using one-way ANOVA with Bonferroni's multi comparison post-test. $P < 0.05$ taken as significant and denoted by * and $p > 0.05$ was taken as non-significant and denoted by ns.

Figure 3.12 Pilot study of COX-1 and COX-2 immunoreactivity in human pulmonary artery endothelium (from one human donor)

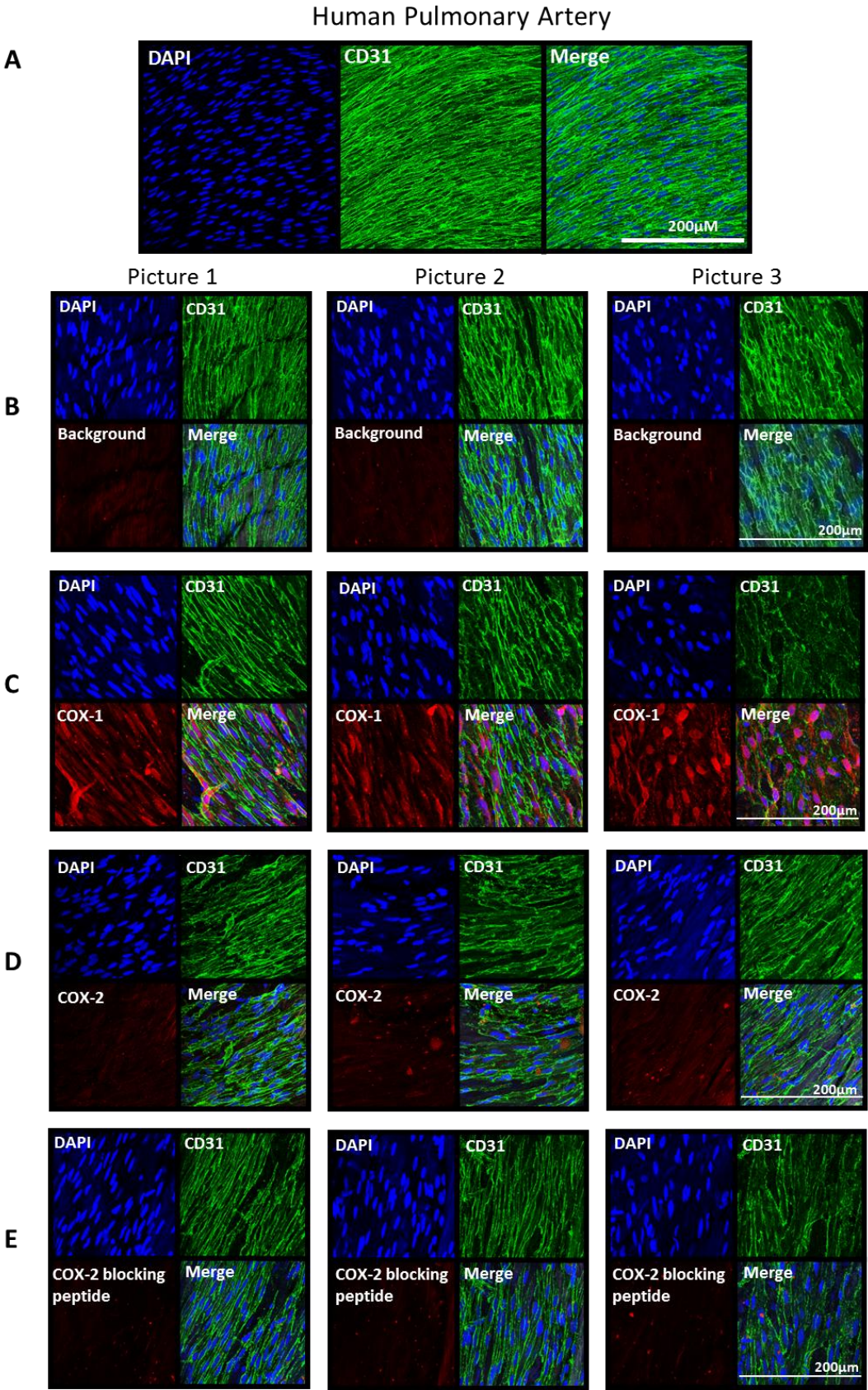


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Figure 3.12 (Continued) Pilot study of COX-1 and COX-2 immunoreactivity in human pulmonary artery endothelium (from one human donor)

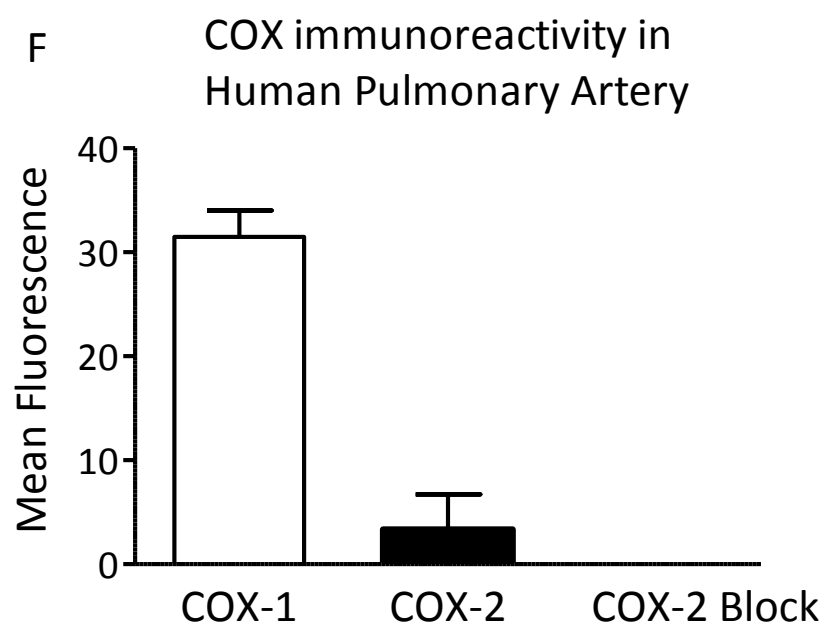


Figure 3.12 Pilot study of COX-1 and COX-2 immunoreactivity in human pulmonary artery endothelium (from one human donor). (A) A full field picture (387.5x387.5µm) of CD31 positive (green) endothelium from a human pulmonary artery. Nuclei visualised by DAPI (blue). (B) 3 representative cropped images (200x200µm) showing background immunoreactivity (red) from the secondary antibody used (goat anti-rabbit Alexa Fluor 594). (C) 3 separate pictures showing human endothelial COX-1 immunoreactivity (red), from the same human donor, using the anti-human COX-1 anti-serum (Cayman 160108). (D) 3 separate pictures showing human endothelial COX-2 immunoreactivity from the same human donor, using the same COX-2 antibody as previously (Cayman 160126 which also recognises human COX-2) (red). (E) COX-2 blocking peptide (the precise epitope which the Cayman 160126 COX-2 antibody was raised against) was added to test the specificity of the COX-2 immunoreactivity. (F) Quantified COX-1 and COX-2 immunoreactivity (with or without COX-2 blocking peptide), as mean fluorescence from n=3 separate images from n=1 human donor (See details about donor in General Methods Chapter 2). Secondary control background was subtracted from each image before analysis.

Figure 3.13 Summary of non-specific background immunoreactivity from the secondary control Goat anti rabbit Alexa Fluor 594

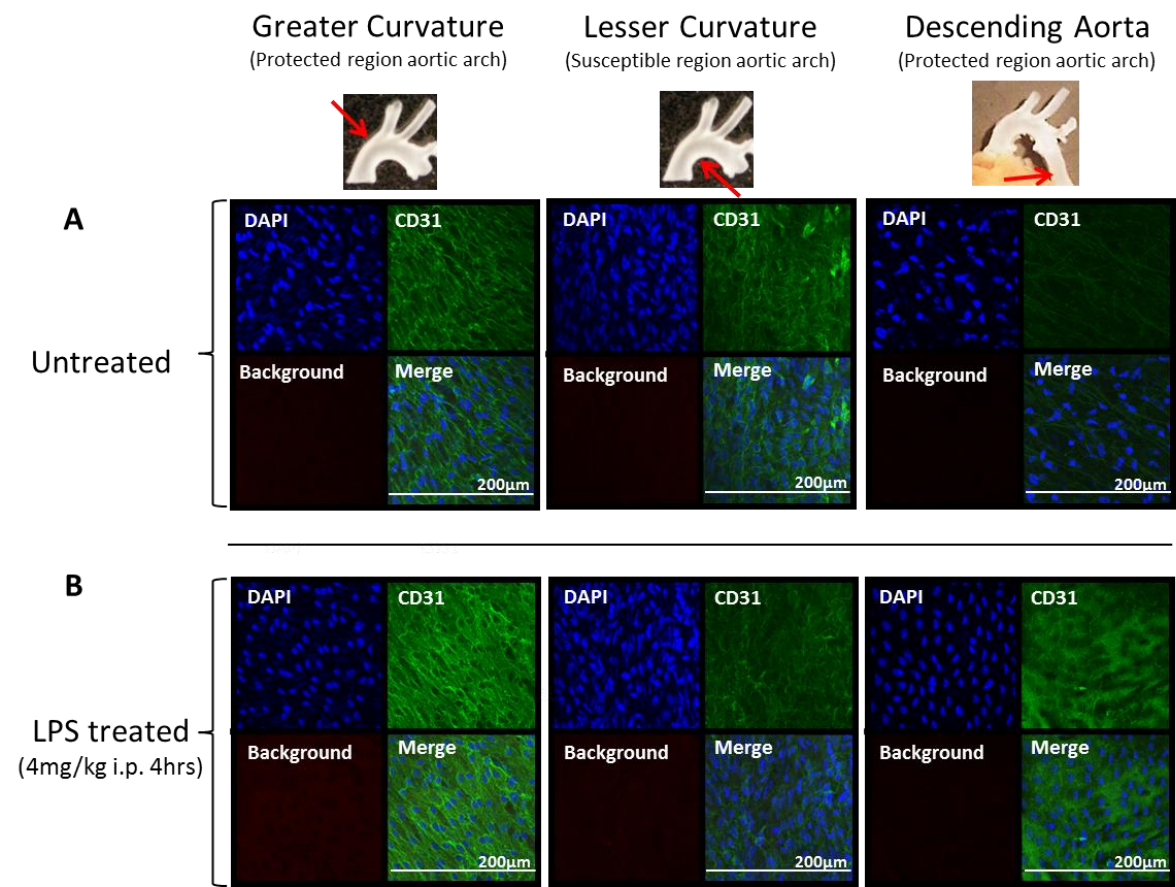


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Figure 3.13 (Continued) Summary of non-specific background immunoreactivity from the secondary control Goat anti rabbit Alexa Fluor 594

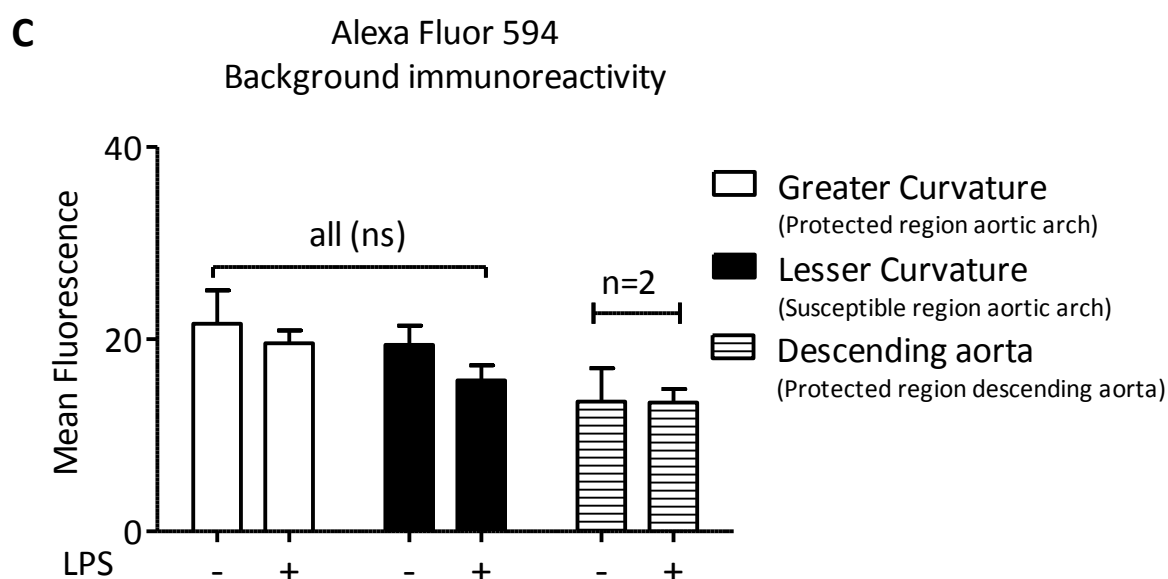


Figure 3.13 Summary of non-specific background immunoreactivity from Alexa Fluor 594. Secondary antibody Goat anti-rabbit Alexa Fluor 594 (Invitrogen A-11012) used to detect the COX-1 and COX-2 primary antibodies (raised in rabbit). Representative cropped images (200x200µm) of non-specific background immunoreactivity in the greater and lesser curvature of the aortic arch from **(A)** untreated and **(B)** LPS treated (*E.coli* serotype 0111:B4, 4mg/kg i.p. for 4hrs) 3 month old (young adult) male WT mice (C57BL/6J, Harlan, UK) Aortic arches were fixed according to the transcardial perfusion fixation procedure. **(C)** Background immunoreactivity quantified as mean fluorescence from the greater and lesser curvature and the descending aorta, from untreated (-) and LPS treated (+) mice. Data is the mean $\pm$ S.E.M of $n=12$ (2 separate images were taken per region from $n=6$ mice), except for the descending aorta where it was based on $n=2$ (2 separate images from $n=1$ mouse). Data was analysed using one-way ANOVA with Bonferroni's multi comparison post-test. $P>0.05$ was taken as non-significant and denoted by ns.

Figure 3.14 Use of blocking peptide to test the specificity of the COX-1 immunoreactivity

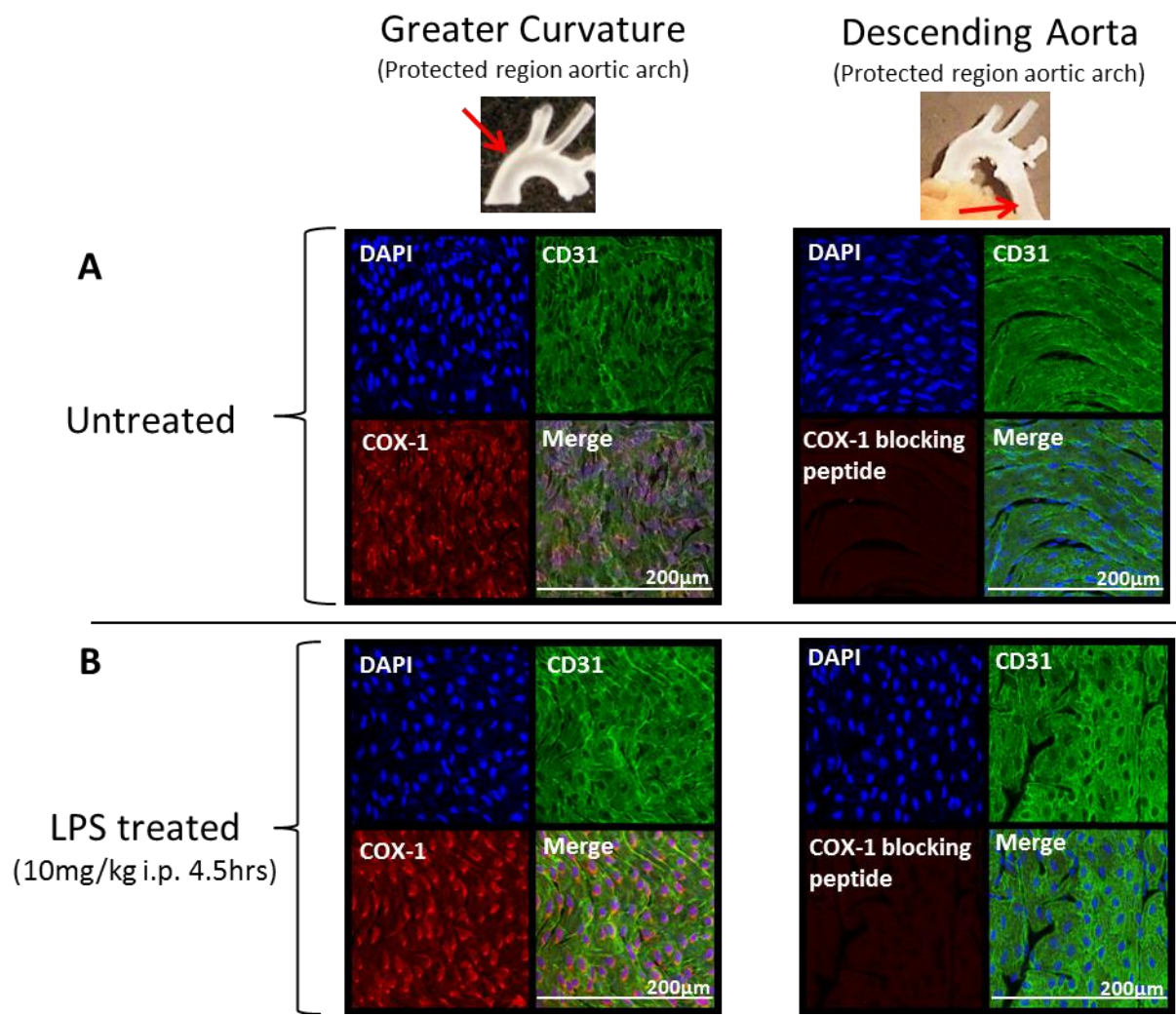


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Figure 3.14 (Continued) Use of blocking peptide to test the specificity of the COX-1 immunoreactivity

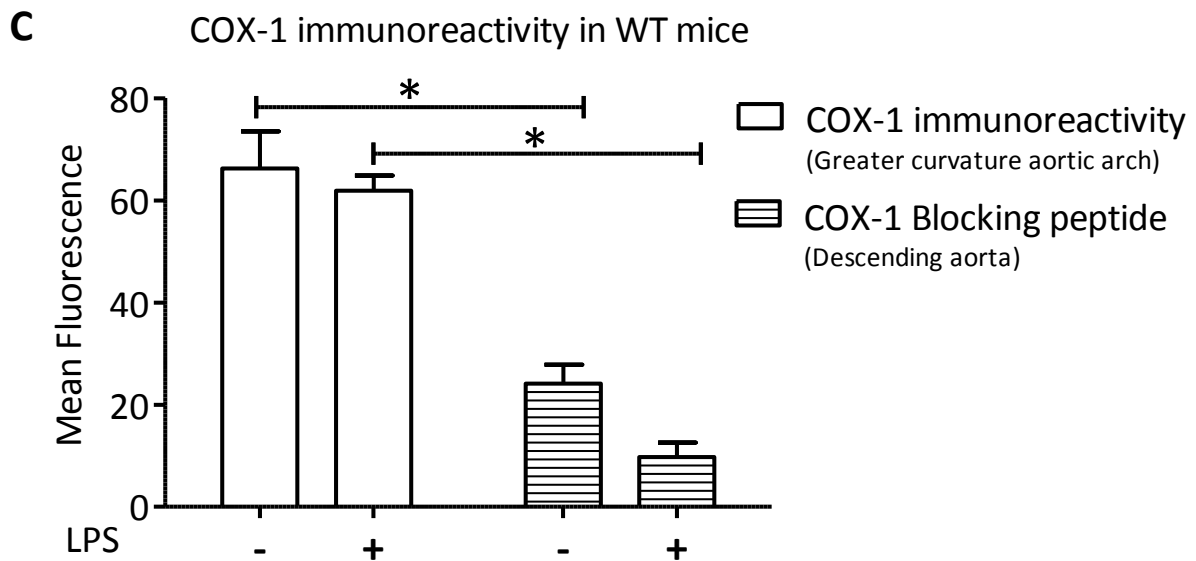


Figure 3.14 Use of blocking peptide to test the specificity of the COX-1 immunoreactivity. Representative cropped images (200x200µm) of COX-1 immunoreactivity (**red**) in the greater curvature of aortic arches from **(A)** untreated and **(B)** LPS treated (*E.coli* serotype 055:B5, 10mg/kg i.p. for 4.5 hours) WT mice. Aortic arches (including the descending aorta) were fixed according to the ex vivo semi-perfusion fixation procedure. The descending aorta from the same animal was incubated with COX-1 blocking peptide (the precise epitope which the Cayman 160109 murine COX-1 antibody was raised against). Nuclei visualised with DAPI (**blue**) and endothelial cells outlined with CD31 (**green**). **(C)** COX-1 immunoreactivity quantified as mean fluorescence, data is the mean $\pm$ S.E.M from $n=4-8$ pictures, 1-2 separate images from $n=4$ young adult (3 month old), male WT mice (C57BL/6J bred in-house). Secondary control background was subtracted from each image before analysis. Data was analysed using unpaired Student's t-test and $p<0.05$ taken as significant and denoted by *.

Figure 3.15 Use of blocking peptide to test the specificity of the COX-2 immunoreactivity

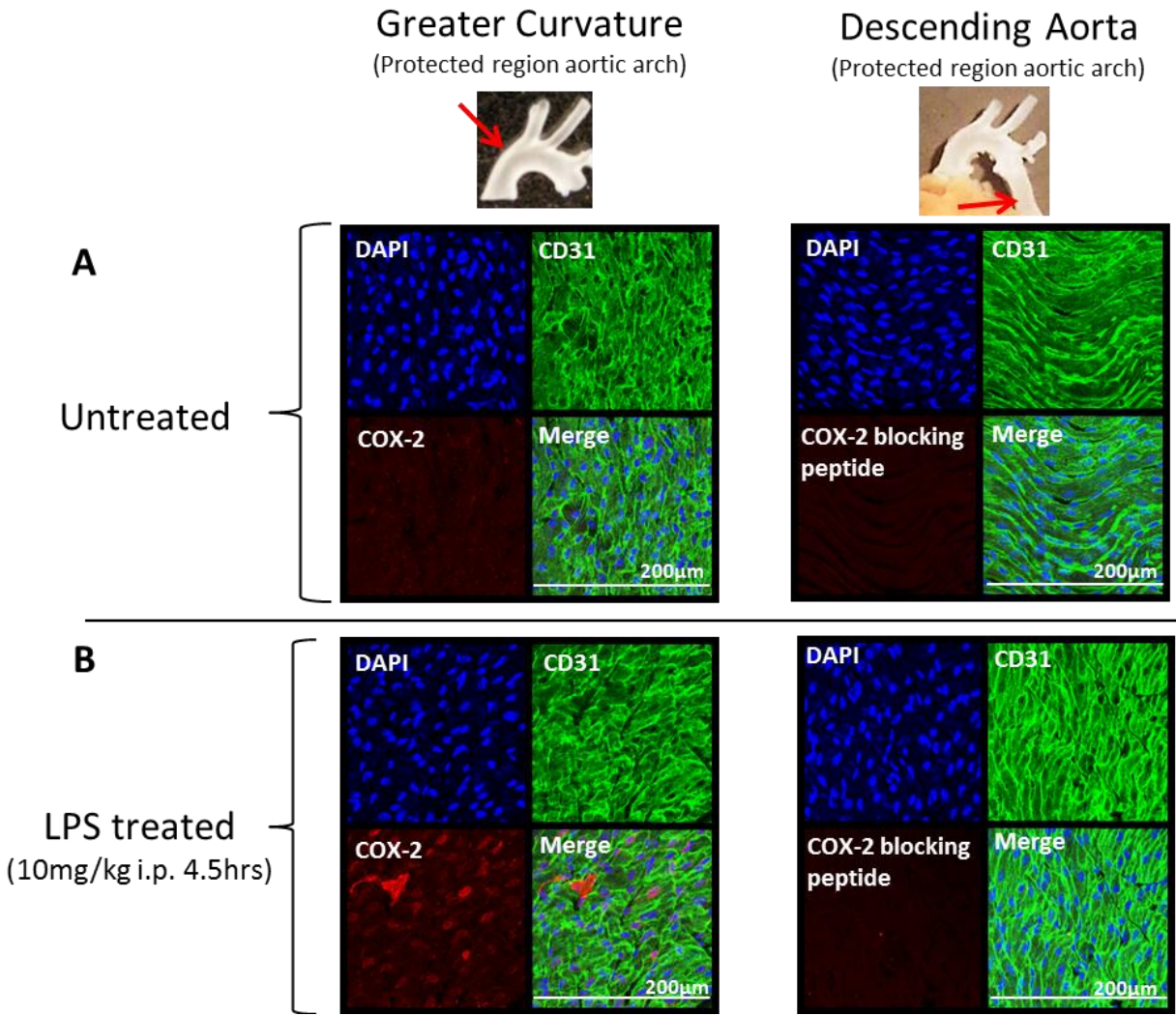


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Figure 3.15 (Continued) Use of blocking peptide to test the specificity of the COX-2 immunoreactivity

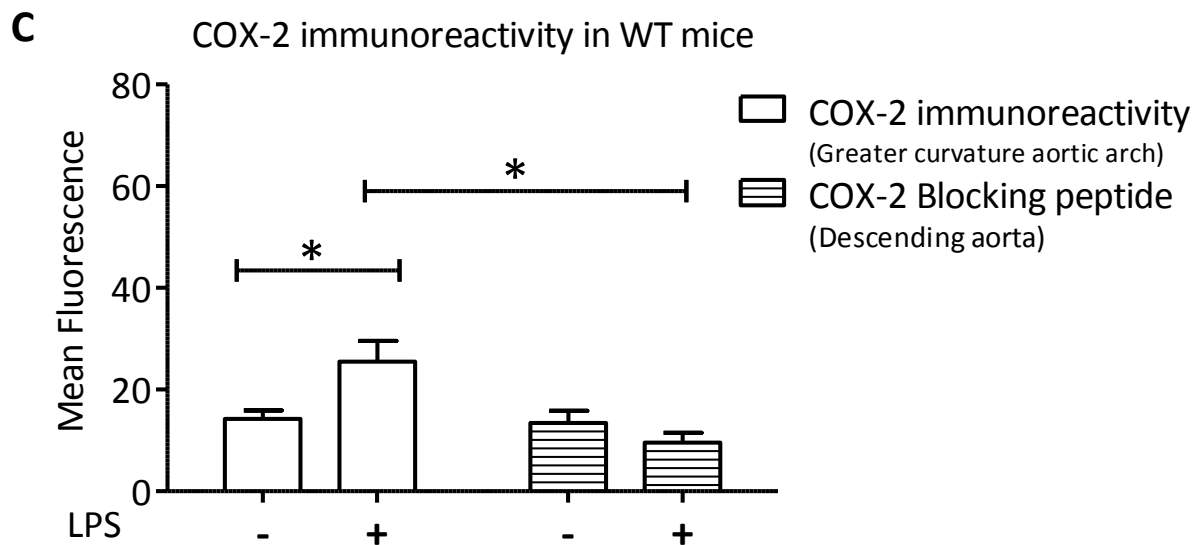


Figure 3.15 Use of blocking peptide to test the specificity of the COX-2 immunoreactivity. Representative cropped images (200x200 μ m) of COX-2 immunoreactivity (**red**) in the greater curvature of aortic arches from **(A)** untreated and **(B)** LPS treated (*E.coli* serotype 055:B5, 10mg/kg i.p. for 4.5 hours) WT mice. Aortic arches (including the descending aorta) were fixed according to the ex vivo semi-perfusion fixation procedure. The descending aorta from the same animal was incubated with COX-2 blocking peptide (the precise epitope which the Cayman 160126 murine COX-2 antibody was raised against). Nuclei visualised with DAPI (**blue**) and endothelial cells outlined with CD31 (**green**). **(C)** COX-2 immunoreactivity quantified as mean fluorescence, data is the mean $\pm$ S.E.M from $n=4-8$ pictures, 1-2 separate images from $n=4$ young adult (3 month old), male WT mice (C57BL/6J bred in-house). Secondary control background was subtracted from each image before analysis. Data was analysed using unpaired Student's t-test and $p<0.05$ taken as significant and denoted by *.

Figure 3.16 COX-1 immunoreactivity in the greater and the lesser curvature of aortic arches from young adult male WT, COX-1^{-/-} and COX-2^{-/-} mice

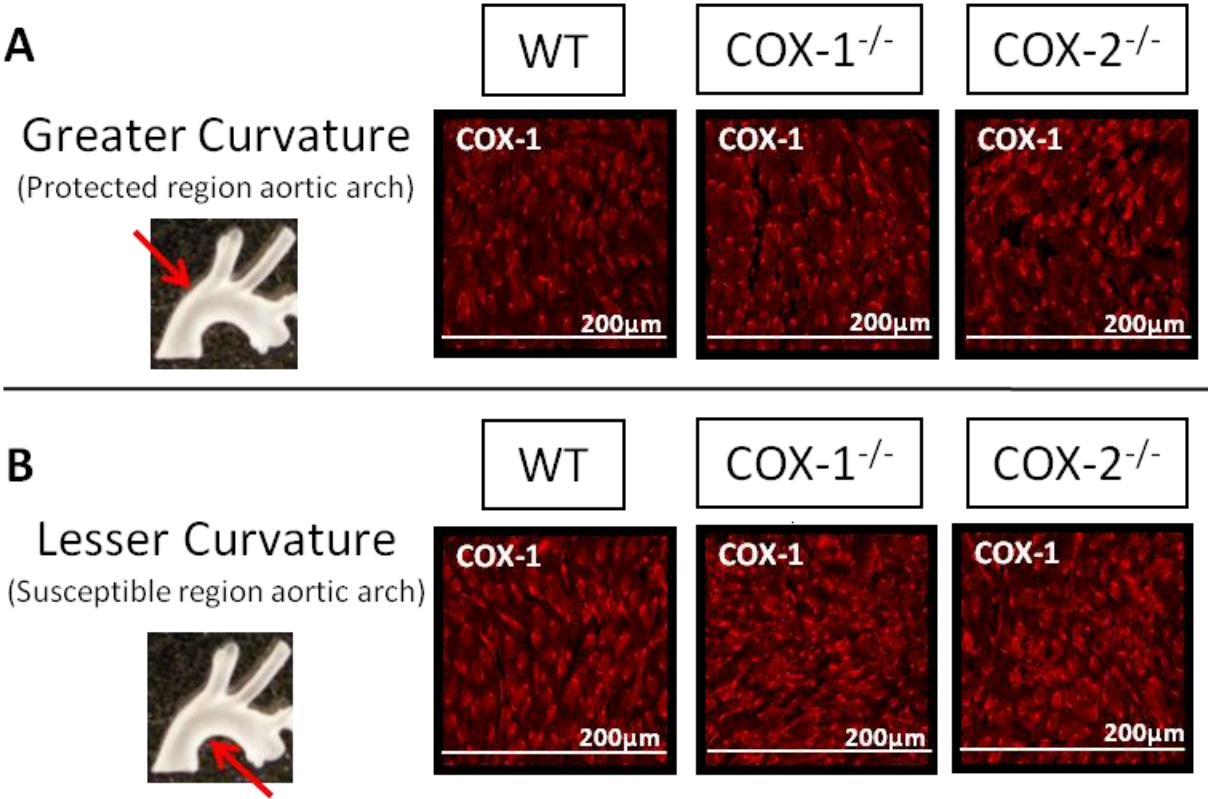


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Figure 3.16 (Continued) COX-1 immunoreactivity in the greater and the lesser curvature of aortic arches from young adult male WT, COX-1^{-/-} and COX-2^{-/-} mice

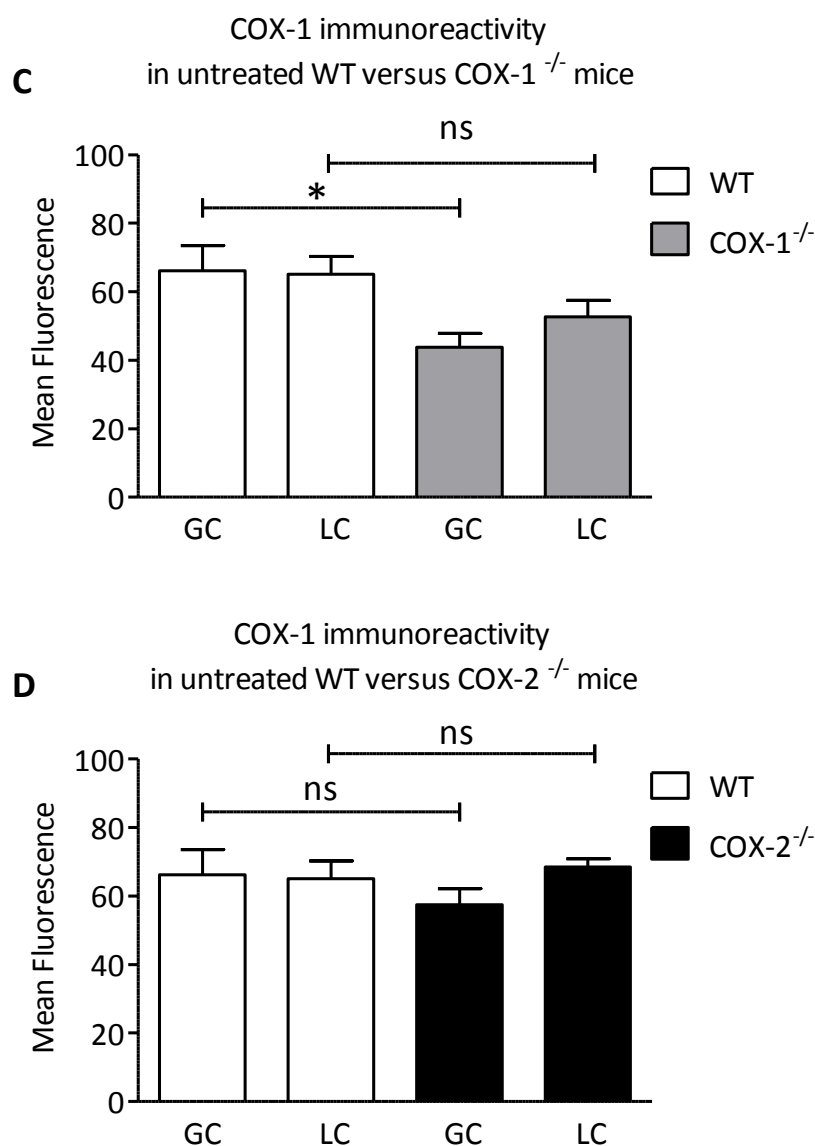


Figure 3.16 COX-1 immunoreactivity in the greater and the lesser curvature of aortic arches from young adult male WT, COX-1^{-/-} and COX-2^{-/-} mice. Representative cropped images (200x200μm) of COX-1 immunoreactivity (red) in aortic arches from untreated WT, COX-1^{-/-} and COX-2^{-/-} mice in (A) the greater curvature and (B) the lesser curvature. The aortic arches were fixed according to the ex vivo semi-perfusion fixation procedure. COX-1 immunoreactivity in the Greater Curvature (GC) and Lesser Curvature (LC) was quantified in untreated (C) WT versus COX-1^{-/-} mice and (D) WT versus COX-2^{-/-} as mean fluorescence. Data is the mean ± S.E.M from n=6-8 pictures, 2 separate images from each region from n=4 young adult (3 month old), male WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice. Secondary control background was subtracted from each image before analysis. Data was analysed using unpaired Student's t-test and $p < 0.05$ taken as significant and denoted by * and $p > 0.05$ was taken as non-significant and denoted by ns.

Figure 3.17 COX-1 immunoreactivity in COX-1^{-/-} with or without blocking peptide

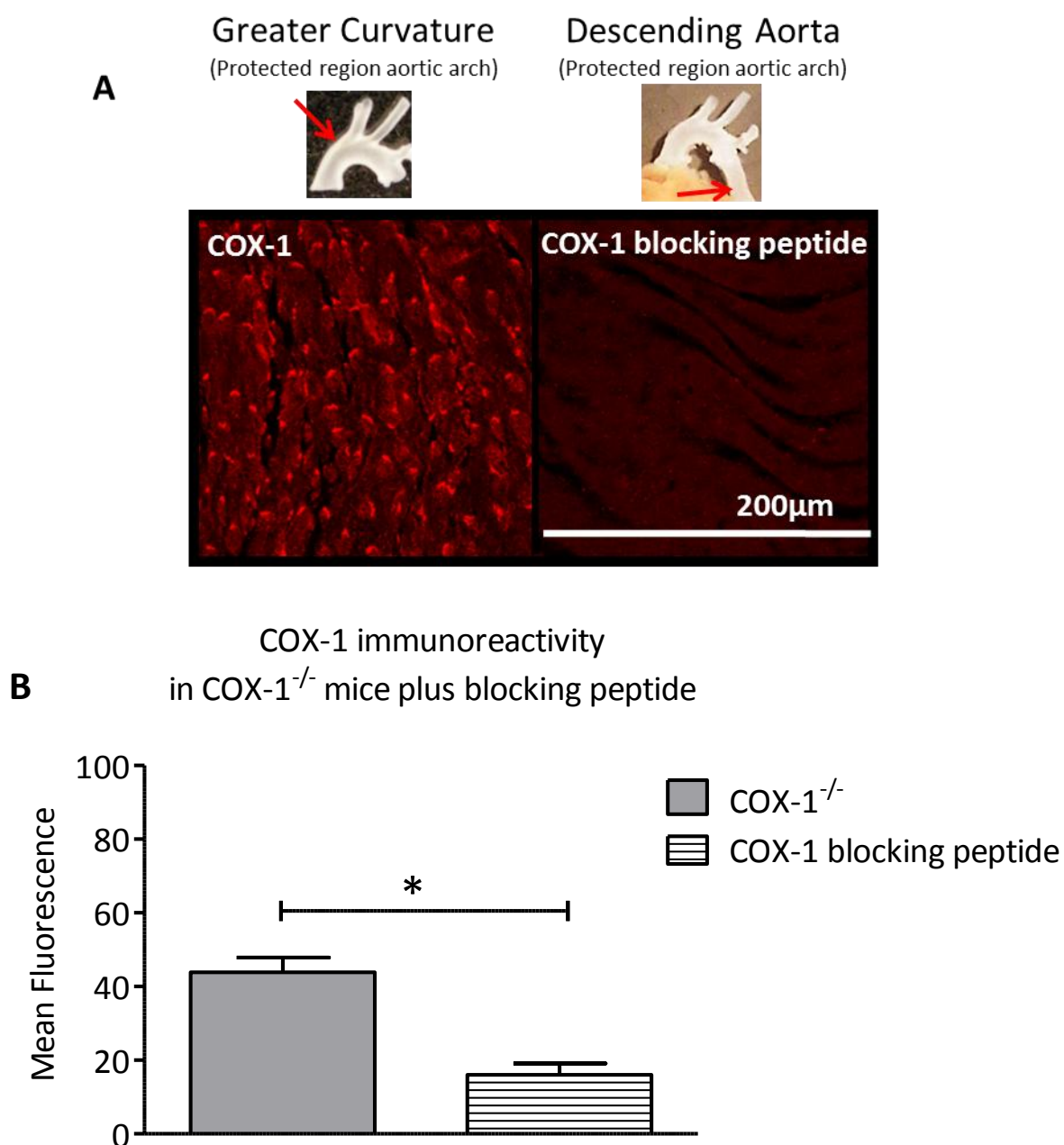


Figure 3.17 COX-1 immunoreactivity in COX-1^{-/-} with or without blocking peptide. (A) Representative cropped image (200x200μm) of COX-1 immunoreactivity (red) in the greater curvature of an aortic arch from an untreated COX-1^{-/-} mice and the descending aorta from the same animal after incubation with COX-1 blocking peptide (the precise epitope which the Cayman 160109 murine COX-1 antibody was raised against). Aortic arches (including the descending aorta) were fixed according to the ex vivo semi-perfusion fixation procedure. (B) COX-1 immunoreactivity quantified as mean fluorescence. Data is the mean ± S.E.M from n=8 pictures, 2 separate images from n=4 young adult (3 month old), male COX-1^{-/-} mice. Secondary control background was subtracted from each image before analysis. Data was analysed using unpaired Student's t-test and p<0.05 taken as significant and denoted by *.

Figure 3.18 COX-2 immunoreactivity in the greater and the lesser curvature of aortic arches from LPS treated young adult male WT, COX-1^{-/-} and COX-2^{-/-} mice

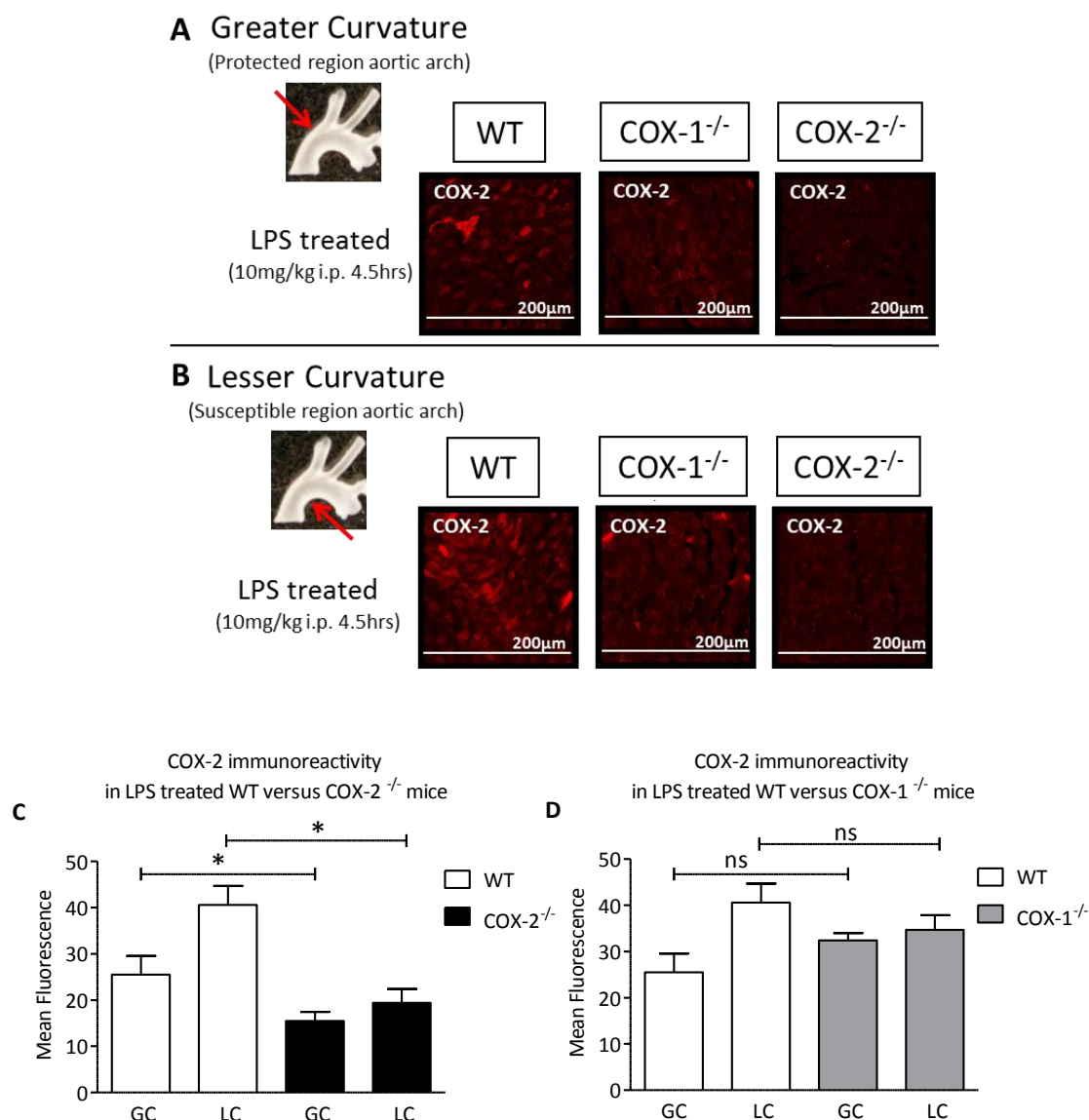


Figure 3.18 COX-2 immunoreactivity in the greater curvature of aortic arches from LPS treated young adult male WT, COX-1^{-/-} and COX-2^{-/-} mice. Representative cropped images (200x200μm) of COX-2 immunoreactivity (red) in aortic arches from untreated WT, COX-1^{-/-} and COX-2^{-/-} mice in (A) the greater curvature and (B) the lesser curvature in LPS treated conditions (*E.coli* serotype 055:B5, 10mg/kg i.p. for 4.5 hours). The aortic arches were fixed according to the ex vivo semi-perfusion fixation procedure. COX-2 immunoreactivity in the Greater Curvature (GC) and Lesser Curvature (LC) was quantified as mean fluorescence in LPS treated (C) WT versus COX-2^{-/-} mice and (D) WT versus COX-1^{-/-} mice. Data is the mean ± S.E.M from n=8 pictures, 2 separate images from each region from n=4 young adult (3 month old), male WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice. Secondary control background was subtracted from each image before analysis. Data was analysed using unpaired Student's t-test and p<0.05 taken as significant and denoted by * and p>0.05 was taken as non-significant and denoted by ns.

Figure 3.19 'Equilibrated study'; stimulated prostacyclin release from aortic arches and thoracic aorta from young adult WT, COX-1^{-/-} and COX-2^{-/-} mice

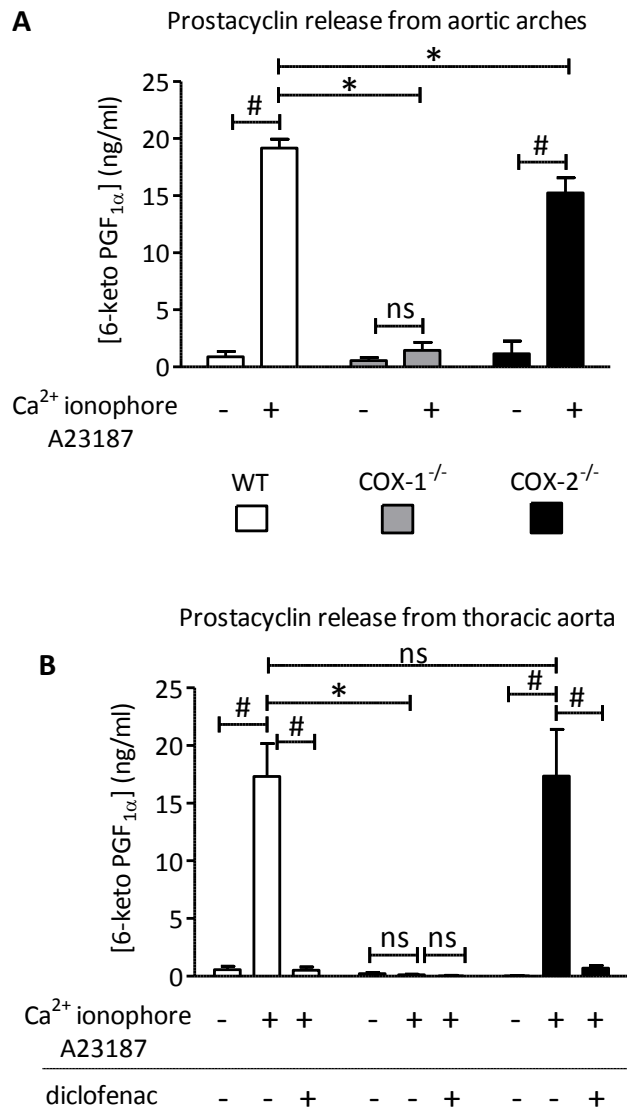


Figure 3.19 'Equilibrated study'; stimulated prostacyclin release from aortic arches and thoracic aorta from young adult WT, COX-1^{-/-} and COX-2^{-/-} mice. Prostacyclin release after 1.5 hours equilibration (-), and after 30 minutes calcium ionophore (A23187, 50μM) stimulation (+), from aortic arches (**A**) and thoracic aorta (**B**) from young adult (3 month old), male and female WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice, measured as the prostacyclin stable breakdown product 6-ketoPGF_{1α} by RIA. 100 μM diclofenac was added to the wells of some thoracic aorta segments during the equilibration phase (1.5 hours) and for the stimulation phase (30 minutes). Data is the mean ± S.E.M from n=6-7 animals. Within group analysis was performed using unpaired Student's t-test and p<0.05 taken as significant and denoted by #. Between groups analysis was performed using one-way ANOVA with Bonferroni's multi comparison post-test, p<0.05 was taken as significant and denoted by * and p>0.05 was taken as non-significant and denoted by ns.

Figure 3.20 'Acute study'; stimulated prostacyclin release from aortic arches and thoracic aorta from young adult WT, COX-1^{-/-} and COX-2^{-/-} mice

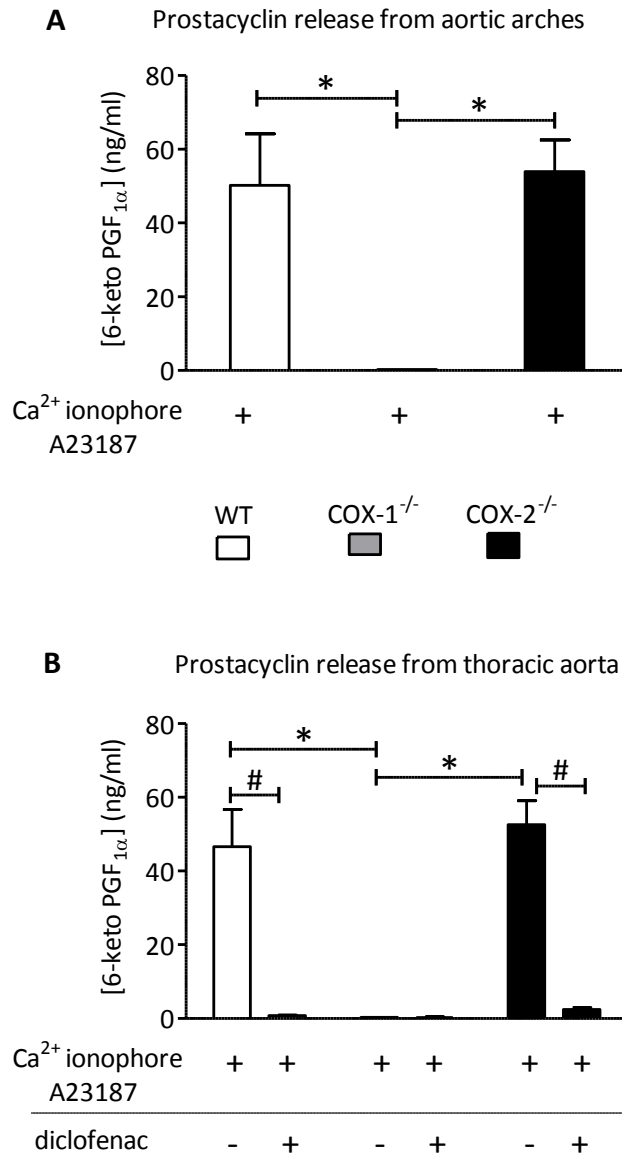


Figure 3.20 'Acute study'; stimulated prostacyclin release from aortic arches and thoracic aorta from young adult WT, COX-1^{-/-} and COX-2^{-/-} mice. Prostacyclin release after 30 minutes calcium ionophore (A23187, 50μM) stimulation (+), from aortic arches (**A**) and thoracic aorta (**B**) from young adult (3 month old), male and female WT (C57BL/6J, bred in-house) COX-1^{-/-} and COX-2^{-/-} mice, measured as the prostacyclin stable breakdown product 6-ketoPGF_{1α} by EIA. 100 μM diclofenac was added to the wells of some thoracic aorta segments during the stimulation phase (30 minutes). Data is the mean ± S.E.M from n=4 animals. Within group analysis was performed using unpaired Student's t-test and p<0.05 taken as significant and denoted by #. Between groups analysis was performed using one-way ANOVA with Bonferroni's multi comparison post-test, p<0.05 was taken as significant and denoted by*.

Figure 3.21 'Equilibrated study'; Pilot study ($n=2$) of stimulated prostacyclin and thromboxane release from aortic arches and thoracic aorta from untreated and LPS treated young adult WT, COX-1^{-/-} and COX-2^{-/-} mice

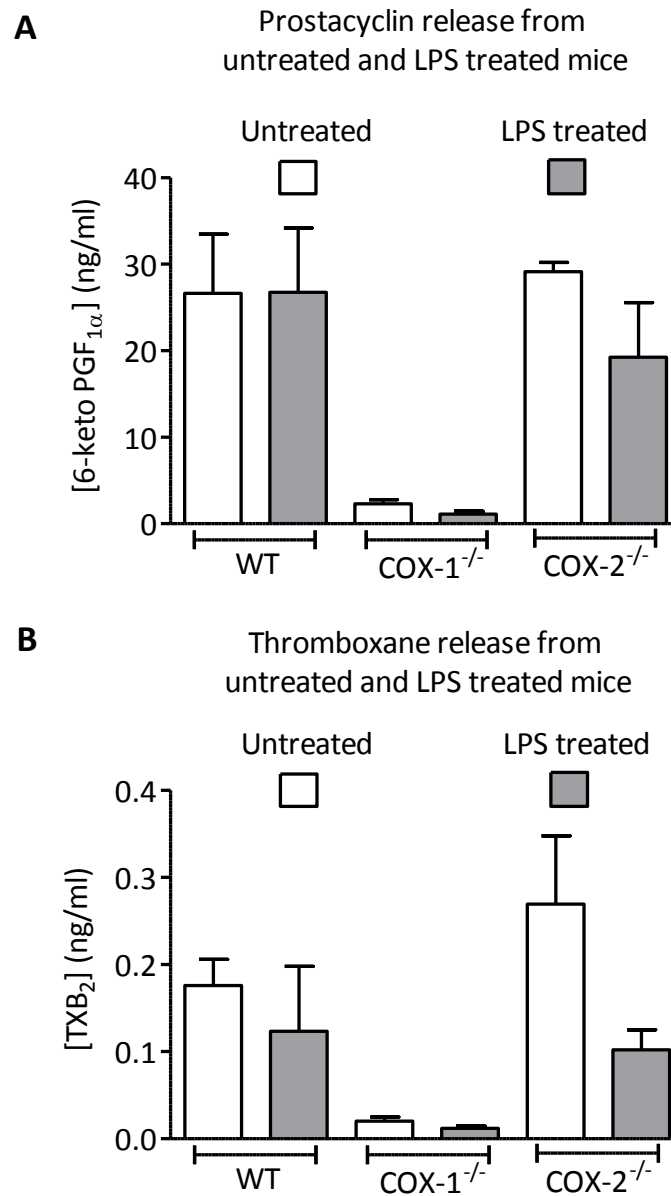


Figure 3.21 'Equilibrated study'; pilot study ($n=2$) of stimulated prostacyclin release from aortic arches and thoracic aorta from young adult WT, COX-1^{-/-} and COX-2^{-/-} mice. Stimulated prostacyclin (A) and thromboxane (B) release after 1.5 hours equilibration plus 30 minutes calcium ionophore (A23187, 50 μ M) stimulation, from thoracic aorta from young adult (3 month old), untreated and LPS treated (*E.coli* serotype 055:B5, 10mg/kg i.p. for 4.5 hours) male WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice. Prostacyclin release was measured as the stable breakdown product 6-ketoPGF_{1α} and thromboxane release was measured as the stable breakdown product TXB₂ by EIA. Data is the mean $\pm$ S.E.M from $n=2$ animals.

Summary of Chapter 3

Below is a summary of the results from this chapter and how they relate to the research questions that I set out to study:

How is the morphology of the endothelium and the underlying smooth muscle defined in the gross area of the mouse aortic arch?

Using tissue from young healthy mice, without cardiovascular disease, I have in this chapter characterized morphological phenotypes of endothelial cells enriched in areas of the aortic arch that correlates with different types of shear stress (Suo *et al.*, 2007). Cells in the greater curvature displayed characteristic elongated nuclei and were evenly spread out and aligned. In contrast, endothelial cells lining the lesser curvature had polygonally shaped nuclei and were randomly orientated in a more cobblestone fashion. The endothelial cells lining the 'interface region' (situated between the greater and the lesser curvature displayed a mixed morphology.

There was a distinct difference in nuclear density and of the endothelial cell marker, CD31 immunoreactivity, between the two regions of the arch. Nuclear density of the endothelial cells increased progressively from the greater curvature via the interface region to the lesser curvature whereas the opposite was shown for the CD31 immunoreactivity which declined in parallel. The pattern of the CD31 immunoreactivity also differed between the two regions, where the CD31 appeared more evenly expressed throughout the cytoplasm in the cells of the greater curvature it was more localised at the intracellular junctions in cells in the lesser curvature. There depth of the CD31 positive endothelial cell layer was not significantly different between the two regions.

The smooth muscle cell nuclei (directly underneath the endothelial cell layer) appeared to be more aligned in the greater curvature and randomly oriented in the lesser curvature, although the nuclear density was not different between the regions.

Which COX isoform predominates in vascular endothelium in situ and how does this relate to endothelial cell morphology?

This *en face* model was used to study relative COX expression in different areas of endothelium of the aortic arch, in order to examine how different shear stress profiles (laminar shear stress in the greater curvature and oscillatory shear stress in the lesser curvature), may affect constitutive COX expression and activity in young healthy mice.

- Tile composites covering a large area of aortic arch endothelium showed that COX-1 immunoreactivity was highly abundant with an intense perinuclear staining pattern in every endothelial cells lining both the atherosclerosis protected (greater curvature) and the atherosclerosis prone (lesser curvature) regions of the mouse aortic arch and thereby in each shear responsive endothelial phenotype represented in this vascular structure.
- There was no significant difference in COX-1 immunoreactivity in endothelial cells of the two regions in untreated mice. COX-1 immunoreactivity was, however increased in the endothelium of the lesser curvature region in mice treated with LPS.
- COX-1 immunoreactivity was enriched in the endothelial layer compared to the smooth muscle layer.
- COX-2 immunoreactivity in the endothelium of the aortic arch was overall very sparse and displayed a punctate staining pattern. However, COX-2 appeared to be modestly enriched in the atherosclerosis prone, lesser curvature of the mouse aortic arch compared to any other region of the arch.
- Similarly, preliminarily pilot data using human pulmonary artery showed that, COX-1 immunoreactivity was abundant and COX-2 immunoreactivity was sparse in the endothelium of this human vessel.
- Whilst COX-2 was low in endothelium of healthy, control mice, it was, as expected, induced when animals were treated with LPS and this COX2 induction was also significantly higher in the lesser curvature compared to the greater curvature.
- Specificity of COX primary antibodies was validated using respective blocking peptides.

- Some COX-1 immunoreactivity was detected in endothelium of COX-1^{-/-} mice which was reduced with the COX-1 blocking peptide, suggesting a truncated COX-1 protein persists in these mice.
- Levels of COX-1 immunoreactivity were not altered in COX-2^{-/-} mice compared to WT mice.
- COX-2 immunoreactivity in LPS treated COX-2^{-/-} mice was significantly reduced compared to WT mice.
- Counterpart COX isoforms were not up-regulated in endothelium of the COX^{-/-} mice.

Which COX isoform drives endothelial prostacyclin production in healthy blood vessels?

- Prostacyclin ‘basal’ release was very low in unstimulated tissues.
- Calcium ionophore stimulated prostacyclin release in thoracic and aortic arch tissue from WT and COX-2^{-/-} mice. Thromboxane release was low under all conditions and in tissue from each type of mouse.
- This bioassay protocol was validated using the COX-1 and COX-2 inhibitor (NSAID) diclofenac, which completely inhibited the stimulated prostacyclin release.
- Prostacyclin release was very low or absent in thoracic aorta or aortic arch tissue from COX-1^{-/-} mice even after stimulation with calcium ionophore.
- Similar results were obtained using a second protocol, without the equilibration step, ‘acute’ protocol, where prostacyclin release from either thoracic aorta or aortic arch was abundant in tissue from WT and COX-2^{-/-} mice, but abolished in tissue from COX-1^{-/-} mice.
- This pattern of COX-1 bioactivity remained the same in tissue from mice treated with LPS.

These experiments indicate that the enzyme responsible for prostacyclin production in aortic tissue from young healthy mice is mainly COX-1. The implications of these observations are discussed in detail in my General Discussion chapter (Chapter 6).

Chapter 4

Effect of age, gender and heart disease on expression and activity of COX isoforms in the aortic endothelium

Rationale behind Chapter 4

The results from the previous chapter indicate that COX-1 immunoreactivity predominates in the endothelium of both the atherosclerosis protected (greater curvature) and inflammation and atherosclerosis susceptible (lesser curvature) regions of the aortic arch from healthy young adult male mice and that COX-1 enzyme activity accounts for the majority of the prostacyclin production in blood vessels from these mice. COX-1 therefore seems to be the main constitutively expressed endothelial COX isoform, responsible for prostacyclin production in healthy tissues.

It is however important to consider that COX activity and isoform expression may differ between males and females (Akarasereenont *et al.*, 2000; Egan *et al.*, 2004). Furthermore, cardiovascular homeostasis and risk of disease is known to be influenced by the sex of the animal. In humans, for example women are somewhat protected from heart attack and stroke up until the age of menopause (Kannel *et al.*, 1976). This chapter therefore includes experiments where I examined COX immunoreactivity in tissue from healthy female mice and sub analysed data for COX bioactivity between male versus female mice.

There is also literature that suggests that COX-2 in the endothelium is increased as we age and/or with endothelial dysfunction associated with chronic cardiovascular conditions (Heymes *et al.*, 2000; Wu *et al.*, 1998). This is a relevant argument considering that the target patient group for chronic dosing of NSAIDs and COX-2 selective inhibitors are often elderly patients with arthritis. It has been shown that the negative effect of age on endothelial function in mice is apparent by 12 months (Gendron *et al.*, 2007). I therefore compared results in tissue from young (3 month old) as well as mature (12 month old) mice to see if there is any age related change in endothelial COX-1 and COX-2 expression and prostacyclin production.

Finally, in order to understand how gross cardiovascular disease/inflammation may affect the relative expression and activity of COX isoforms in the endothelium I studied vascular tissue from animal models of (i) heart failure and (ii) atherosclerosis.

In summary, I have in this chapter specifically compared the role of COX-1 and COX-2 in blood vessels from young adult versus mature adult mice, in male versus female mice, in tissue from control rats versus rats with chronic heart failure and in WT mice versus ApoE^{-/-} mice fed either normal chow or an atherogenic diet.

The aims and research questions of this chapter can be summarised as follows:

Which COX isoform(s) predominate in vascular endothelium in female mice and in mature adult (12 month old) WT mice?

- Examine COX-1 and COX-2 immunoreactivity in endothelial cells lining the atherosclerosis-protected greater curvature and the atherosclerosis-prone lesser curvature of the mouse aortic arch from young adult (3 month old) female WT mice and from mature adult (12 month old) WT mice.

Which COX isoform drives endothelial prostacyclin production in female mice and in mature adult (12 month old) WT mice?

- Establish the functional relevance of COX-1 versus COX-2 in the aorta in terms of prostacyclin releasing capacity by using tissue from young (3 month old) and mature adult (12 month old) male versus female, WT versus COX-1^{-/-} and COX-2^{-/-} mice.

Does the relative expression pattern of endothelial COX-1 and COX-2 or prostanoid profile released from vessels change following cardiovascular disease (rat model of chronic heart failure or mouse model of atherosclerosis)?

- Repeat the COX *en face* immunohistochemistry protocol and stimulated prostanoid release in rats with chronic heart failure and in mice with early markers of atherogenesis and vascular inflammation (in ApoE^{-/-} mice fed a normal chow diet) and with established atherosclerotic plaques (in ApoE^{-/-} mice fed an atherogenic diet).

Methods

Animals

WT, COX-1^{-/-} and COX-2^{-/-} mice

WT, COX-1^{-/-} and COX-2^{-/-} mice were bred, maintained and genotyped as described in the General Methods Chapter 2. For this chapter, animals were male and female and used at two age groups; young adult (3-4 month old) and mature adult (11-16 months old) mice. All WT mice were from the C57BL/6J strain; some WT were purchased from Harlan, UK, whilst other WT were bred in-house with a shared background to match the COX-1^{-/-} and COX-2^{-/-} mice (backcrossed to C57BL/6J, Harlan, UK, more than 7 times) see General Methods Chapter 2. Purchased WT mice will be referred to as 'C57BL/6J, Harlan, UK' and the other COX colony WT controls will be referred to as 'C57BL/6J, bred in-house'. Mice were killed by CO₂ asphyxiation and aortic arches were dissected out and fixed with 2% formaldehyde according to the '*ex vivo semi-perfusion fixation procedure*' or the '*transcardial perfusion fixation procedure*' and staining protocols described in the General Methods Chapter 2 (Table 2.18). COX and CD31 immunoreactivity was measured in the endothelium of the aortic arch as described in Chapter 3 and as detailed in the General Methods Chapter 2. COX activity was also measured in tissue from WT and COX-1^{-/-} and COX-2^{-/-} mice according to the '*equilibrated study*' or '*acute study*' protocol as described in Chapter 3 and as detailed in the General Methods Chapter 2. Stimulated prostacyclin release data from Chapter 3 (Figure 3.19 and Figure 3.20) including the '*equilibrated study*' and the '*acute study*' protocol were included for post-hoc analysis of male versus female prostacyclin release from aortic arches and thoracic aorta.

ApoE^{-/-} mice

In this thesis I have performed experiments in both normal chow fed ApoE^{-/-} mice (ApoE^{tm1Unc} from a C57BL/6J background) as well as ApoE^{-/-} mice that have been fed an atherogenic diet, in order to consider a model of early atherogenesis as well as more advanced and established atherosclerosis respectively (for more details see General methods Chapter 2).

Normal chow fed ApoE^{-/-} mice as a model of early atherogenesis

The normal chow fed ApoE^{-/-} mice and WT controls (C57BL/6J, bred in-house) were killed by CO₂ asphyxiation and aortic arches were dissected out and fixed with 2% formaldehyde according to the '*transcardial perfusion fixation procedure*' and staining protocols for COX immunoreactivity as described in the General Methods Chapter 2 (Table 2.18).

High cholesterol chow fed ApoE^{-/-} mice as a model of accelerated atherosclerosis

These ApoE^{-/-} mice were fed an atherogenic high fat and high cholesterol diet (21% fat, 1.25% cholesterol, cholate-free; Research Diets, D12108) starting from 12 weeks of age for 10 weeks in order to accelerate the rate of atherosclerotic plaque formation.

For illustrative purposes and as a pilot study, I was able to image and quantify some of these functional changes in the aortic arch of a high cholesterol chow fed ApoE^{-/-} mouse which had gone on to develop profound atherosclerosis (due to the high fat feeding). This first high cholesterol chow fed ApoE^{-/-} mouse (female) was perfused according to the '*transcardial perfusion fixation procedure*' whereas due to an animal share, the remaining n=4 male and female high cholesterol chow fed ApoE^{-/-} mice were perfused according to the '*ex vivo semi-perfusion fixation procedure*'.

Heart failure model; Rats

In this chapter I have examined COX activity and expression in a heart failure model in rats. The full details of the model are given in the General Methods Chapter 2. The surgery, maintenance and husbandry of the rats were performed by our collaborator Dr Alexander Lyon (Imperial College). In brief, male Sprague Dawley rats (250-300g, 2-3 month old) were anaesthetized and the left proximal coronary artery was ligated 1mm below the left atrial appendage, encompassing both the proximal left anterior descending artery and first diagonal coronary arteries, giving a large infarct size (~30% left ventricular myocardium) to induce chronic myocardial infarction. Animals were allowed to recover and were followed for approximately 4 months after the procedure, where markers of heart failure manifest.

These are described in detail in the General Methods Chapter 2. For this study, rats with established chronic heart failure rats or age-matched controls (male Sprague Dawley rats) were anaesthetised with isoflurane (5% induction and 3% maintenance) and buprenorphine (opioid for pain relief, 0.05ml of 1mg/ml) was administered subcutaneously. Unfractionated heparin (100-200 units) was injected intravenously prior to dissection. The aortic arch (including carotid artery branches) and the thoracic aorta was removed immediately after death and placed in PBS. The thoracic aorta was prepared for the '*equilibrated study*' protocol of stimulated prostanoid release, whilst the aortic arch fixed in 2% paraformaldehyde for 20 minutes before the start of the COX immunoreactivity staining protocol according to details in the General Methods Chapter 2.

For antibody validation purposes, segments of an abdominal aorta (from a control rat, n=1) were incubated overnight in 37°C +/- LPS (E.coli serotype 0111:B4, 10µg/ml) in order to serve as a positive control for COX-2 induction. These segments were then subject to the '*equilibrated study*' protocol of stimulated prostanoid release, whilst the aortic arch fixed in 2% paraformaldehyde for 20 minutes before the start of the COX immunoreactivity staining protocol according to details in the General Methods Chapter 2 (Table 2.18).

En Face imaging of the arch lumen

In this chapter I have imaged CD31, COX-1 and COX-2 using antibodies and the stain DAPI to visualise nuclei as described in the General Methods Chapter 2 (Table 2.18). Mean fluorescence was quantified using LAS AF software. COX-1 and COX-2 immunoreactivity was measured in the endothelium of the aortic arch, the descending thoracic aorta, the carotid branches (brachiocephalic artery, left common carotid artery and the left subclavian artery) according to protocols described in the General Methods Chapter 2.

Measurement of COX activity

COX activity was measured in the male versus female mice, mature adult mice and rats with or without chronic heart failure, after equilibration (according to the '*equilibrated study*' protocol, see Chapter 2), in thoracic aortic tissue stimulated with calcium ionophore (A23187, 50 μ M) for 30 minutes. COX activity was indexed by measuring levels of prostacyclin (as the stable breakdown product 6-ketoPGF_{1 α} ; by EIA assay from Cayman Chemical), PGE₂ by HTRF assay (Cisbo), or TXA₂ (measured as the stable breakdown product TXB₂; by EIA assay from Cayman Chemical).

Analysis and statistics

Data is presented as the mean $\pm$ S.E.M and was analysed using Graph Pad Prism 5 using statistical tests described in the figure legends. A p value of <0.05 was taken as significant and denoted by * and p>0.05 was considered non-significant and denoted by ns.

Results

COX isoform expression and activity in endothelium of male versus female young adult mice

Similar to the observations made in male mice in the previous chapter, I found that COX-1 immunoreactivity predominated vastly over COX-2 immunoreactivity in female mice and the COX-2 immunoreactivity (although sparse) was significantly higher in the lesser curvature compared to the greater curvature (Figure 4.1A+B+C). In a sub-analysis of data presented in the previous chapter, prostacyclin release from aortic arches or thoracic aortas from young adult (3 month old) female mice (n=2) was virtually absent in tissue from COX-1^{-/-} mice and being unaffected by COX-2 gene deletion (Figure 4.2). This was true using both the 'equilibrated' and the 'acute' bioassay protocols (Figure 4.2A+B+C+D). No discernible difference was seen in prostacyclin release from tissue of male versus female young adult (3 month old) mice (Figure 4.2) or older mature adult (12 month old) mice (Figure 4.3).

COX isoform expression in aortic arch endothelium from mature adult (12 month old) mice

Mice reach sexual maturity at approximately 2 months. At 3-6 months mice are considered mature but still young with no particular signs of cardiovascular ageing. At 12 plus months mice are considered mature/middle aged with significant signs of vascular aging (Gendron *et al.*, 2007). As was seen in the endothelium of the aortic arch of young adult (3 month old) mice, COX-1 was readily detected in tissue from mature adult (12 month old) mice in a similar perinuclear staining pattern as observed in young adult (3 month old) mice (Figure 4.4C+D and Figure 4.6). Again, as was seen in tissue from young adult (3 month old) mice, COX-2 immunoreactivity was sparse in the endothelium of the aortic arch from mature adult (12 month old) mice and showed a similar punctate staining pattern (Figure 4.5C+D and Figure 4.6). Levels of mean fluorescence were comparable for COX-1 and COX-2 in tissue from young adult (3 month old) versus mature adult (12 month old) mice (Figure 4.6C)

COX activity in endothelium from mature adult (12 month old) mice

In a pilot study at the beginning of my PhD, where we had WT mice and COX-1^{-/-} mice, but not COX-2^{-/-} mice, I was able to measure prostacyclin release from thoracic aorta from mature adult (12 month old) mice, using the same ‘equilibrated’ protocol I described in Chapter 3. In this early study I found that prostacyclin releasing capacity of aortic tissue was greatly reduced in tissue from COX-1^{-/-} mice (Figure 4.7). Once we had enough animals from the COX-2^{-/-} colony, I then repeated this entire experiment and compared the ability of thoracic aortic tissue from WT, COX-1^{-/-} and COX-2^{-/-} mature adult (12 month old) mice (Figure 4.8). Again, I found that prostacyclin release was virtually absent in aortic tissue from COX-1^{-/-}, but not COX-2^{-/-} mature adult (12 month old) mice (Figure 4.8). Figure 4.8 confirms that, in this second data set, COX-1 again predominates over COX-2 in the aortic endothelium of 12 month aged mice and additionally shows no discernible compensatory effects on COX-1 or COX-2 expression in tissue from COX-1^{-/-} and COX-2^{-/-} mice (Figure 4.9).

COX isoform expression in endothelium from aged matched control rats versus rats with chronic heart failure

As with endothelium from mouse aortic tissue, COX-1 predominates over COX-2 immunoreactivity in aortic arch endothelium from aged matched control rats and rats with established heart failure, and both the COX-1 and COX-2 immunoreactivity was blocked by the addition of COX-1 and COX-2 blocking peptides (Figure 4.10). Similar observations were made in the carotid artery branches of the aortic arch (Figure 4.11). No significant effect of heart failure was seen on COX-1 or COX-2 immunoreactivity (Figure 4.10 and Figure 4.11).

Validation studies for the COX immunoreactivity in rat endothelium

There was no significant difference between the non-specific background immunoreactivity between aged matched control rats and rats with chronic heart failure (Figure 4.12). In order to be sure that the COX-2 antibody (Cayman 160126) was indeed able to detect COX-2 in rat tissue, I performed a validation study where segments of abdominal aorta from an aged matched control rat were incubated with or without LPS overnight and COX-2 immunoreactivity imaged. In these experiments, COX-2 immunoreactivity was easily detectable, increased by LPS and blocked by COX-2 blocking peptide (Figure 4.13).

COX prostanoid profile bioactivity in endothelium from aged matched control rats versus rats with chronic heart failure

In line with COX immunoreactivity, COX activity, measured by prostacyclin, thromboxane and PGE₂ release, was similar in tissue from aged matched control and heart failure animals (Figure 4.14A). In a post-hoc analysis of the rats with the highest myocardial infarction score (n=2 out of n=4 rats with heart failure), there was still no difference compared to n=2 aged matched controls in terms of COX immunoreactivity or prostanoid profile. As was seen with the mouse tissue, prostacyclin was the major COX product released from these vessels. Although, PGE₂ release was as expected increased from the abdominal aorta that had been treated over night with LPS treated (Figure 4.14B).

COX isoform expression in aortic endothelium from WT versus normal chow fed ApoE^{-/-} mice

As was found in tissue from WT mice; COX-1 immunoreactivity was highly abundant in endothelium from the descending aorta from ApoE^{-/-} mice (when fed a normal chow diet), whereas COX-2 immunoreactivity in endothelium from the adjacent mid arch part of the aortic arch was very sparse (Figure 4.15). Furthermore, there was no significant difference in COX-1 or COX-2 immunoreactivity between the WT mice and ApoE^{-/-} mice in both the endothelial and underlying smooth muscle layers of these tissues (Figure 4.16A+B).

En face imaging of a large plaque like structure in the aortic arch from an ApoE^{-/-} mouse fed an atherogenic diet

I was able to repeat the *en face* imaging protocol In an ApoE^{-/-} mouse fed an atherogenic diet, which upon micro dissection had distinct yellow/white plaque like structures inside the lesser curvature of the aortic arch, the mid arch and the proximal part of the descending aorta as well as dense and calcified aortic valves (Figure 4.17A).

The superficial layer of the plaque had CD31 positive endothelium covering most of the plaque (although somewhat fainter in intensity and more localised to intracellular junctions compared to plaque free 'normal looking' adjacent areas of endothelium). There was also a strikingly higher nuclear density (visualised by the nuclear stain DAPI) (Figure 4.17C). Since DAPI does not distinguish between different cell types, other more specific markers would

be needed for closer examination of the cellular and non-cellular composite of this plaque like structure.

COX isoform expression in aortic endothelium from ApoE^{-/-} mice fed an atherogenic diet

When the mid arch and the proximal descending aorta from the same ApoE^{-/-} mouse (fed an atherogenic diet) was stained with COX-1 and COX-2, there was clear COX-1 and COX-2 immunoreactivity enriched on top and inside the plaque (Figure 4.18 and 4.19). When this study was repeated in 4 other mice and the data from these mice were quantified, there was a trend for enriched COX-1 and COX-2 immunoreactivity in the plaque like structure, but this did not reach statistical significance (Figure 4.19C+D).

It is relevant and important to note that in experiments performed by my colleague, Dr Nicholas Kirkby, prostacyclin release by COX-2 knock out ApoE^{-/-} mice (double knockout mice; ApoE^{-/-} COX-2^{-/-}) show that, even in atherosclerotic enriched vascular tissue, COX-1 drives gross prostacyclin production.

Figures

Figure 4.1 COX-1 versus COX-2 immunoreactivity in the greater and the lesser curvature of the mouse aortic arch from young adult *female* WT mice

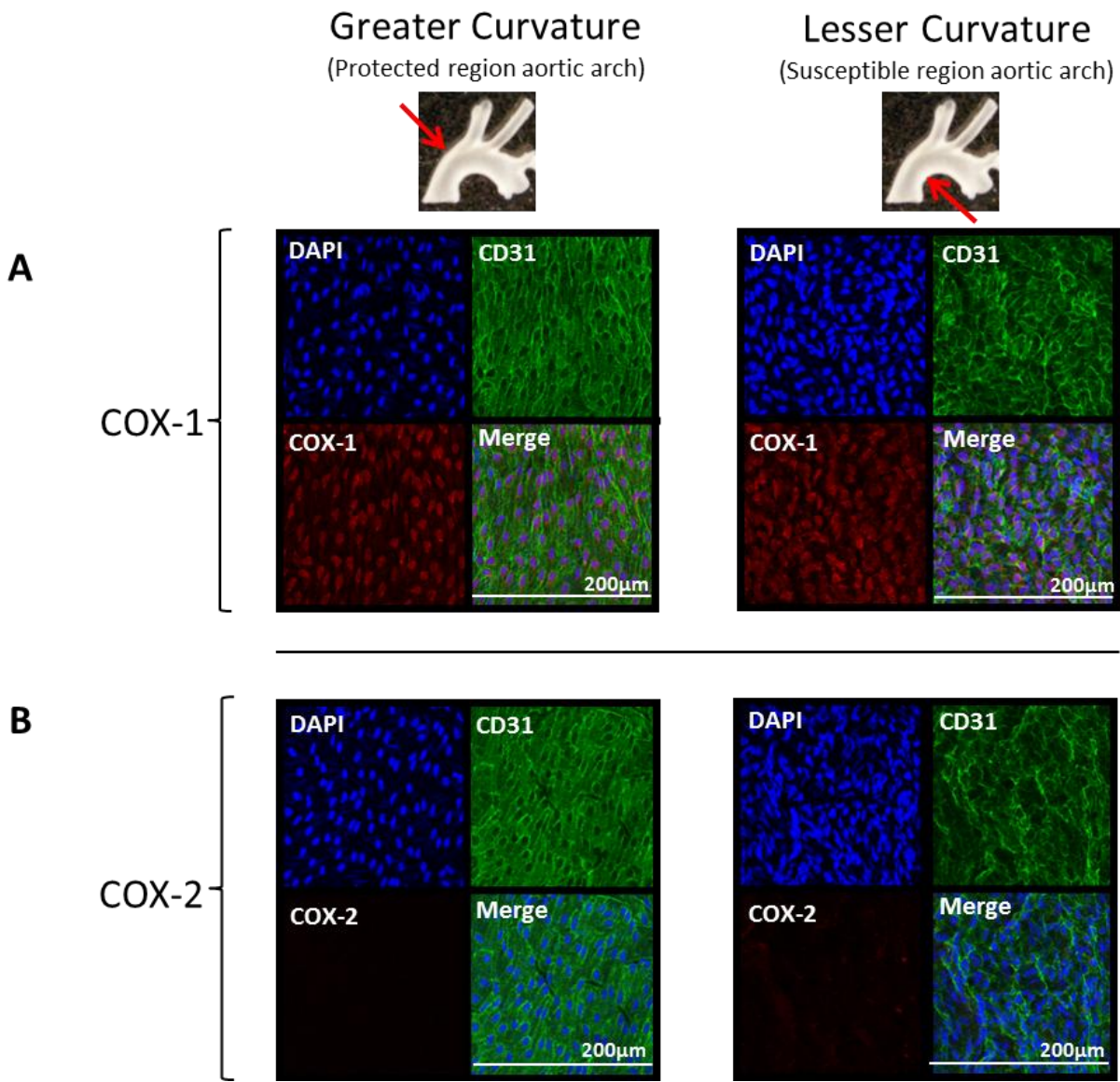


Figure 4.1 continues on the next page.

Figure 4.1 (Continued) COX-1 versus COX-2 immunoreactivity in the greater and the lesser curvature of the mouse aortic arch from young adult **female** WT mice

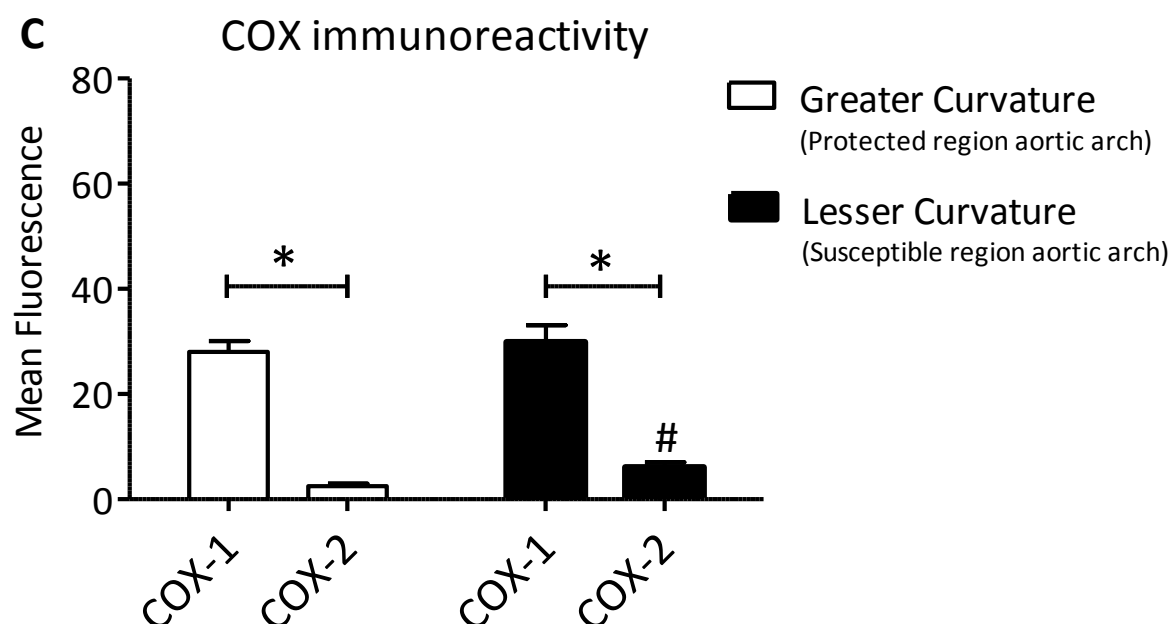


Figure 4.1 COX-1 versus COX-2 immunoreactivity in the greater and the lesser curvature of the mouse aortic arch from young adult female WT mice. Representative cropped images (200x200 μ m) of (A) COX-1 immunoreactivity and (B) COX-2 immunoreactivity (red), from the greater and the lesser curvature of the mouse aortic arch. Aortic arches were fixed according to the transcardial perfusion fixation procedure. Nuclei visualised with DAPI (blue) and endothelial cells outlined with CD31 (green). (C) Pooled data comparing COX-1 and COX-2 immunoreactivity in $n=4$ female WT mice from head to head experiments (same experimental conditions, staining protocol and secondary antibody Alexa Fluor 594 was used to capture both COX-1 and COX-2 immunoreactivity). 3 unique images were captured from the greater curvature and the lesser curvature respectively, using identical confocal settings throughout the study. COX immunoreactivity was quantified as mean fluorescence. Secondary control background was subtracted from each image before analysis. Data is presented as the mean $\pm$ S.E.M of $n=9-12$ images from separate regions of tissue from $n=4$ WT mice young adult (3 month old), female mice (C57BL/6J, bred in-house). Within group analysis (greater curvature versus the lesser curvature) was performed using paired Student's t -test and $p<0.05$ was taken as significant and denoted by #. Between groups analysis was analysed using one-way ANOVA with Bonferroni's multi comparison post-test and $*p<0.05$ was taken as significant and denoted by *.

Figure 4.2 'Equilibrated study' and 'Acute study'; stimulated prostacyclin release from aortic arches and thoracic aorta from young adult **male versus female** WT, COX-1^{-/-} and COX-2^{-/-} mice

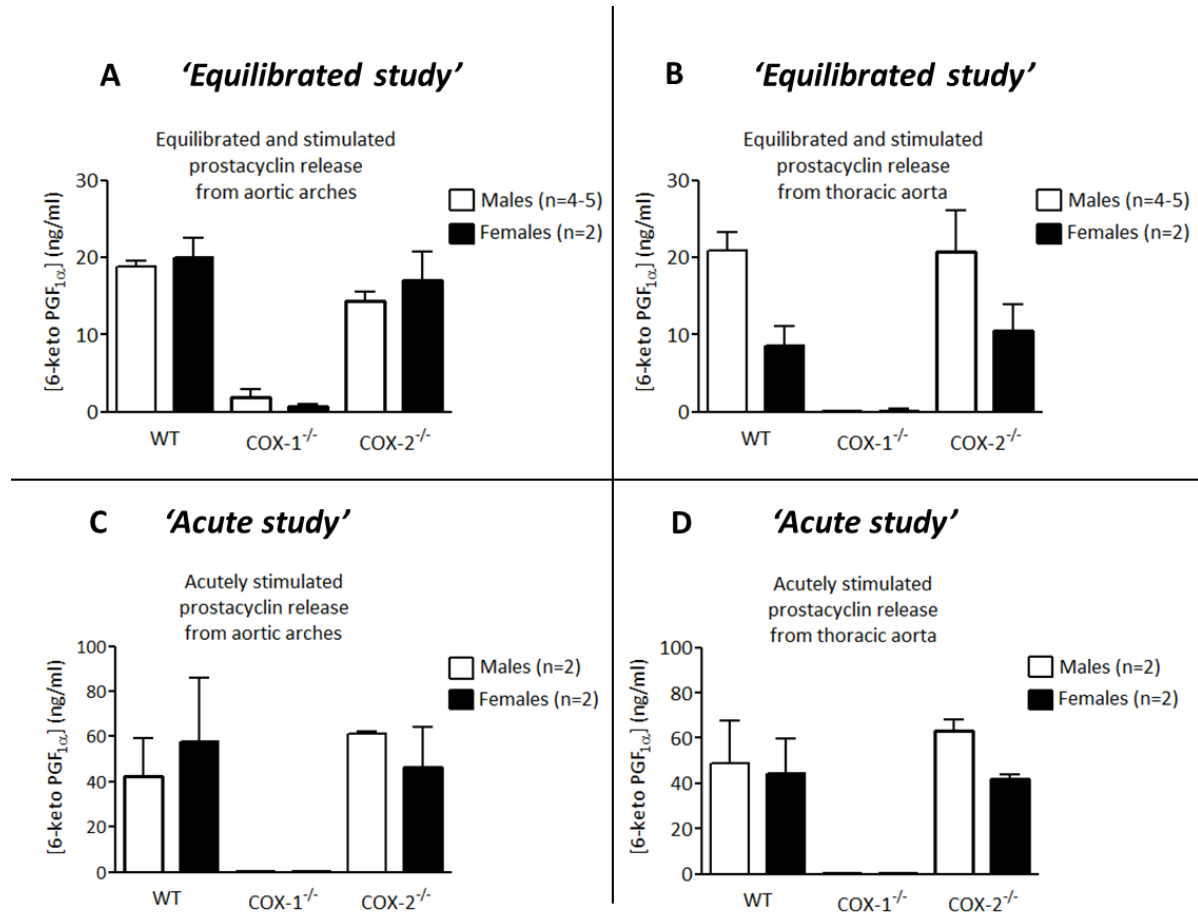


Figure 4.2 'Equilibrated study' and 'Acute study'; stimulated prostacyclin release from aortic arches and thoracic aorta from young adult **male versus female** WT, COX-1^{-/-} and COX-2^{-/-} mice.

'Equilibrated study': Stimulated prostacyclin release measured from (A) aortic arches and (B) thoracic aorta after 1.5 hours equilibration and 30 minutes calcium ionophore (A23187, 50μM) stimulation, measured as the prostacyclin stable breakdown product 6-ketoPGF_{1α} by RIA. Data is presented as the mean ± S.E.M from n=4-5 male and n=2 female young adult (3 month old) WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice.

'Acute study': Stimulated prostacyclin release measured from (B) aortic arches and (C) thoracic aorta after 30 minutes calcium ionophore (A23187, 50μM) stimulation, measured as the prostacyclin stable breakdown product 6-ketoPGF_{1α} by EIA. Data is the mean ± S.E.M from n=2 male and n=2 female young adult (3 month old) WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice.

Data represents post hoc analysis of prostacyclin release from male versus female mice from pooled data (presented in Figure 3.18 and 3.19 in Chapter 3).

Figure 4.3 'Equilibrated study' stimulated prostacyclin release from thoracic aorta from mature adult, male versus female WT mice

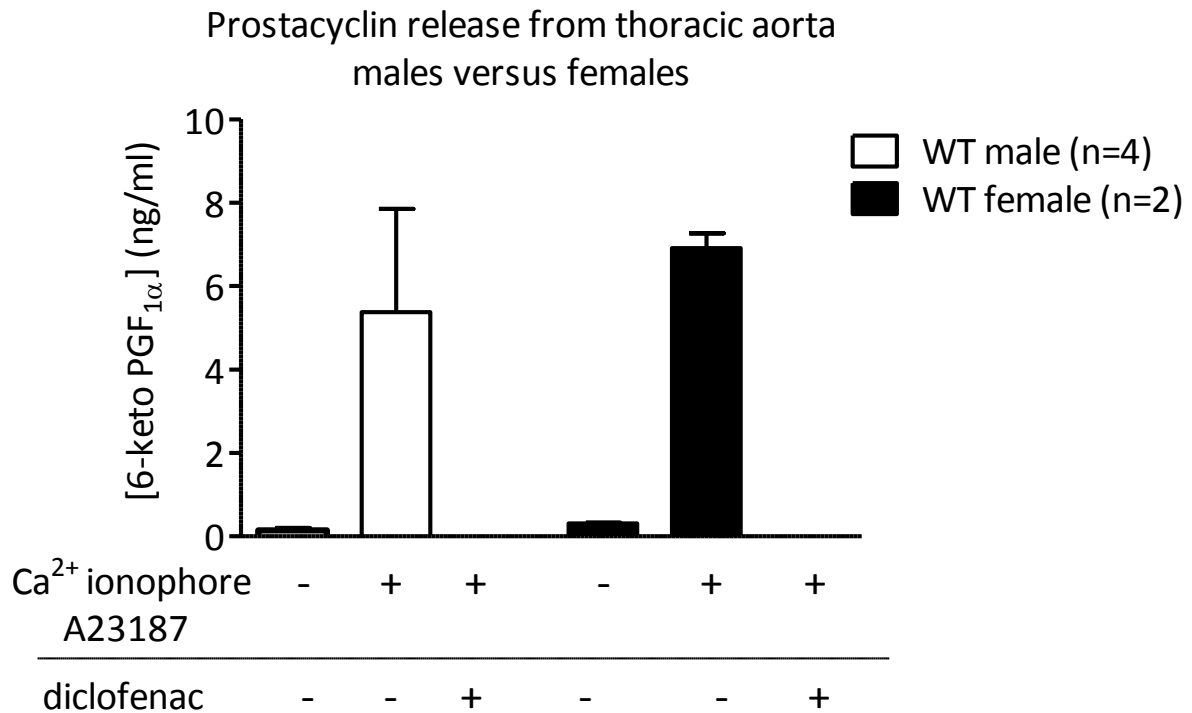


Figure 4.3 'Equilibrated study' stimulated prostacyclin release from thoracic aorta from mature adult, male versus female WT mice. Stimulated prostacyclin release measured from thoracic aorta after (-) 1.5 hours equilibration and (+) after 30 minutes calcium ionophore (A23187, 50 μ M) stimulation, measured as the prostacyclin stable breakdown product 6-ketoPGF_{1 α} by EIA. 100 μ M diclofenac was added to the wells of some thoracic aorta segments during the stimulation phase (30 minutes). Data is presented as the mean $\pm$ S.E.M from n=4 male and n=2 female mature adult (10-16 month old) WT mice (C57BL/6J, bred in-house). Data represents post hoc analysis of prostacyclin release from male versus female mice from pooled data (Figure 4.8).

Figure 4.4 Tile composite of 4x4 images showing COX-1 immunoreactivity in the greater and the lesser and curvature of the aortic arch from a mature adult WT mouse

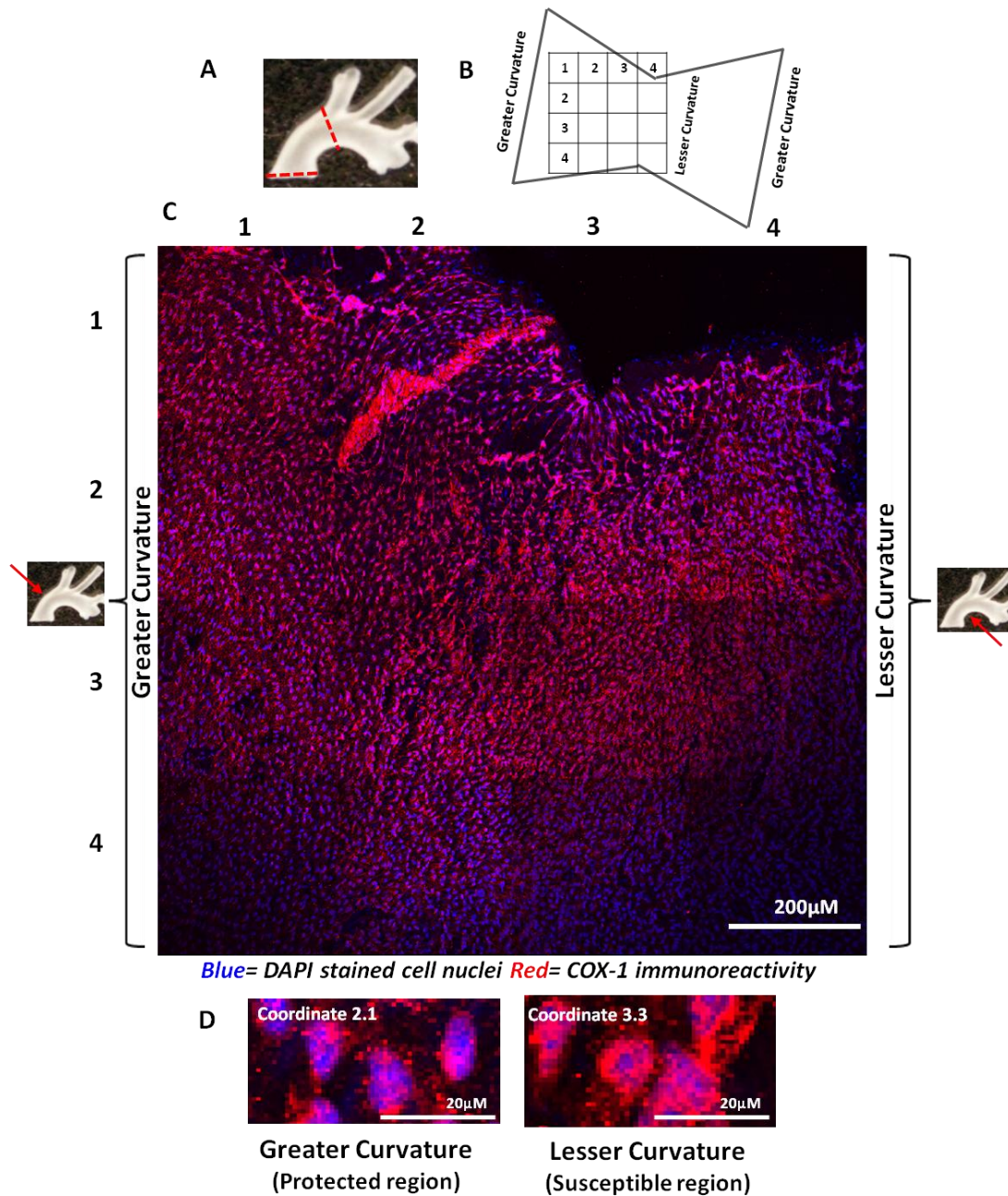


Figure 4.4 Tile composite of 4x4 images showing COX-1 immunoreactivity in the lesser and greater curvature of the aortic arch from a mature mouse. Images from a perfusion fixed (according to the transcatheter perfusion fixation procedure) aortic arch dissected from a mature adult (12 month old) male WT mouse (C57BL/6J, Harlan, UK). **(A)** Red dotted line, mark dissection cutting points. **(B)** A schematic of the mounted (opened and flattened) aortic arch, marking the location of the greater and the lesser curvature as well as the position of the tile composite (4x4 images, coordinates 1.1-4.4). **(C)** The entire tile of 4x4 images. DAPI stained nuclei (**blue**) and COX-1 immunoreactivity (**red**). **(D)** Zoomed in images (from coordinates 2.1 and 3.3) showing intracellular localisation of COX-1 immunoreactivity.

Figure 4.5 Tile composite of 4x4 images showing COX-2 immunoreactivity in the greater and the lesser curvature of the aortic arch from a mature adult WT mouse

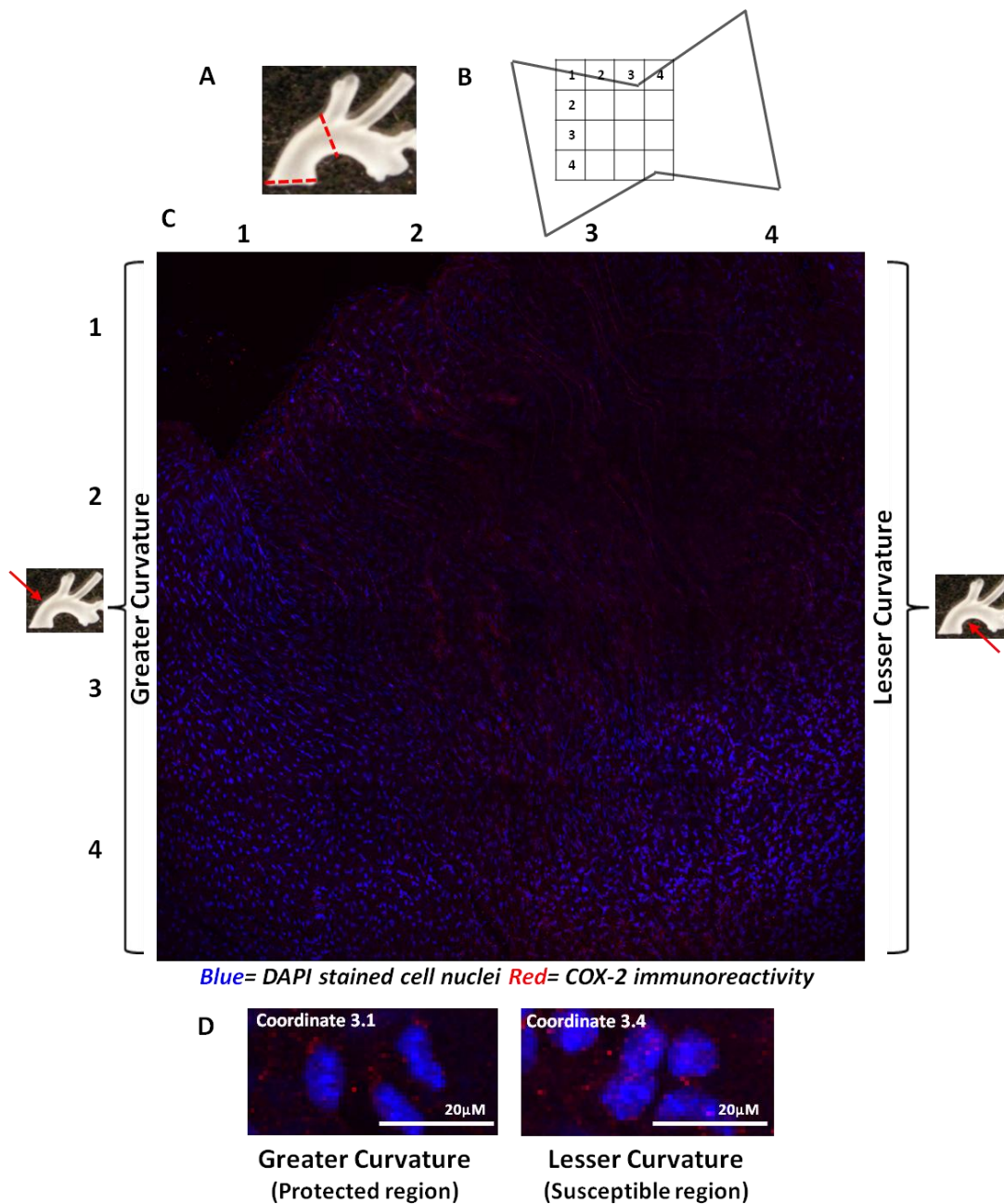


Figure 4.5 Tile composite of 4x4 images showing COX-2 immunoreactivity in the greater and the lesser curvature of the aortic arch from a mature mouse. Images from a perfusion fixed (according to the transcardial perfusion fixation procedure) aortic arch dissected from a mature adult (12 month old) male WT mouse (C57BL/6J, Harlan, UK). **(A)** Red dotted line, mark dissection cutting points. **(B)** A schematic of the mounted (opened and flattened) aortic arch, marking the location of the greater and the lesser curvature as well as the position of the tile composite (4x4 images, coordinates 1.1-4.4). **(C)** The entire tile of 4x4 images. DAPI stained nuclei (**blue**) and COX-2 immunoreactivity (**red**). **(D)** Zoomed in images (from coordinates 3.1 and 3.4) showing intracellular localisation of COX-2 immunoreactivity.

Figure 4.6 Comparison of COX-1 and COX-2 immunoreactivity in aortic arches from male young adult (3 month old) versus mature adult (12 month old) WT mice

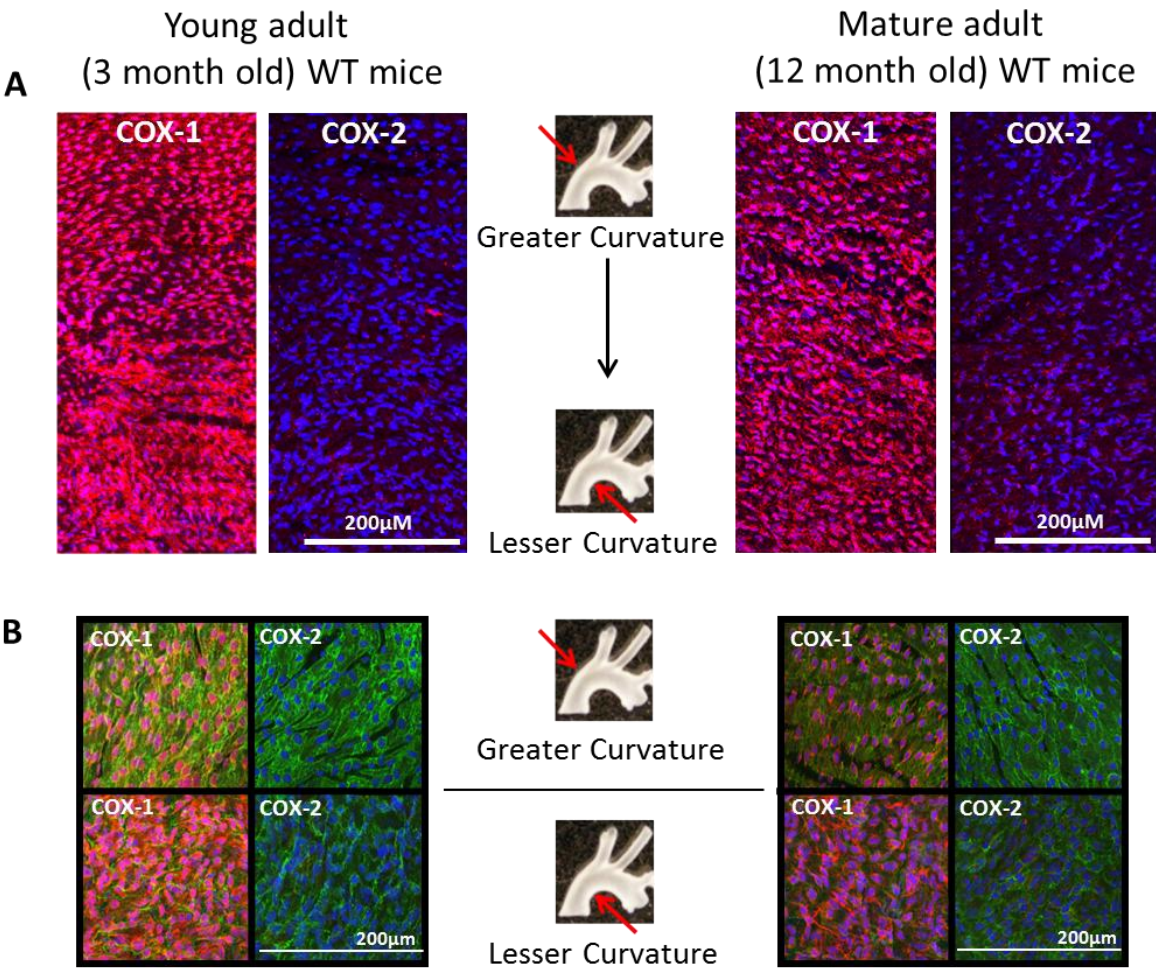


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Figure 4.6 (Continued) Comparison of COX-1 and COX-2 immunoreactivity in aortic arches from male young adult (3 month old) versus mature adult (12 month old) WT mice

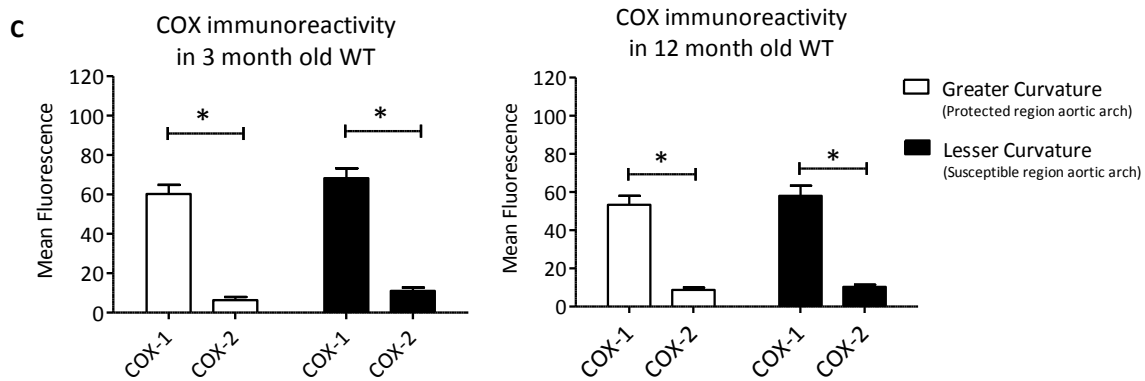


Figure 4.6 Comparison of COX-1 and COX-2 immunoreactivity in aortic arches from male young adult (3 month old) versus mature adult (12 month old) WT mice. (A) Part of tiles showing COX-1 and COX-2 immunoreactivity in the greater and lesser curvature of aortic arches from young versus mature male WT mice. **(B)** Representative cropped images (200x200µm) showing COX-1/COX-2 immunoreactivity (red), in merged images with DAPI stained nuclei (blue) and endothelial cell marker CD31 (green), in the greater and lesser curvature of aortic arches from young adult (3 month old) versus mature adult (12 month old) male WT mice (C57BL/6J, Harlan, UK). **(C)** Quantified COX immunoreactivity as mean fluorescence from the greater and lesser curvature of the aortic arch. Data is presented as the mean $\pm$ S.E.M from n=14-18 images from separate regions of tissue from n=6 young adult (3 month old), male WT mice (C57BL/6J, Harlan, UK) and n=8-11 images from n=4 mature adult (12 month old), male WT mice (C57BL/6J, Harlan, UK). Secondary control background was subtracted from each image before analysis. Data was analysed using one-way ANOVA with Bonferroni's multi comparison post-test and $*p<0.05$ was taken as significant and denoted by*.

Figure 4.7 '**Equilibrated study**'; stimulated prostacyclin release from thoracic aorta from male **mature adult** WT and COX-1^{-/-} mice

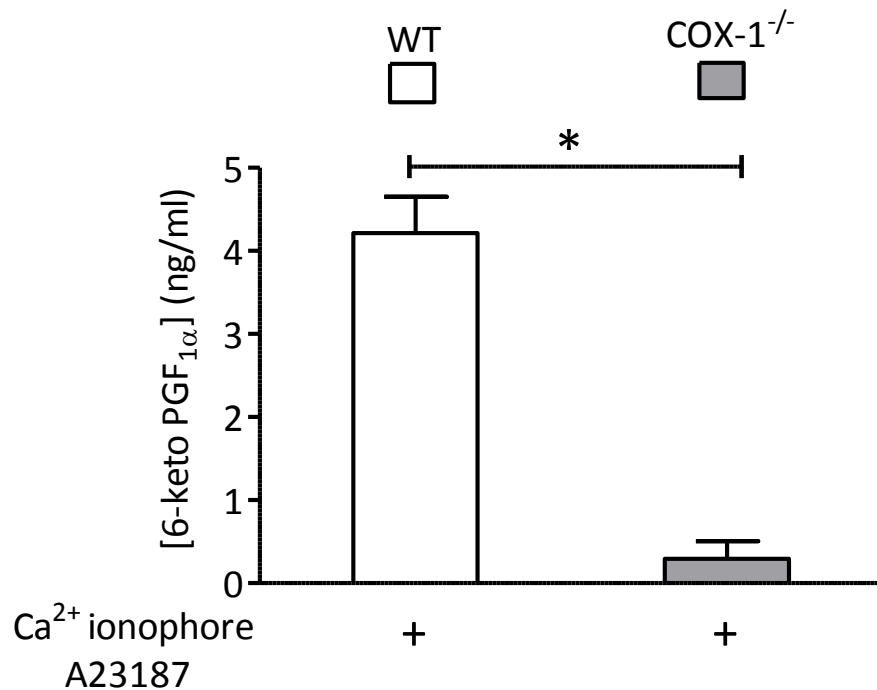


Figure 4.7 '**Equilibrated study**'; stimulated prostacyclin release from thoracic aorta from male **young adult** WT and COX-1^{-/-} mice. Stimulated prostacyclin release after 1.5 hours equilibration and after 30 minutes calcium ionophore (A23187, 50μM) stimulation (+), from thoracic aorta from mature adult (12 month old), male WT and COX-1^{-/-} mice, measured as the prostacyclin stable breakdown product 6-ketoPGF_{1α} by RIA. Data is presented as the mean ± S.E.M from n=4-5 animals and was analysed using unpaired Student's t-test and p<0.05 taken as significant and denoted by*.

Figure 4.8 'Equilibrated study'; stimulated prostacyclin release from thoracic aorta from male and female **mature adult** WT, COX-1^{-/-} and COX-2^{-/-} mice

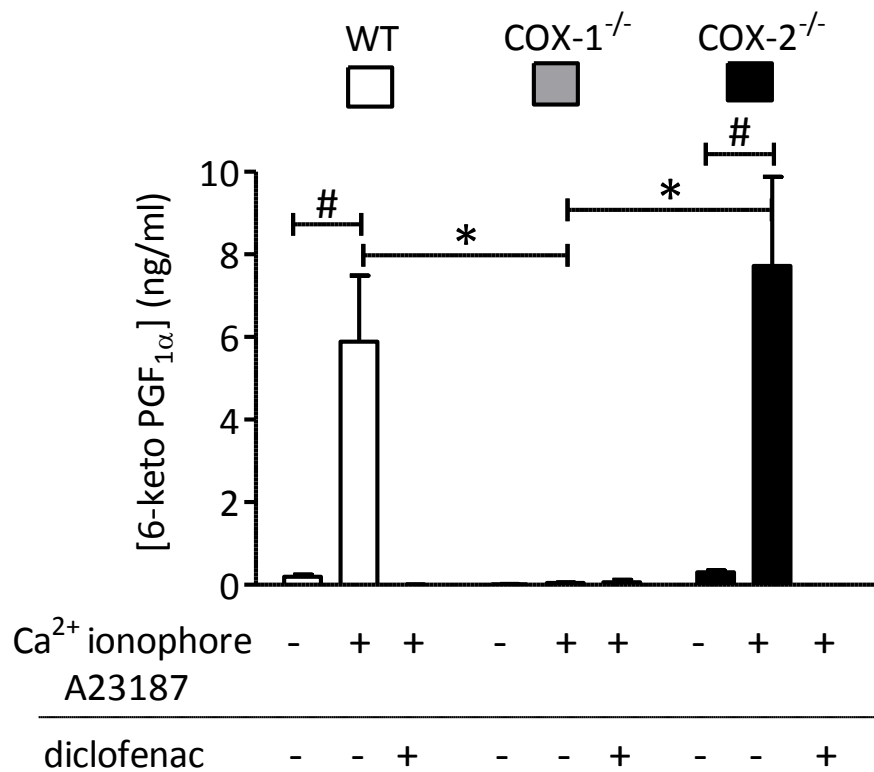


Figure 4.8 'Equilibrated study'; stimulated prostacyclin release from thoracic aorta from male and female **mature adult** WT, COX-1^{-/-} and COX-2^{-/-} mice. Prostacyclin release after 1.5 hours equilibration (-), and after 30 minutes calcium ionophore (A23187, 50μM) stimulation (+), from thoracic aorta from 12 month old, mature adult, male and female WT, COX-1^{-/-} and COX-2^{-/-} mice, measured as the prostacyclin stable breakdown product 6-ketoPGF_{1α} by EIA. 100 μM diclofenac was added to the wells of some thoracic aorta segments during the equilibration phase (1.5 hours) and for the stimulation phase (30 minutes). Data is presented as the mean ± S.E.M from n=6 animals. Within group analysis was performed using unpaired Student's t-test and p<0.05 taken as significant and denoted by #. Between groups analysis was performed using one-way ANOVA with Bonferroni's multi comparison post-test, p<0.05 was taken as significant and denoted by *.

Figure 4.9 COX-1 and COX-2 immunoreactivity in the greater and the lesser curvature of aortic arches from mature adult WT, COX-1^{-/-} and COX-2^{-/-} mice

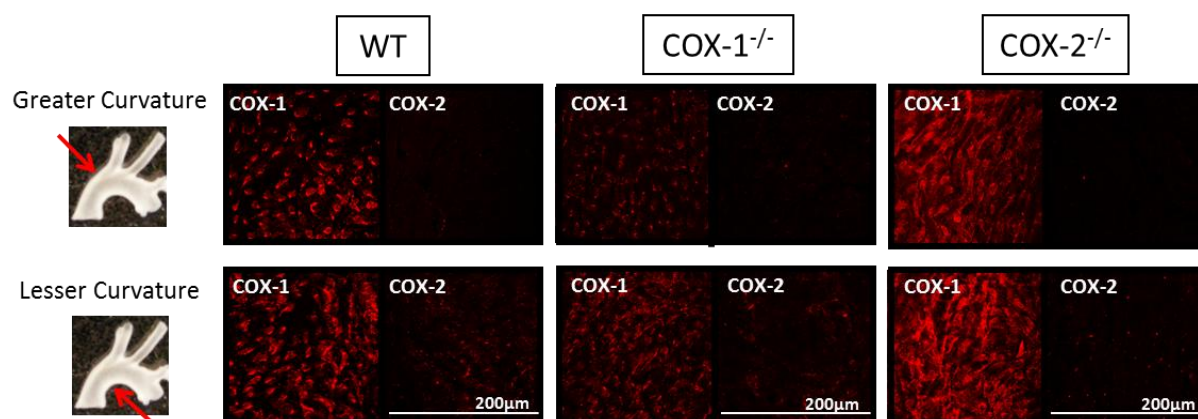


Figure 4.9 COX-1 and COX-2 immunoreactivity in the greater and the lesser curvature of aortic arches from mature adult WT, COX-1^{-/-} and COX-2^{-/-} mice. Representative cropped images (200x200µm) of COX-1 and COX-2 immunoreactivity (red) in the greater and the lesser curvature of aortic arches from untreated WT, COX-1^{-/-} and COX-2^{-/-} mice. All of the images have been treated the same in terms of alterations in brightness and contrast for clarity of viewing purposes (+40% brightness and +40% contrast).

Figure 4.10 COX-1 and COX-2 immunoreactivity in aortic arch endothelium from aged matched control rats versus rats with chronic heart failure

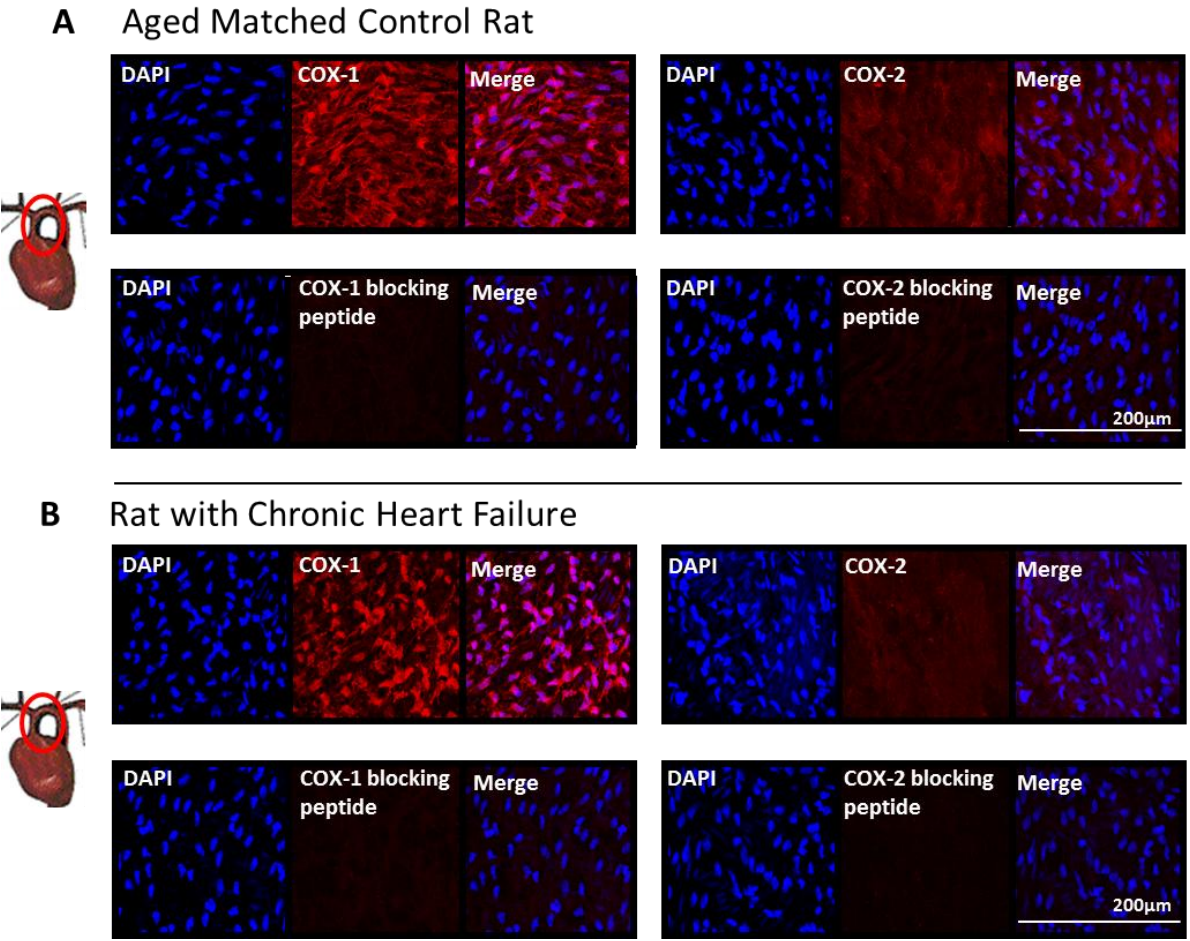


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Figure 4.10 (Continued) COX-1 and COX-2 immunoreactivity in aortic arch endothelium from aged matched control rats versus rats with chronic heart failure

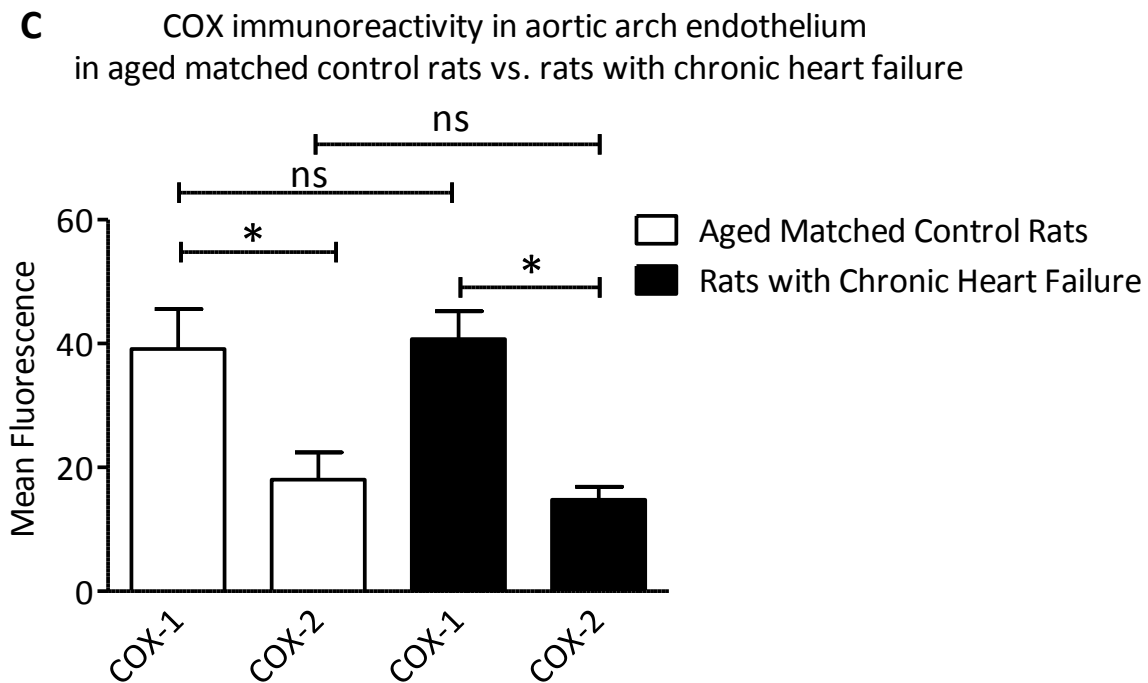


Figure 4.10 COX-1 and COX-2 immunoreactivity in aortic arch endothelium from aged matched control rats versus rats with chronic heart failure. Representative cropped images (200x200µm) of COX-1 and COX-2 immunoreactivity aortic arch segments from **(A)** aged matched control rats (6-7 months old) and **(B)** rats with chronic heart failure (6-7 month old). Nuclei was visualised by DAPI (**blue**) and merge (**blue and red**) represents both DAPI stained nuclei (**blue**) and COX-2 immunoreactivity (**red**). Some aortic arch segments were incubated with COX-1 or COX-2 blocking peptides (the precise epitopes which the Cayman COX-1 160109 or 160126 COX-2 antibodies was raised against). **(C)** Quantified COX immunoreactivity as mean fluorescence from n=4 heart failure rats and n=5 aged matched control rats (average values from 2 pictures per aortic arch tissue imaged). Data is presented as the mean ± S.E.M and was analysed using one-way ANOVA with Bonferroni's multi comparison post-test and $p < 0.05$ was taken as significant and denoted by * and $p > 0.05$ was taken as non-significant and denoted by ns.

Figure 4.11 COX-1 and COX-2 immunoreactivity in the endothelium of the carotid artery branches of the aortic arch from aged matched control rats versus rats with chronic heart failure

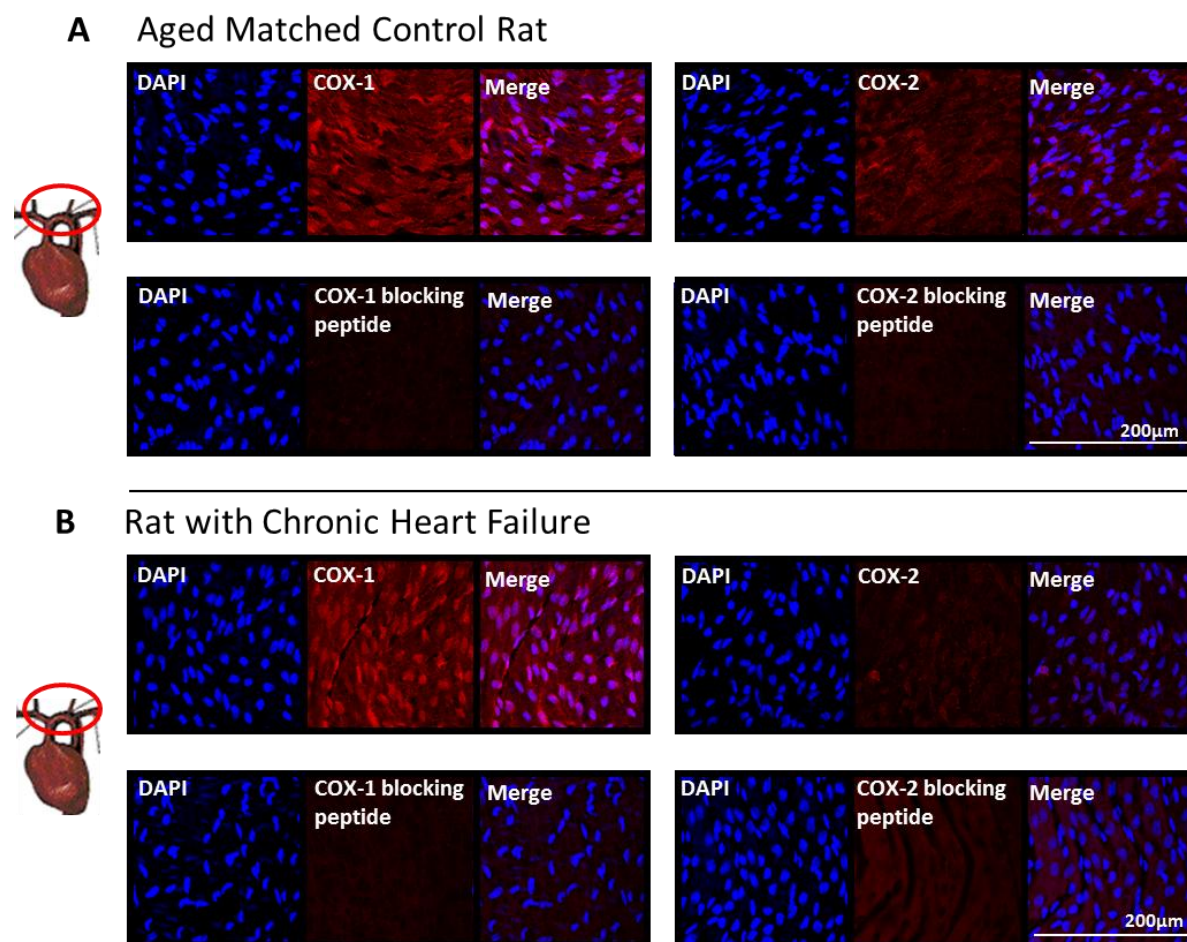


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Figure 4.11 (Continued) COX-1 and COX-2 immunoreactivity in the endothelium of the carotid artery branches of the aortic arch from aged matched control rats versus rats with chronic heart failure

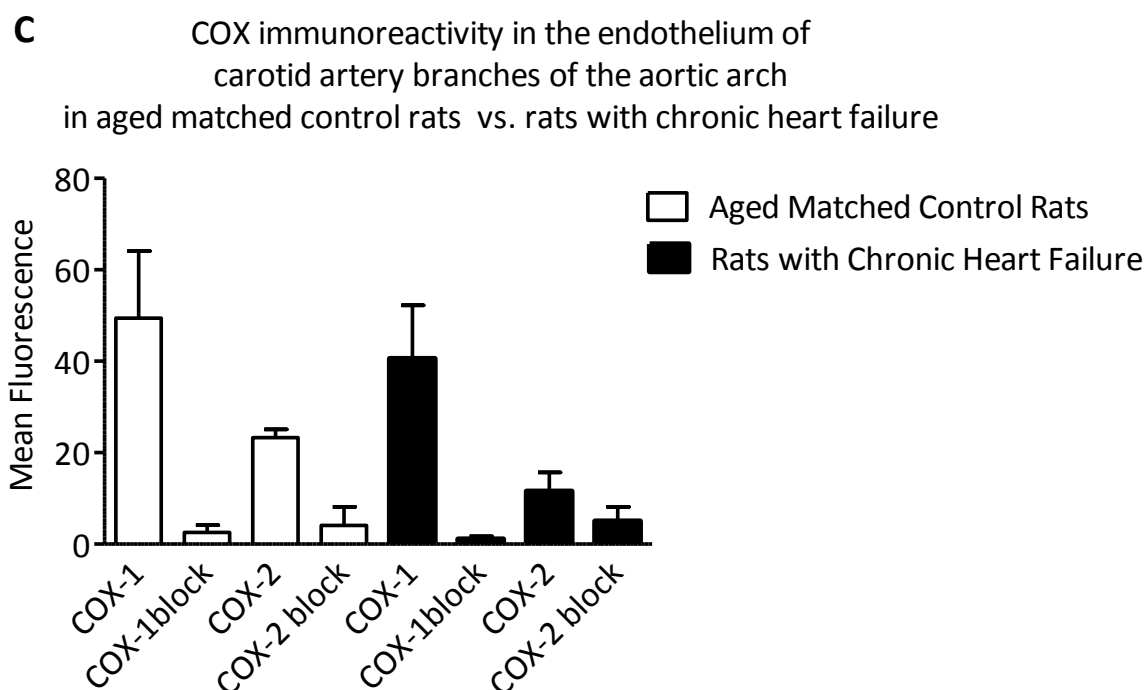


Figure 4.11 COX-1 and COX-2 immunoreactivity in the endothelium of the carotid artery branches of the aortic arch from aged matched control rats versus rats with chronic heart failure. Representative cropped images (200x200 μ m) of COX-1 and COX-2 immunoreactivity in carotid artery branches (brachiocephalic artery, left common carotid artery and left subclavian artery) from **(A)** aged matched control rats (6-7 months old) and **(B)** rats with chronic heart failure (6-7 months old). Some segments of the carotid artery branches of the aortic arch were incubated with COX-1 or COX-2 blocking peptides (the precise epitopes which the Cayman COX-1 160109 or 160126 COX-2 antibodies was raised against). Nuclei was visualised by DAPI (**blue**) and merge (**blue and red**) represents both DAPI stained nuclei (**blue**) and COX-2 immunoreactivity (**red**). **(C)** Quantified COX immunoreactivity as mean fluorescence from $n=2$ heart failure rats and $n=3$ aged matched control rats (average values from 2 pictures per tissue imaged). Data is presented as the mean $\pm$ S.E.M.

Figure 4.12 Non-specific background immunoreactivity from Alexa Fluor 594 secondary control in the endothelium of aortic arch tissue and carotid artery branches from aged matched control rats versus rats with chronic heart failure

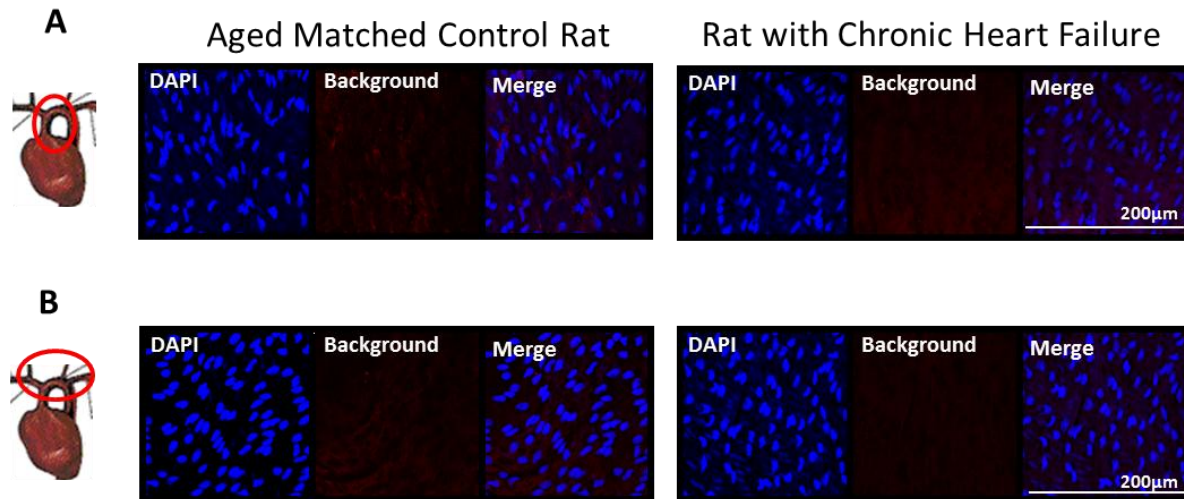


Figure 4.12 Non-specific background immunoreactivity from Alexa Fluor 594 secondary control in the endothelium of aortic arch tissue and carotid artery branches from aged matched control rats versus rats with chronic heart failure. Representative cropped images (200x200µm) of background immunoreactivity (red) in (A) aortic arch tissue and (B) carotid artery branches (brachiocephalic artery, left common carotid artery and left subclavian artery) from aged matched control rats and rats with chronic heart failure (all rats were 6-7 month old). Nuclei was visualised by DAPI (blue) and merge (blue and red) represents both DAPI stained nuclei (blue) and background immunoreactivity (red).

Figure 4.13 Validation experiment; positive control for the COX-2 antibody- abdominal aorta incubated in vitro overnight +/- LPS

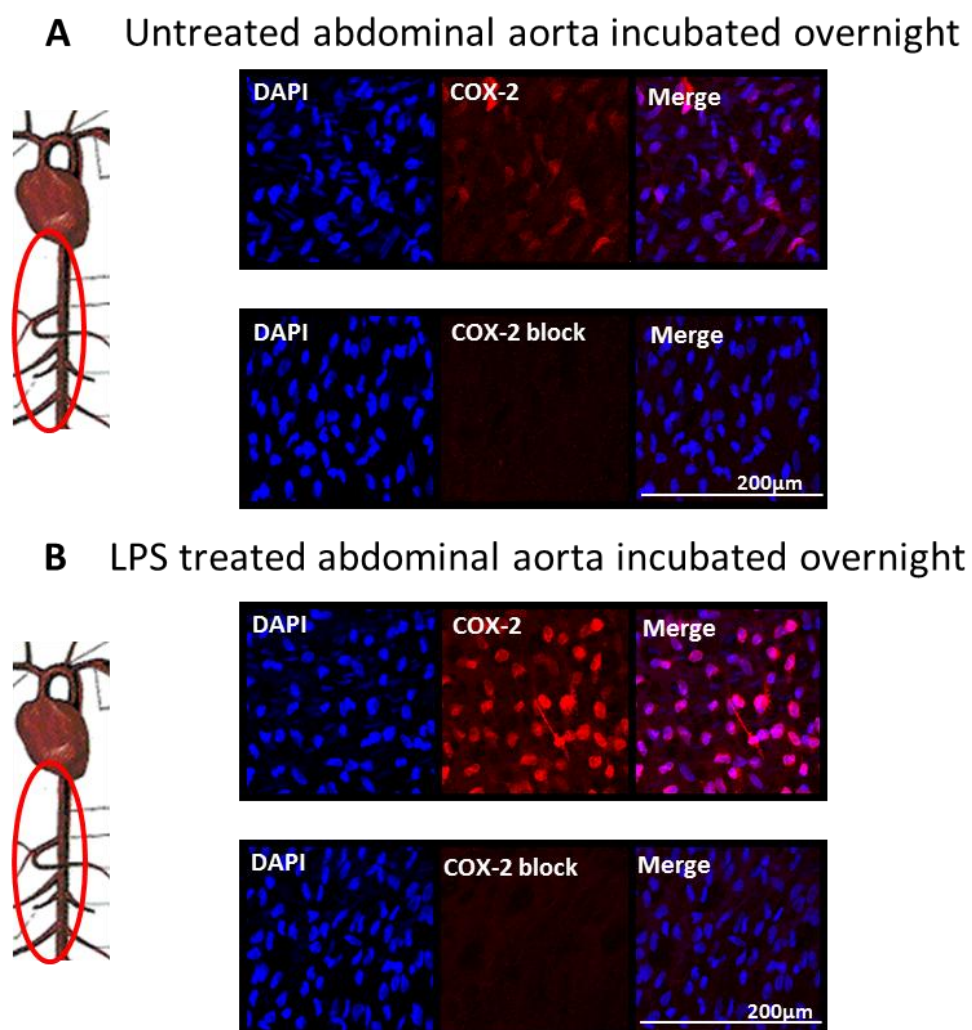


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Figure 4.13 (Continued) Validation experiment; positive control for the COX-2 antibody- abdominal aorta incubated in vitro overnight +/- LPS

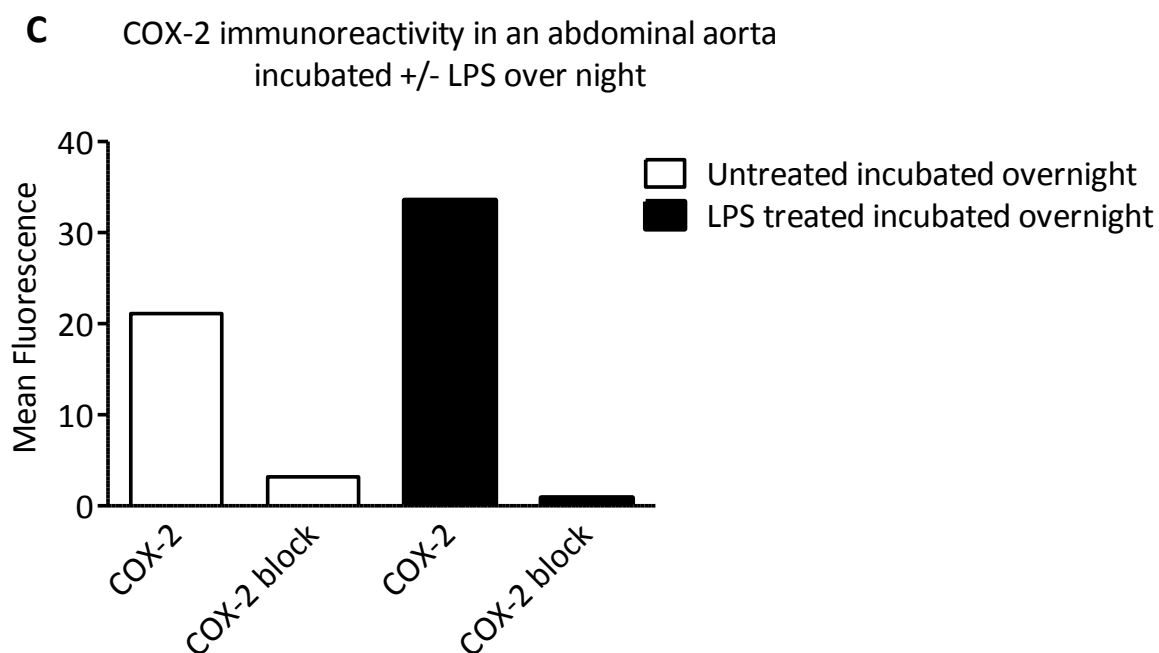


Figure 4.13 Positive control validation experiments for the COX-2 antibody- abdominal aorta incubated in vitro overnight +/- LPS. Representative cropped images (200x200µm) of COX-2 immunoreactivity (red) in segments of an abdominal aorta incubated overnight in 37°C +/- LPS (*E.coli* serotype 0111:B4, 10µg/ml). Some segments of abdominal aorta were incubated with the COX-2 blocking peptide (the precise epitope which the Cayman 160126 COX-2 antibody was raised against). Nuclei was visualised by DAPI (**blue**) and merge (**blue and red**) represents both DAPI stained nuclei (**blue**) and COX-2 immunoreactivity (**red**). **(C)** Quantified COX-2 immunoreactivity as mean fluorescence from *n*=1 abdominal aorta (average values from 2 pictures per tissue imaged) from an aged matched control rat (6-7 months old).

Figure 4.14 Prostanoid profile in aged matched control rats versus rats with chronic heart failure and from an *in vitro* assay with overnight incubation +/- LPS

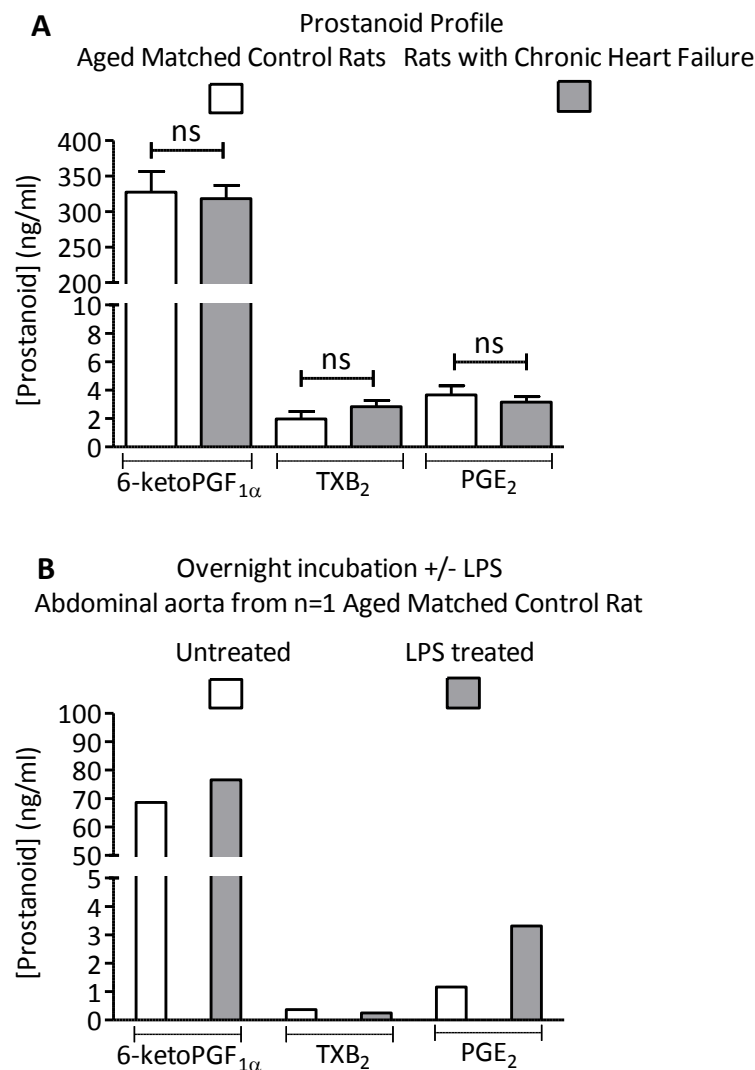


Figure 4.14 Prostanoid profile in aged matched control rats versus rats with chronic heart failure and from an *in vitro* assay with overnight incubation +/- LPS. Stimulated prostacyclin (measured as the stable breakdown product 6-ketoPGF_{1α} by EIA), thromboxane (measured as the stable breakdown product TXB₂ by EIA) and prostaglandin E₂ (by HTRF) release from rat aorta after 1.5 hours equilibration and 30 minutes calcium ionophore (A23187, 50μM) stimulation. 100 μM diclofenac was added to the wells of some thoracic aorta segments during the stimulation phase (30 minutes). **(A)** Data is presented as the mean ± S.E.M from n=5 thoracic aortic rings from aged matched control rats and rats with chronic heart failure (all rats were 6-7 month old). **(B)** Data is presented as the mean ± S.E.M from n=1 an aged matched control rat abdominal aorta incubated overnight in 37°C +/- LPS (*E.coli* serotype 0111:B4, 10μg/ml). Data was analysed by using one-way ANOVA with Bonferroni's multi comparison post-test and $p > 0.05$ was taken as non-significant and denoted by ns.

Figure 4.15 COX-1 and COX-2 immunoreactivity in aortic arch endothelium from normal chow fed WT versus ApoE^{-/-} mice

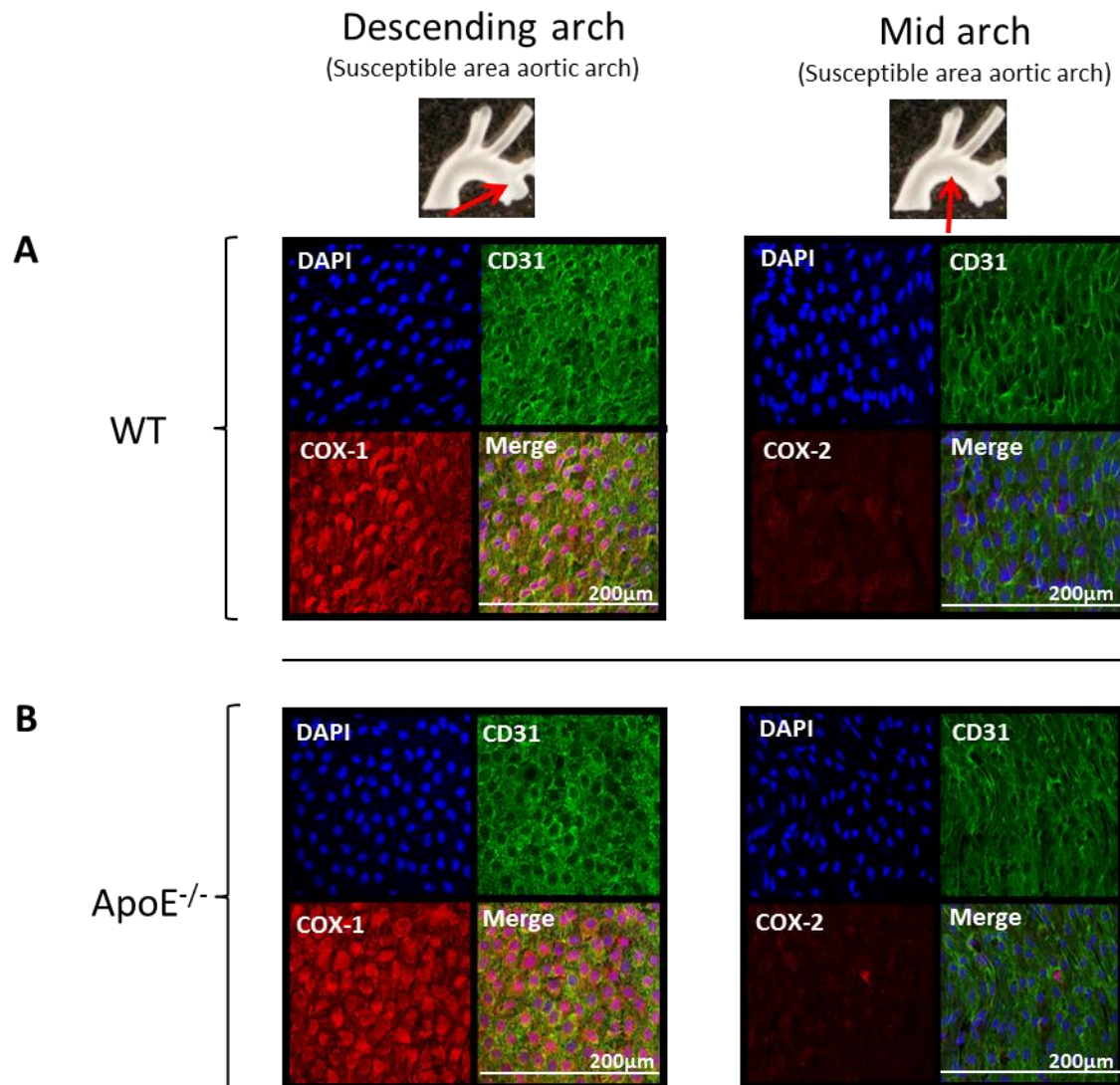


Figure 4.15 COX-1 and COX-2 immunoreactivity in aortic arch endothelium from normal chow fed WT versus ApoE^{-/-} mice. Representative cropped images (200x200µm) of COX-1 and COX-2 immunoreactivity (red) in normal chow fed **(A)** WT mice (C57BL/6J, bred in-house) and **(B)** ApoE^{-/-} mice (C57BL/6J background). Aortic tissue was fixed according to the transcatheter perfusion fixation procedure. Red arrow indicate the proximal part of the descending arch that were stained with COX-1 and mid aortic arches that were stained with COX-2. Nuclei visualised with DAPI (**blue**) and endothelial cells outlined with CD31 (**green**). Merge represent blue, green and red channels merged.

Figure 4.16 Quantified COX-1 and COX-2 immunoreactivity in the endothelial versus smooth muscle layer of aortic arch endothelium from normal chow fed WT versus ApoE^{-/-} mice

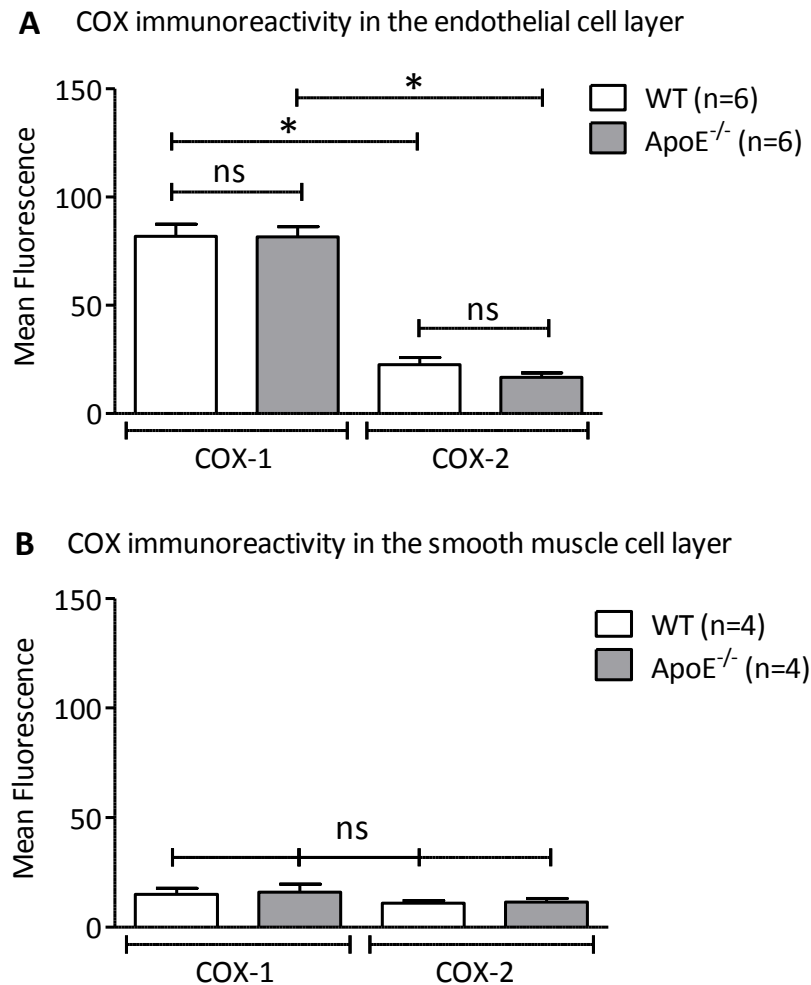


Figure 4.16 Quantified COX-1 and COX-2 immunoreactivity in the endothelial versus smooth muscle layer of aortic arch endothelium from normal chow fed WT versus ApoE^{-/-} mice. Pooled data comparing COX-1 and COX-2 immunoreactivity in **(A)** the endothelial layer and **(B)** the underlying smooth muscle layer in normal chow fed WT mice (C57BL/6J, bred in-house) and ApoE^{-/-} mice (C57BL/6J background). Aortic tissue was fixed according to the transcardial perfusion fixation procedure. 1 image was taken from every COX-1 stained descending aortic arch and 1 image was taken from every COX-2 stained mid aortic arch (see representative images in Figure 4.15). COX immunoreactivity was quantified as mean fluorescence. Secondary control background was subtracted from each image before analysis. Data is presented as the mean $\pm$ S.E.M of $n=6$ images of the endothelial layer and $n=4$ images of the underlying smooth muscle layer from $n=6$ young adult (3 month old), 3 female and 3 male WT mice (C57BL/6J, bred in-house) and 2 female and 4 male ApoE^{-/-} mice. Data was analysed using one-way ANOVA with Bonferroni's multi comparison post-test and $*p<0.05$ was taken as significant and denoted by * and $p>0.05$ was taken as non-significant and denoted by ns.

Figure 4.17 Manually made tile composite of 3x4 images showing the superficial morphology of a plaque like structure in an $ApoE^{-/-}$ mouse fed an atherogenic diet

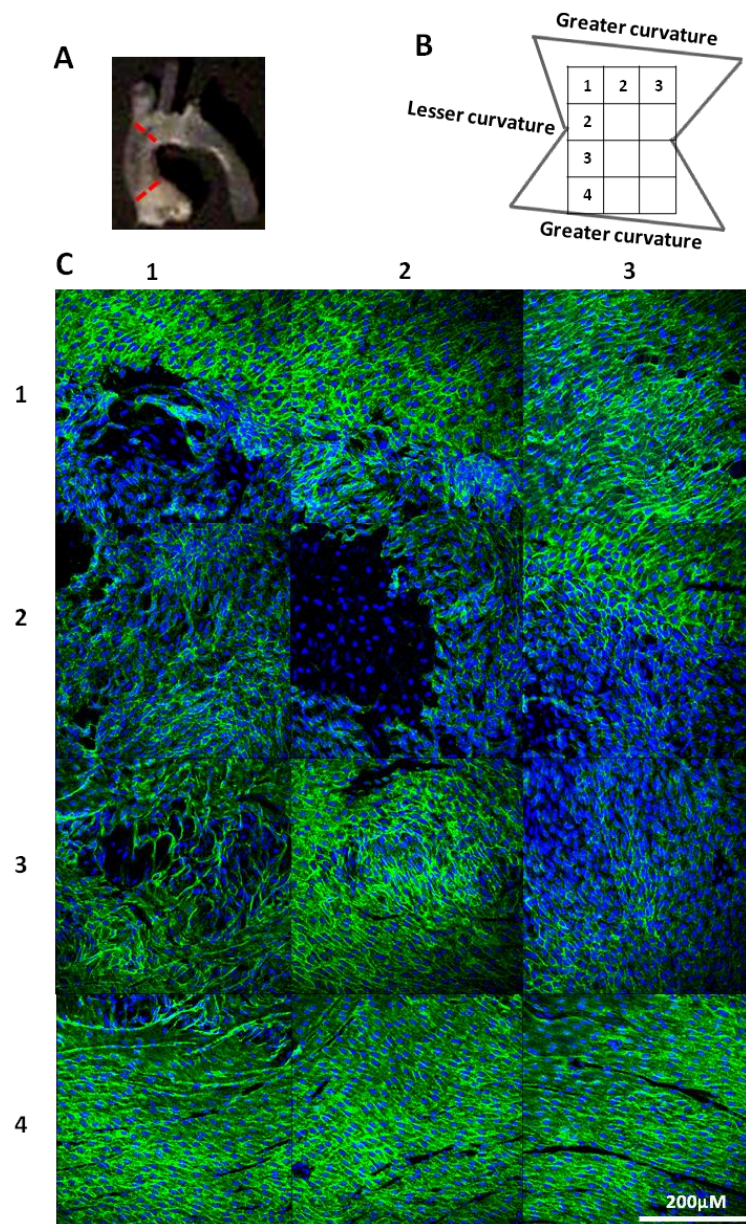


Figure 4.17 Manually made tile composite of 3x4 images showing morphology of a plaque in an $ApoE^{-/-}$ mouse fed an atherogenic diet. Images from a perfusion fixed (according to the transcardial perfusion fixation procedure) aortic arch dissected from a female $ApoE^{-/-}$ mouse. The tile was manually created by moving the field of view to the next field along and then puzzled together as a tile. **(A)** Photo of the vessel that was imaged. The plaques show up in the photo as white as do the aortic root/valves (that was calcified and hardened). Red dotted line marks dissection cutting points. **(B)** A schematic of the mounted (opened and flattened) aortic arch, marking the location of the greater and the lesser curvature as well as the position of the tile composite (3x4 images, coordinates 1.1-4.3). **(C)** The entire tile of 3x4 images. DAPI stained nuclei (**blue**) and CD31 immunoreactivity (**green**). The brightness and contrast has been enhanced for viewing purposes (+20 and +40).

Figure 4.18 COX-1 and COX-2 immunoreactivity in plaques in the descending arch and mid arch endothelium from an ApoE^{-/-} mouse fed an atherogenic diet

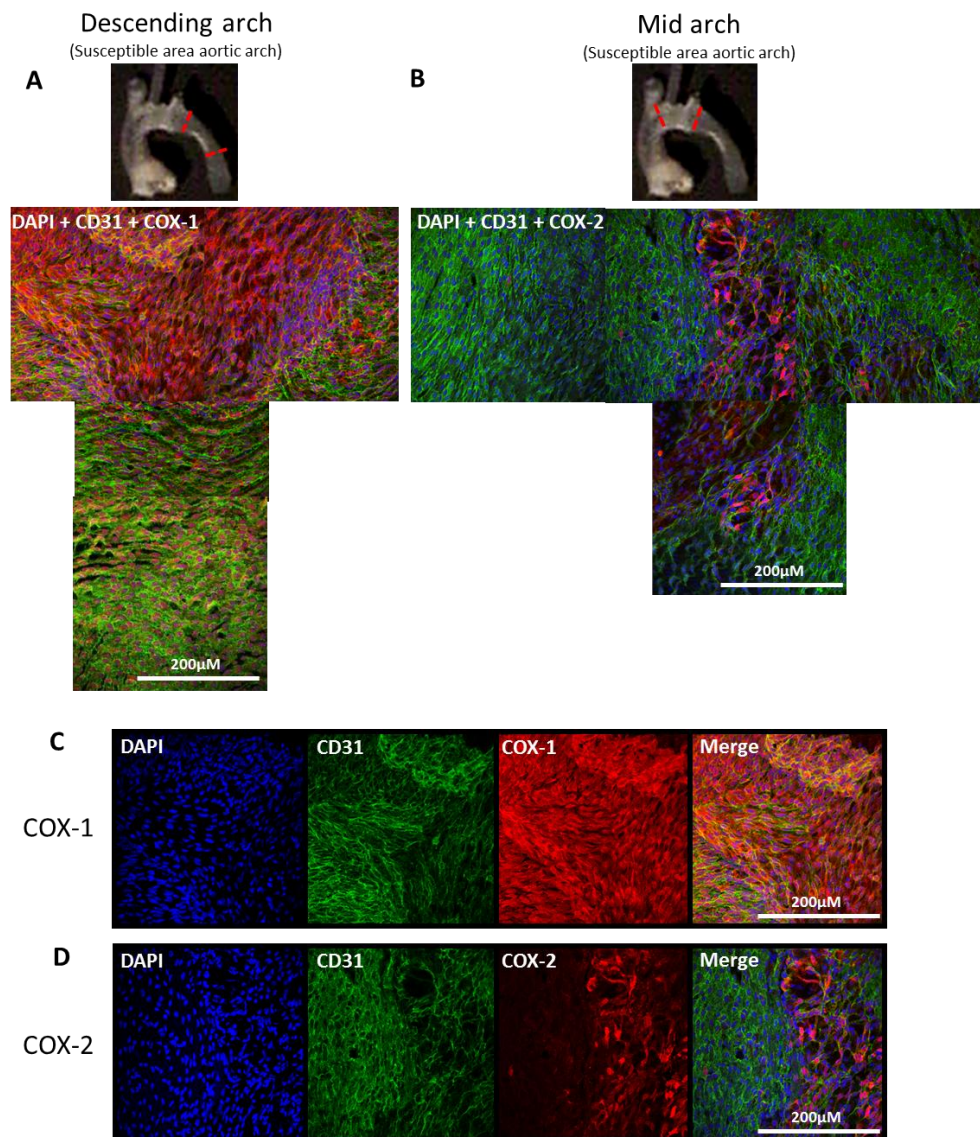


Figure 4.18 COX-1 and COX-2 immunoreactivity in plaques in the descending arch and mid arch endothelium from an ApoE^{-/-} mouse fed an atherogenic diet. Images from a perfusion fixed (according to the transcatheter perfusion fixation procedure) aortic arch dissected from a female ApoE^{-/-} mouse (fed an atherogenic diet). The tile was manually created by moving the field of view to the next field along and then puzzled together as a tile. Photos of the vessel that was imaged where the plaques show up as white and red dotted line marks dissection cutting points to the descending arch and the mid arch respectively. Nuclei was visualised with DAPI (**blue**), endothelial cells outlined with CD31 (**green**), COX-1 and COX-2 immunoreactivity (**red**) and the three merged together (merge). **(A)** COX-1 immunoreactivity in the endothelium of the descending arch in a manual tile composite of 4 images. **(B)** COX-2 immunoreactivity in the endothelium of the mid arch in a manual tile composite of 4 images. One of the images shown in (A) and (B) (387.5x387.5 μm) was selected for more detailed view of **(C)** COX-1 immunoreactivity and **(D)** COX-2 immunoreactivity.

Figure 4.19 COX-1 and COX-2 immunoreactivity in the plaque versus normal endothelium and smooth muscle cell layers in $ApoE^{-/-}$ mice fed an atherogenic diet

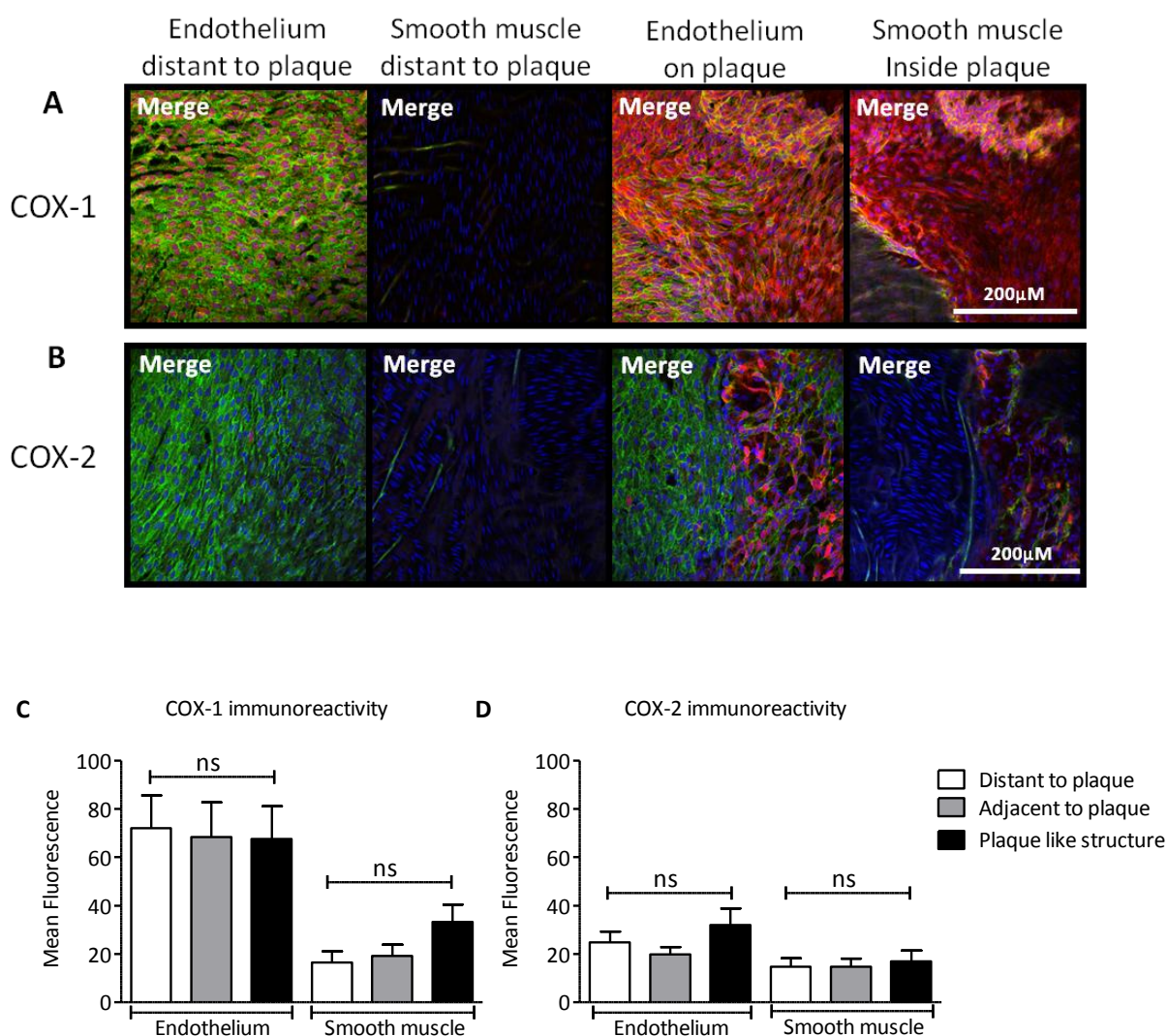


Figure 4.19 COX-1 and COX-2 immunoreactivity in the plaque versus normal endothelium and smooth muscle cell layers in $ApoE^{-/-}$ mice fed an atherogenic diet. Representative images from a perfusion fixed (according to the transcardial perfusion fixation procedure) aortic arch dissected from a female $ApoE^{-/-}$ mouse (fed an atherogenic diet). The other $n=4$ mice were perfused according to the ex vivo semi-perfusion fixation procedure. **(A)** COX-1 immunoreactivity in the endothelium of the descending arch. **(B)** COX-2 immunoreactivity in the endothelium of the mid arch. Nuclei was visualised with DAPI (blue), endothelial cells outlined with CD31 (green), COX-1 and COX-2 immunoreactivity (red) and the three merged together (merge). **(C)** COX-1 and **(D)** COX-2 immunoreactivity data was presented as mean fluorescence and gathered by using a $130 \times 137 \mu$ m region of interest (ROI) box in the LAS confocal software program which was moved to endothelium and underlying smooth muscle distant, adjacent and directly on top the plaque like structures. Data represent 1 ROI per region from images from $n=5$ $ApoE^{-/-}$ mice (males and females) and was analysed using one-way ANOVA with Bonferroni's multi comparison post-test and $p > 0.05$ was taken as non-significant and denoted by ns.

Summary of Chapter 4

Using tissue from female young adult (3 month old) and male mature adult (12 month old) WT mice, I have shown that the relative COX expression and activity pattern did not change with the female sex or with age. Therefore the conclusion remains the same as shown in Chapter 3; COX-1 greatly predominates over COX-2 both in terms of immunoreactivity and in bioactivity and appears to be responsible for the majority of prostacyclin production in vessels from male and female, young and mature mice.

Using tissue from a chronic heart failure model in rats or from a mouse model of atherosclerosis, I was able to show that these types of cardiovascular disease/insult was not associated with gross changes in either COX isoform expression or profile of COX products released from vessels. Specifically, I found in tissue from rats with heart failure, no alteration in COX expression profile (COX-1 still predominated over COX-2) or in bioactivity compared to age matched controls. The profile of prostanoids released from blood vessels of control and heart failure rats was similar and showed the following rank order; prostacyclin >>> PGE₂ >= thromboxane. In tissue from a mouse model of early atherosclerosis (normal chow fed ApoE^{-/-} mice), where inflammation markers and fatty streaks are present, but plaques have not yet formed (Daugherty, 2002; Zhang *et al.*, 1992), again there was no difference in COX-1 or COX-2 immunoreactivity. At more advanced stages of this atherosclerosis model (ApoE^{-/-} fed an atherogenic diet for 10 weeks) where plaques are readily detectable, COX-1 and COX-2 immunoreactivity was found to be enriched in areas of atherosclerotic plaques. Despite profound plaque presence in aortic tissue from these mice it was found by my colleague Dr Nicholas Kirkby that COX-1, and not COX-2 was still responsible for driving gross prostacyclin production in vessel segments *ex vivo*.

In summary, these experiments indicate that, in terms of total vessel activity, COX-1 still predominates over COX-2 in the endothelium as animal's age as well as when serious cardiovascular disease is present. The implications of these observations are discussed in detail in my General Discussion chapter (Chapter 6).

Chapter 5

*Functional consequences of COX-1 and COX-2 gene deletion
in aortic arch endothelium*

Rationale behind Chapter 5

The results from the previous chapters indicate that COX-1 is the predominant COX isoform in aortic arch endothelium in terms of expression levels (immunoreactivity) and bioactivity (prostacyclin production) during both health and disease (in the mice and rat models tested). There was however detectable, albeit sparse, COX-2 immunoreactivity in the lesser curvature of the mouse aortic arch. Therefore, even though COX-1 appears to be the predominant, constitutively expressed isoform, responsible for the majority of the endothelial prostacyclin production, COX-2 may still play an important and bioactive role localised to regions of the vasculature susceptible to inflammation and atherosclerosis such as the lesser curvature of the aortic arch.

In this chapter, I have applied this information and investigated the functional consequences of COX-1 and COX-2 gene deletion in aortic arch endothelium of COX-1^{-/-} and COX-2^{-/-} mice.

In order to get an overview of any morphological differences in the aortic endothelium between the genetically modified animals and the WT animals studied in this chapter, I have compared endothelial CD31 immunoreactivity and DAPI stained nuclear density in the greater and lesser curvature of aortic arches from WT, COX-1^{-/-} and COX-2^{-/-} and normal chow fed ApoE^{-/-} mice.

Diminished or reduced prostacyclin release in the vessels of COX^{-/-} animals may influence NF-κB activity since prostacyclin analogues have been reported to inhibit NF-κB translocation/activation in macrophages (Raychaudhuri *et al.*, 2002), T-cells (Zhou *et al.*, 2007a) and dendritic cells (Zhou *et al.*, 2007b). This is important and relevant to my thesis since activated NF-κB is thought to play a key role in the development of vascular inflammation and atherosclerosis (Collins *et al.*, 2001). Interestingly, the endothelium lining the lesser curvature of the aortic arch has been shown to be 'primed for activation' with elevated levels of nuclear NF-κB and cell adhesion molecules, which may have relevance to the increased susceptibility of atherosclerotic plaques forming in this area (Hajra *et al.*, 2000). I have therefore investigated how NF-κB activity in the endothelium is affected by COX-1 or COX-2 gene deletion. For these studies I have used untreated and LPS treated mice in order to compare control versus inflammatory conditions.

Prostacyclin is also known to greatly influence platelet function and adhesion and thereby contribute to the anti-thrombotic surface of the endothelium (Moncada *et al.*, 1977). In this chapter I have therefore investigated platelet adhesion to the endothelium of the greater and lesser curvature regions of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Platelets express CD31; however, as illustrated extensively in this thesis, CD31 is also present on endothelial cells. In order to selectively identify platelets adhered to endothelium using imaging, I used an antibody against CD41 (or platelet glycoprotein IIb), which is a selective marker for platelets and platelet precursors, that is reported to be expressed on the platelet plasma membrane, intraluminal side of the platelet open canalicular system and on the platelet α -granule membrane in platelets (Schmitt *et al.*, 2001).

The aims and research questions of this chapter can be summarised as follows:

What are the functional consequences of COX-1 and COX-2 gene deletion in aortic arch endothelium?

- Compare CD31 immunoreactivity and DAPI nuclear density in the greater and lesser curvature of aortic arches from WT, COX-1^{-/-} and COX-2^{-/-} and normal chow fed and high cholesterol chow fed ApoE^{-/-} mice using immunohistochemistry and *enface* confocal microscopy.
- Investigate the relative role of COX-1 and COX-2 deletion in expression and activation of NF- κ B in the endothelium of the greater and lesser curvature of the aortic arch using tissue from WT, COX-1^{-/-} and COX-2^{-/-} mice.
- Study the adherence of platelets to the endothelium in the greater and lesser curvature in aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed and high cholesterol chow fed ApoE^{-/-} mice.

Methods

Animals

WT, COX-1^{-/-} and COX-2^{-/-} mice

WT, COX-1^{-/-} and COX-2^{-/-} mice were bred, maintained and genotyped as described in the General Methods Chapter 2. For this chapter, animals were male and female young adult (3-4 month old) mice. All WT mice were from the C57BL/6J strain and were bred in-house with a shared background to match the COX-1^{-/-} and COX-2^{-/-} mice (backcrossed to C57BL/6J, Harlan, UK more than 7 times) see General Methods Chapter 2. These COX colony WT controls will be referred to as 'C57BL/6J, bred in-house'. Mice either left untreated or injected with LPS (serotype 055:B5, 4mg/kg; i.p for 30 minutes) to activate nuclear translocation of NF-κB (Hajra *et al.*, 2000). The mice were then killed by CO₂ asphyxiation and aortic arches were dissected out and fixed with 2% formaldehyde according to the '*transcardial perfusion fixation procedure*' and NF-κB, CD31 and CD41 immunoreactivity was measured in the endothelium of the aortic arch as detailed in the General Methods Chapter 2 (Tables 2.18, 2.19, 2.20). The mice in the NF-κB study were 3-4 month old n=3 male and n=2 female mice. The mice in the CD31/nuclear density/CD41 study were all ~3 months old n=3 female and n=3 male WT, COX-1^{-/-} and COX-2^{-/-} mice (n=6 animals in total).

ApoE^{-/-} mice

ApoE^{-/-} mice

In this thesis I have performed experiments in both normal chow fed ApoE^{-/-} mice (ApoE^{tm1Unc} from a C57BL/6J background) as well as ApoE^{-/-} mice that have been fed an atherogenic diet, in order to consider a model of early atherogenesis as well as more advanced and established atherosclerosis respectively (for more details see General methods Chapter 2).

Normal chow fed ApoE^{-/-} mice as a model of early atherogenesis

The normal chow fed ApoE^{-/-} mice (2 female mice and 4 male mice) and WT controls (C57BL/6J, bred in-house, 3 females, 3 males) were killed by CO₂ asphyxiation and aortic arches were dissected out and fixed with 2% formaldehyde according to the '*transcardial*

perfusion fixation procedure' and staining protocols for CD41 immunoreactivity as described in the General Methods Chapter 2 (Table 2.20).

High cholesterol chow fed ApoE^{-/-} mouse in a pilot study of CD31 immunoreactivity, nuclear density and CD41 immunoreactivity in a model of accelerated atherosclerosis

For illustrative purposes and as a pilot study, I was able to image and quantify CD31 immunoreactivity, nuclear density and CD41 immunoreactivity in the aortic arch of a high cholesterol chow fed ApoE^{-/-} mouse which had gone on to develop profound atherosclerosis (after being fed an atherogenic diet for 10 weeks). This female mouse was perfused according to the '*transcardial perfusion fixation procedure*' and staining protocols for CD41 immunoreactivity as described in the General Methods Chapter 2 (Table 2.20).

Blood smears

Blood smears were prepared on glass slides as described in the General Methods Chapter 2. In brief, heparinised blood was withdrawn from mice by venopuncture of the vena cava. One small drop of blood was placed on an electrostatic coated slide and carefully smeared across using the edge of another slide (at a ~45° angle), to evenly spread a thin layer of blood across the slide (this procedure was performed by my colleague Dr Nicholas Kirkby). The slides were then fixed in 100% methanol followed by 2% formaldehyde and washing steps. The slides then underwent the same CD41 staining protocol as the aortic arches.

En Face imaging of the arch lumen

In this chapter I have imaged NF-κB, CD31 and CD41 using antibodies and the stain DAPI to visualise nuclei as described in the General Methods Chapter 2.

NF-κB (as p65) was imaged in male and female 3 month old WT, COX-1^{-/-} or COX-2^{-/-} mice that were left untreated or treated with LPS (serotype 055:B5, L16529, Sigma) 4mg/kg; i.p for 30 minutes which is enough time for nuclear translocation/activation of NF-κB in endothelial cells (Hajra *et al.*, 2000). The mice were sacrificed (by CO₂ asphyxiation) and subject to the systemic transcardial perfusion fixation procedure and staining protocol outlined in General Methods Chapter 2 (Table 2.19).

CD41 positive platelets were imaged in 3 month old male WT, COX-1^{-/-} or COX-2^{-/-} mice and normal chow fed ApoE^{-/-} mice (and one high cholesterol chow fed ApoE^{-/-} mouse) that were left untreated and subject to the systemic transcatheter perfusion fixation procedure and staining protocol outlined in General Methods Chapter 2 (Table 2.20). Dr Nicholas Kirkby performed the *in vivo* part of the experiment, which made it practically possible to perform the experiment fully blinded to the identities of the mice (all vials were labelled with numbers). Once all the study was completed and all the images had been blind scored, the identity of the mice was revealed.

The endothelial layer of aortic arches were imaged *en face* with confocal microscopy (Leica SP5 inverted confocal microscope, 40x oil objective), with the same confocal settings maintained throughout the study and 2-3 images were captured from each greater and lesser curvature region. Immunoreactivity was quantified as mean fluorescence using LAS AF software and Image J Software was used to crop images and to calculate nuclei area and CD31 mean fluorescence and the area/diameter and number of CD41 positive platelets in blood smears and adhered to the endothelium of the aortic arches.

Quantification of endothelial CD31 immunoreactivity and DAPI stained nuclear density

Both the experiment and the quantification procedure were performed blinded (from the same data set as the CD41 study). DAPI and CD31 settings were kept constant at 550Gain and -2% Offset and 880Gain and -2% Offset respectively. 2 images were captured from the greater and the lesser curvature respectively. CD31 immunoreactivity was quantified as mean fluorescence in images that were cropped using Image J software (from 387.5x387.5µm in size to 200x200µm) using a saved region of interest (ROI) box (200x200µm, 1059x1059 pixels) that was dragged to an area in focus selected based on endothelial integrity (in an area devoid of damage from handling). This area was then earmarked for cropping and quantification of the DAPI stained nuclei. The DAPI stained nuclei in the cropped ROI image were thresholded using the 'default' criteria in the thresholding function (thresholded images show the thresholded DAPI positive objects in red) and the DAPI positive nuclear density (or total area), was calculated based on the total sum of the thresholded objects (exceeding a size of 2µm²). The average number of DAPI positive nuclei and average size of these nuclei was estimated based on the same data as

the total sum was calculated from and any object with an area less than $2\ \mu\text{m}^2$ was excluded from the analysis.

Quantification of endothelial NF- κ B immunoreactivity using Velocity 5 software

- 1) Total (global) levels of NF- κ B immunoreactivity; total fluorescence in a cropped area of focus selected for endothelial integrity based on DAPI and CD31 (cropped from $387.5 \times 387.5\ \mu\text{m}$ to $225 \times 225\ \mu\text{m}$) – minus the total fluorescence in the secondary control (non-specific binding) will be referred to as ‘total NF- κ B immunoreactivity’.
- 2) Nuclear NF- κ B immunoreactivity; nuclear bodies, imaged as ‘independent’ DAPI positive objects were quantified in the same cropped images ($225 \times 225\ \mu\text{m}$). At least 50 separate nuclei were analyzed from each image. Using Velocity 5 software the amount of nuclear NF- κ B immunoreactivity, captured as ‘red’ co-localized with ‘blue’ within the selected nuclei, was calculated. An average amount of NF- κ B immunoreactivity per nuclei imaged was then calculated for each image and referred to as ‘Nuclear NF- κ B immunoreactivity (average nuclear fluorescence)’.

Quantification of non-nucleated CD41 positive platelet like bodies in blood smears

The total number and total area taken up by CD41 positive platelets in blood smears from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice was estimated from images taken from a consistent location on the blood smear slide. These images were thresholded using the ‘default’ criteria in Image J and the selected CD41 positive platelets ($1\ \mu\text{m}$ in size to infinity) were included in the analysis.

Quantification of non-nucleated CD41 positive platelet like bodies in the mouse aortic arch

CD41 was used as a platelet marker to stain platelets adhered to the endothelium in aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and ApoE^{-/-} mice. 2 images were taken from each area (greater and lesser curvature) from each aortic arch. My colleague, Ms Claire Potter, helped out with manual blind scoring of the distinct red CD41 positive adhered platelets and the results from both of our blind scores were averaged. The blind scoring was done by using the LAS AF software program to zoom in (with all of the channels zoomed in simultaneously), to manually count the number of DAPI negative, CD31 positive and CD41

positive distinct platelet like bodies close to but clearly separate from other nucleated cells in the aortic endothelium.

The quantification of the CD41 positive platelets were then repeated by a more automated method using Image J software, where a full field (387.5x387.5 μm) was thresholded using the '*intermodes function*' and adjusted to around 70 (out of 255) on the thresholding scale of brightness and saturation. This was manually adjusted for each image (blinded to the identity of the animal), to a threshold level that included the full area of any CD41 positive signal present in the image, but excluded unspecific background noise. At this setting: '*intermodes function*' at 70 out of 255, all of the secondary control images were zero for all measurements. The total number of CD41 positive platelets present in each image was then quantified based on the sum of these thresholded CD41 positive objects that exceeded a size of 1 μm^2 .

The average size in diameter of these more or less round objects was estimated based on the formula for the area of a circle= $\pi \times radius^2$, where the average area (μm^2) was divided with π ($\pi=3.14$), and the square root of this result was multiplied with 2 to get an estimate of the diameter of the circle like (roundish) CD41 positive platelets in the aortic arches and in the blood smears.

Analysis and statistics

Data is presented as the mean $\pm$ S.E.M and was analysed using Graph Pad Prism 5 using statistical tests described in the figure legends. A p value of <0.05 was taken as significant and denoted by * and $p>0.05$ was considered non-significant and denoted by ns.

Results

CD31 immunoreactivity and nuclear density in WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

CD31 positive endothelial cells in all of the WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice displayed the typical morphology associated with each region where endothelial cells lining the greater curvature were evenly spread out and elongated whereas they were more randomly orientated with a cobble stone appearance in the lesser curvature (Figure 5.1 and 5.2). There was abundant CD31 immunoreactivity in both the greater and the lesser curvature in aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice (Figure 5.1, 5.2, 5.3A). The CD31 immunoreactivity did not differ between the two regions within each animal group, except for in COX-2^{-/-} mice where significantly higher levels were detected in the lesser curvature compared to the greater curvature (Figure 5.3A). There was no significant difference in CD31 immunoreactivity in any of the COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice compared to the WT mice (Figure 5.3A).

Furthermore, as reported for tissue from WT animals in data shown in Chapter 3, the nuclear density (quantified as the total area of the DAPI positive thresholded nuclei) was significantly higher in the lesser curvature compared to the greater curvature in all the types of mice studied (Figure 5.1, 5.2, 5.3B). The nuclear density in either region did not significantly vary between the types of mice (WT mice versus the COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice) (Figure 5.3B). The number of DAPI positive thresholded nuclei were significantly increased in the lesser curvature compared to the greater curvature in all of the mice, but again did not vary between the different types of mice (Figure 5.4A). The average size (μm^2) of the thresholded DAPI positive nuclei was also significantly larger in the lesser curvature compared to the greater curvature in all mice tested except for in the normal chow fed ApoE^{-/-} mice (Figure 5.4B).

NF- κ B immunoreactivity in untreated versus LPS treated animals

NF- κ B immunoreactivity was detected in both the greater and the lesser curvature of all types of mice studied (Figure 5.5 and 5.6). Under control (untreated) conditions, NF- κ B immunoreactivity appeared to be predominately located within the cytosol of endothelial

cells (Figure 5.5) and following LPS stimulation (4mg/kg i.p. for 30 minutes), there was a clear translocation of NF- κ B immunoreactivity from the cytosol to the nucleus (Figure 5.6). LPS treatment resulted in significantly higher levels of both total (global cytosolic and nuclear levels) and nuclear NF- κ B immunoreactivity in both the greater and the lesser curvature regions of WT mice (Figure 5.8A+B+C+D) and in COX-1^{-/-} and COX-2^{-/-} mice (Figure 5.8B+C+D) except for total NF- κ B immunoreactivity in the greater curvature which was not significantly different in the COX-1^{-/-} and COX-2^{-/-} mice following LPS treatment (Figure 5.8A).

Non-specific background immunoreactivity

The non-specific background immunoreactivity in the endothelium of WT descending aortas stained only with the secondary antibody (goat anti-rabbit Alexa Fluor 568) was low and without any specific looking pattern (Figure 5.7). This secondary control mean fluorescence (from WT descending aortas) was subtracted from each image before the values were included in graphs and statistical analysis.

Global levels of NF- κ B immunoreactivity in aortic arches from WT versus COX-1^{-/-} and COX-2^{-/-} mice

Global levels of NF- κ B immunoreactivity (total NF- κ B immunoreactivity) were increased in endothelial cells in both the greater and the lesser curvature in aortic arches from untreated COX-1^{-/-} mice, compared to levels detected in WT control animals (Figure 5.5 and 5.8A). A similar pattern of increased NF- κ B immunoreactivity was seen in tissues from untreated COX-2^{-/-} mice compared to WT mice, although this difference did not reach statistical significance in the lesser curvature of untreated COX-2^{-/-} mice compared to WT mice (Figure 5.5 and 5.8B).

Nuclear (NF- κ B immunoreactivity in aortic arches from WT versus COX-1^{-/-} and COX-2^{-/-} mice

Nuclear NF- κ B immunoreactivity was significantly increased in endothelium of both the greater and the lesser curvature in aortic arches from untreated COX-1^{-/-} mice compared to WT mice (Figure 5.5 and 5.8C+D)). A similar pattern of increased NF- κ B immunoreactivity was seen in tissues from untreated COX-2^{-/-} mice compared to WT mice, although this difference did not reach statistical significance in the lesser curvature of untreated COX-2^{-/-} mice compared to WT mice (Figure 5.5 and 5.8C+D)).

Following LPS stimulation, there was a marked increase of nuclear NF- κ B in the greater curvature of COX-2^{-/-} mice, that was significantly much larger than the nuclear levels recorded in the WT mice (Figure 5.8C+D). Nuclear NF- κ B in the greater curvature of COX-1^{-/-} mice was however not different from WT following LPS stimulation (Figure 5.8C). Both the COX-1^{-/-} and the COX-2^{-/-} mice had significantly more nuclear NF- κ B levels in the lesser curvature compared to WT mice following LPS treatment (Figure 5.8D).

Regional differences in NF- κ B immunoreactivity

As previously reported, nuclear NF- κ B immunoreactivity was higher in the lesser curvature than in the greater curvature regions in aortic arches from both untreated and LPS treated, control WT mice (Table 5.10). In my thesis I show this is also the case for NF- κ B immunoreactivity in endothelium of COX-1^{-/-}, but not COX-2^{-/-} mice (Table 5.10). After LPS, both global levels and nuclear NF- κ B immunoreactivity was higher in endothelium of the lesser curvature than the greater curvature regions arch tissue from all genotypes of mice used, including COX-2^{-/-} (Table 5.9 + Table 5.10).

Platelet adhesion to the aortic endothelium visualised by CD41 immunoreactivity
Validation of the CD41 antibody in blood smears

The utility of the CD41 antibody used in this chapter was validated in a pilot study where blood smears from a WT mouse were stained and imaged. These studies confirmed that the platelets were clearly visible with red CD41 immunoreactivity whereas DAPI positive, nucleated cells (blood leukocytes) did not stain with CD41 (Figure 5.11A). When the same CD41 staining protocol was tested in aortic arches from WT mice, there were a few positive CD41 platelet like bodies, that seemed adhered to the endothelium in the vessel wall (Figure 5.11B). This protocol was then repeated using blood from n=2 separate mice from each genotype and images compared head to head. Again, blood from WT as well from COX-1^{-/-}, COX-2^{-/-} and ApoE^{-/-} mice showed clear CD41 positive platelets (Figure 5.11C). There were no CD41 positive platelets detected in the secondary control blood smears or aortic arches (stained only with secondary control).

CD41 positive platelets adhered to the endothelium of aortic arches from WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

Throughout this thesis I have used CD31 as a positive stain for endothelial cells. However, CD31 is also expressed on platelets, and as such is known as platelet endothelial cell adhesion molecule-1 (PECAM-1). In imaging platelets for this part of my thesis I have used CD41, which is also a platelet marker, but unlike CD31 is not expressed on endothelial cells. Platelets have no nucleus and don't stain positively with DAPI (once activated). Therefore, platelets adhered to the endothelium of the aortic arch were characterised as CD41 and CD31 positive, DAPI negative bodies. Platelets adhered to the endothelium (in between or close to nucleated endothelial cells) were observed in both regions of the aortic arch from each genotype of the mouse studied (Figure 5.12 and 5.13). Both manual (Figure 5.14B) and automated (using Image J software) (Figure 5.14A) blinded quantification showed a clear signal for increased platelet adhesion in the lesser curvature region of aortic arches from COX-2^{-/-} mice (Figure 5.14A+B). Within group analysis showed that the number of CD41 positive platelets did not vary between regions of the arch for individual genotypes, except

for in the COX-2^{-/-} and in the normal chow fed ApoE^{-/-} mice where there was a significant increase in the lesser curvature compared to the greater curvature (Figure 5.14A+B).

The average size (in diameter) of the CD41 positive platelets in the aortic arch (Figure 5.15A) were on average around the same size as the average sized of the platelets recorded from the blood smears from these mice (around 3µm) (Figure 5.15B).

Pilot study of CD41 positive platelets adhered to the endothelium of the aortic arch in an ApoE^{-/-} mice fed an atherogenic diet

The data above reports platelet adhesion in tissue from COX^{-/-} mice and from ApoE^{-/-} mice, fed on a normal chow diet, which will display markers of vascular inflammation, but may not yet have developed atherosclerotic plaques (Daugherty, 2002; Zhang *et al.*, 1992). However, during the course of this study, I had the opportunity to image platelets in a lipid enriched, atherosclerotic plaque containing aortic arch from an ApoE^{-/-} mouse fed an atherogenic diet to induce atherosclerosis. In this pilot study I manually made a tile of images from adjacent, consecutive regions of the aortic arch (also in Chapter 4, Figure 4.17). These showed a clear plaque-like structure running along the lesser curvature of the aortic arch (Figure 5.16). When the CD41 positive platelets adhered to the superficial CD31 positive endothelial layer of the plaque was quantified (by the automated protocol in Image J), there appeared to be more CD41 positive platelets adhered to the plaque enriched areas of the arch compared to 'normal looking' adjacent endothelium in the greater curvature regions (Figure 5.16). The plaque enriched areas were also associated with an increased nuclear density (quantified by number of thresholded DAPI positive nuclei and the total area of these nuclei) (Figure 5.17).

There was also CD41 positive (and CD31 positive) platelets inside the core of the plaque and in the deeper layers underneath; where the CD41 immunoreactivity was found associated with a nucleated cell (Figure 5.18).

Figures Chapter 5

Figure 5.1 Representative images of CD31 immunoreactivity and DAPI positive nuclear density in the greater curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

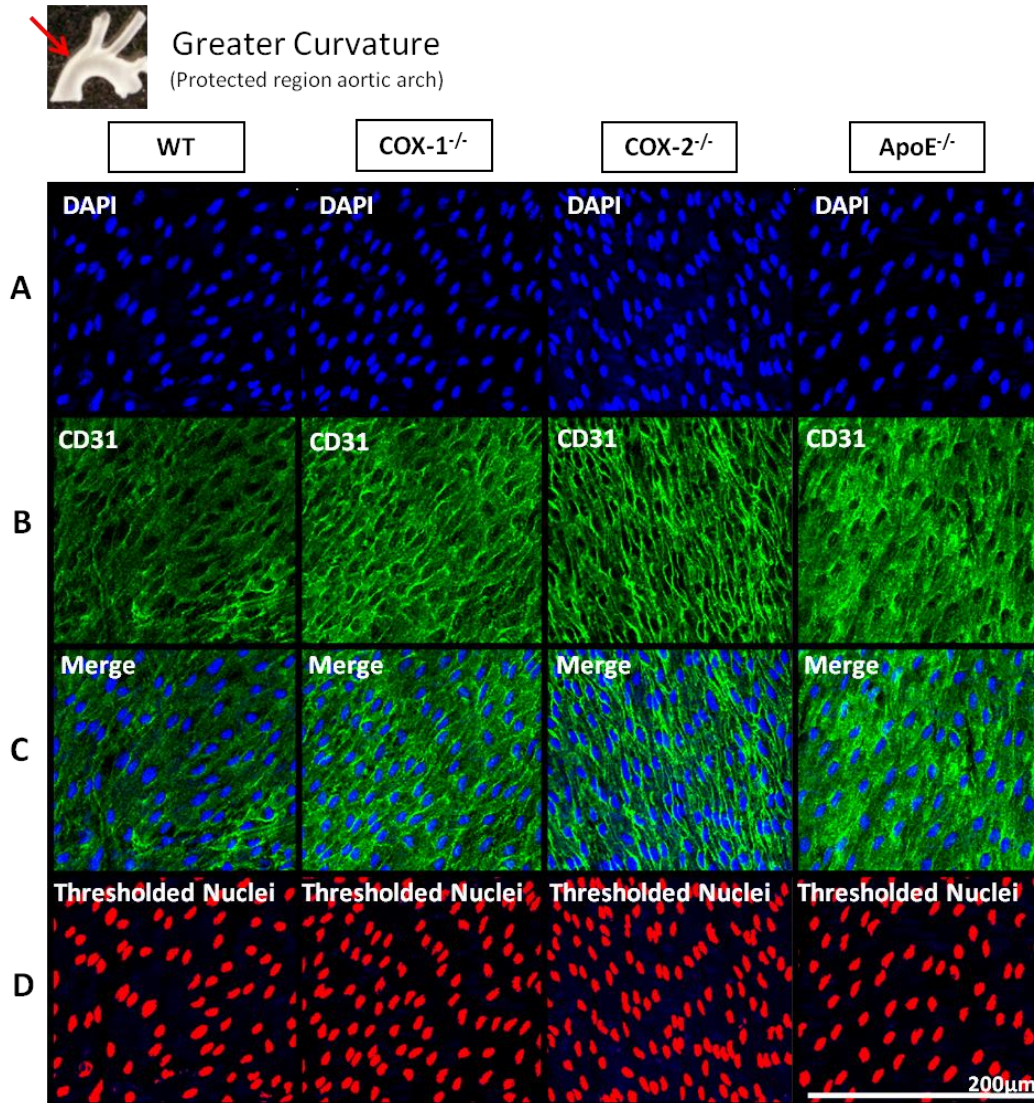


Figure 5.1 Representative images of CD31 immunoreactivity and DAPI positive nuclear density in the greater curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Representative images (180x200µm) showing endothelial morphology in the greater curvature of aortic arches from n=6 male and female 3 month old WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice fixed according to the transcardial perfusion fixation procedure. **(A)** DAPI nuclear stain (**blue**). **(B)** CD31 immunoreactivity (**green**) **(C)** Merge of blue and green. **(D)** DAPI positive nuclei thresholded by the default criteria in Image J (**red**) were selected for quantification of nuclei area and size. All of the images have been enhanced in brightness (+20) and contrast (+40) for clarity of viewing purposes.

Figure 5.2 Representative images of CD31 immunoreactivity and DAPI positive nuclear density in the lesser curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

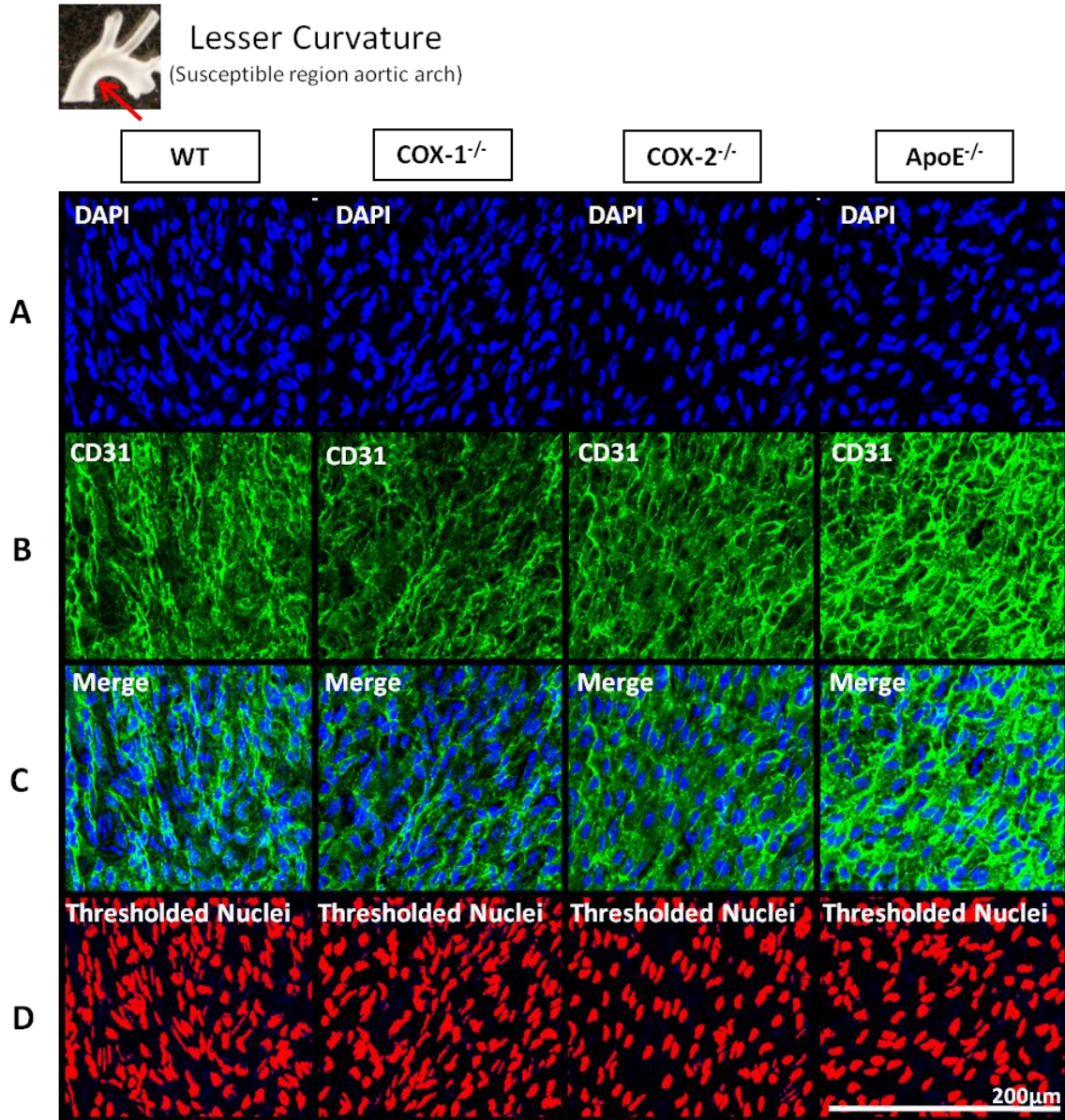


Figure 5.2 Representative images of CD31 immunoreactivity and DAPI positive nuclear density in the lesser curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Representative images (180x200µm) showing endothelial morphology in the lesser curvature of aortic arches from n=6 male and female 3 month old WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice fixed according to the transcardial perfusion fixation procedure. (A) DAPI nuclear stain (blue). (B) CD31 immunoreactivity (green) (C) Merge of blue and green. (D) DAPI positive nuclei thresholded by the default criteria in Image J (red) were selected for quantification of nuclei area and size. All of the images have been enhanced in brightness (+20) and contrast (+40) for clarity of viewing purposes.

Figure 5.3 Quantified CD31 immunoreactivity and DAPI positive nuclear density in the greater and lesser curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

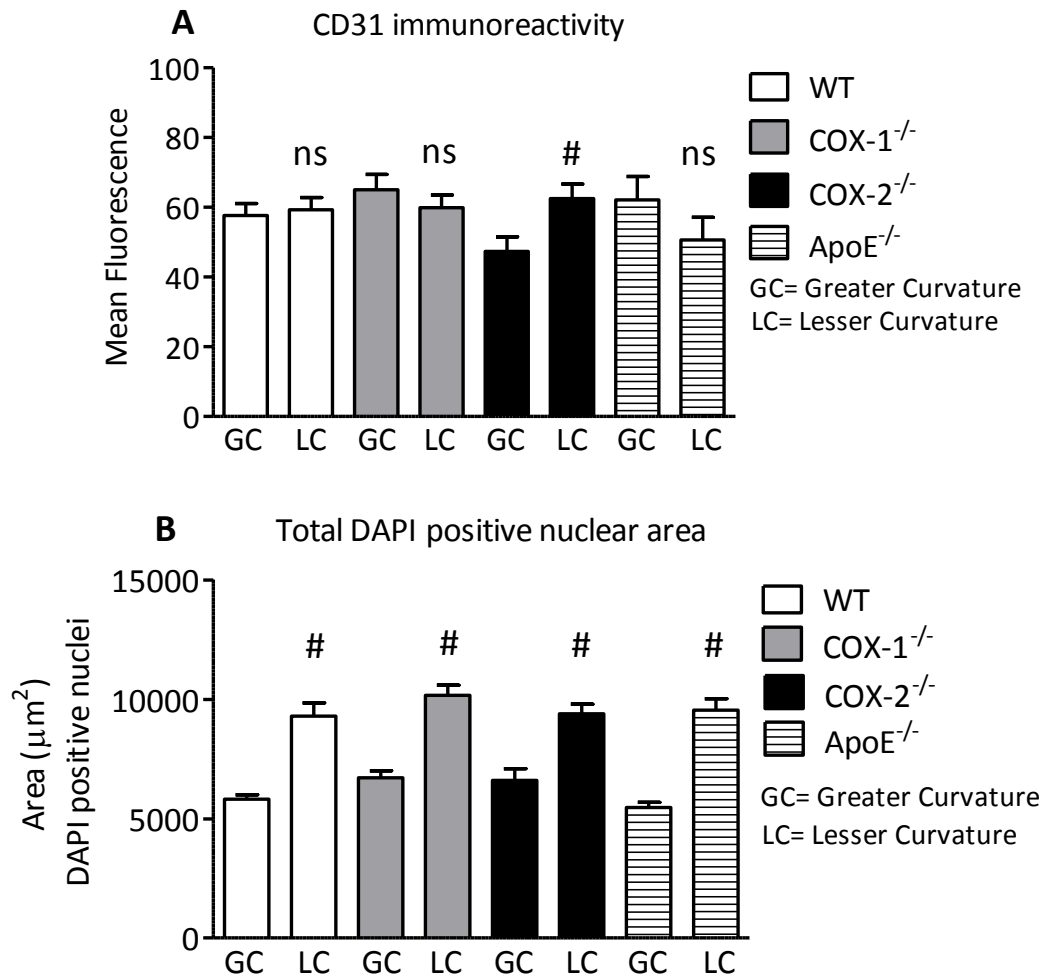


Figure 5.3 Quantified CD31 immunoreactivity and DAPI positive nuclear density in the greater and lesser curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. 12 images (2 per region) were zoomed in (from the full field image 387.5x387.5 to 180x200μm) using LAS confocal software in a blinded fashion from n=6 3 month old male and female WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. The blinded quantification included **(A)** mean fluorescence of CD31 immunoreactivity using LAS confocal software and **(B)** the total DAPI positive nuclear area (μm²) in thresholded images using the default criteria in Image J. Data is presented as mean ± S.E.M. Within group analysis (greater curvature versus the lesser curvature) was performed using paired Student's t-test and p<0.05 was taken as significant and denoted by #. Between groups analysis was analysed using one-way ANOVA with Dunnett's multiple comparison post-test where the WT data from each region was set as the control column. P<0.05 taken as significant and denoted by * and p>0.05 was taken as non-significant and denoted by ns.

Figure 5.4 Quantified number and average size of DAPI positive nuclei in the greater and the lesser curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

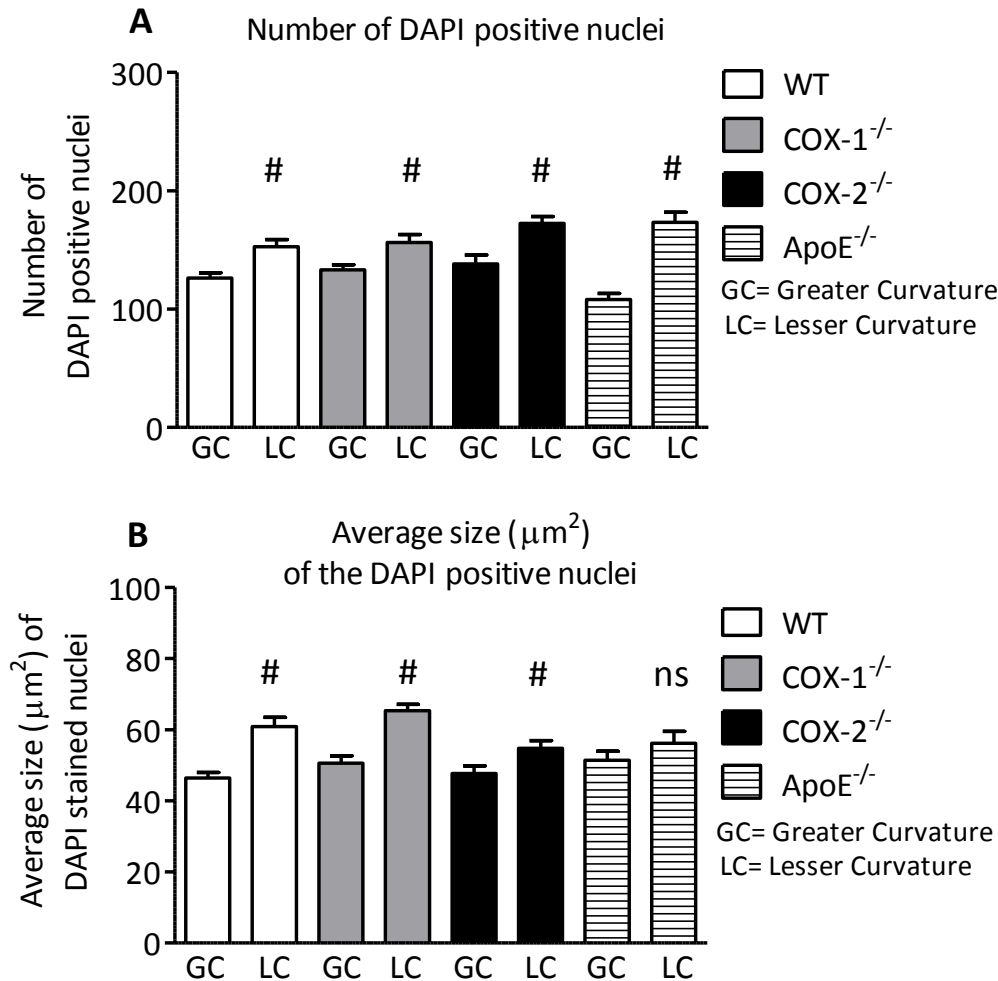


Figure 5.4 Quantified number and average size of DAPI positive nuclei in the greater curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. 12 images (2 per region) were zoomed in (from the full field image 387.5x387.5 to 180x200 μm) using LAS confocal software in a blinded fashion from n=6 3 month old male and female WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Blinded quantification included **(A)** the number and **(B)** the average area size (μm^2) of the DAPI positive nuclei selected by thresholding using the default criteria in Image J, where DAPI positive objects with an area $\leq 2 \mu\text{m}^2$ were excluded from the analysis. Data is presented as mean $\pm$ S.E.M. Within group analysis (greater curvature versus the lesser curvature) was performed using paired Student's t-test and $p < 0.05$ was taken as significant and denoted by #. Between groups analysis was analysed using one-way ANOVA with Dunnett's multiple comparison post-test where the WT data was set as the control column. $P < 0.05$ taken as significant and denoted by * and $p > 0.05$ was taken as non-significant and denoted by ns.

Figure 5.5 NF- κ B immunoreactivity in the greater and lesser curvature of aortic arches from untreated WT, COX-1^{-/-} and COX-2^{-/-} mice

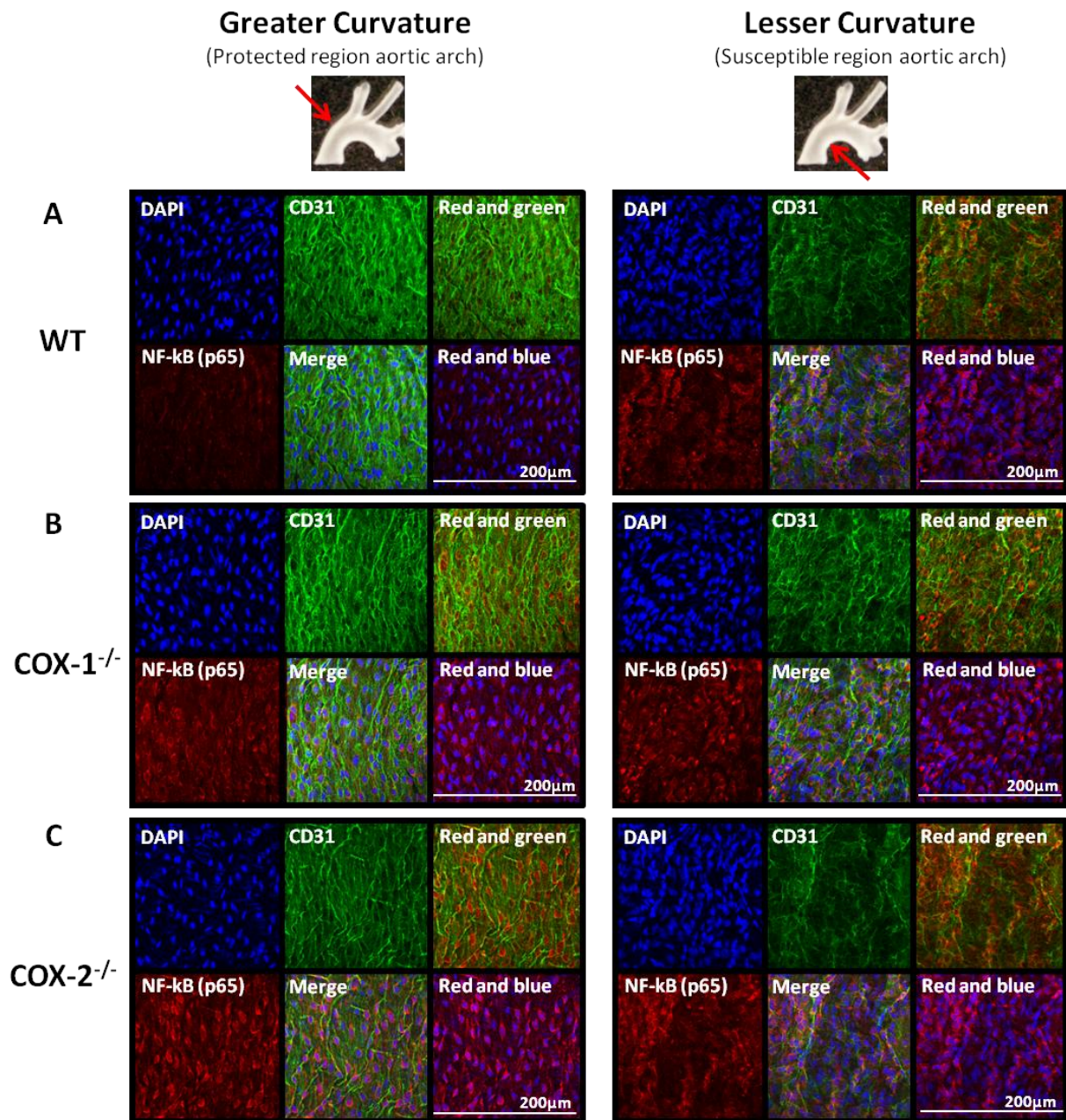


Figure 5.5 NF- κ B immunoreactivity in the greater and lesser curvature of aortic arches from untreated WT, COX-1^{-/-} and COX-2^{-/-} mice. Representative cropped images (200x200 μ m) showing NF- κ B/p65 immunoreactivity (red) in the endothelium of the greater and the lesser curvature of aortic arches from untreated (A) WT mice (C57BL/6J, bred in-house) (B) COX-1^{-/-} mice and (C) COX-2^{-/-} mice (3-4 month old male and female). The aortic arches were fixed according to the transcardial perfusion fixation procedure. Nuclei visualised with DAPI (blue) and endothelial cells outlined with CD31 (green). Merge represent DAPI (blue), CD31 (green) and NF- κ B/p65 (red) channels merged. Red and green represents NF- κ B (red) and CD31 (green) merged. Red and blue channels were merged in order to identify nuclear location of red NF- κ B/p65 in the blue DAPI stained nuclei.

Figure 5.6 NF- κ B immunoreactivity in the greater and lesser curvature of aortic arches from LPS treated WT, COX-1^{-/-} and COX-2^{-/-} mice

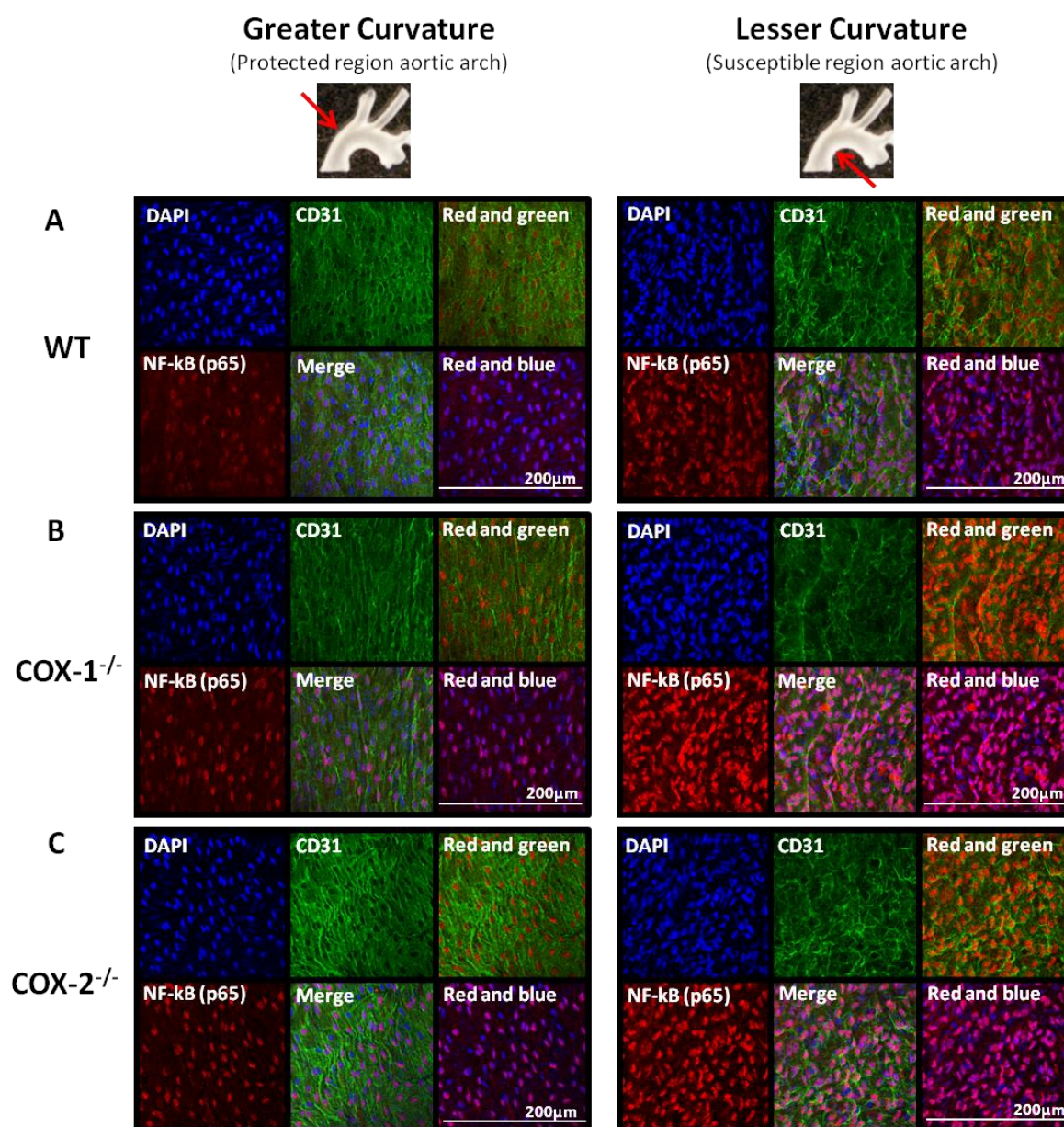


Figure 5.6 NF- κ B immunoreactivity in the greater and lesser curvature of aortic arches from LPS treated WT, COX-1^{-/-} and COX-2^{-/-} mice. Representative cropped images (200x200 μ m) showing NF- κ B/p65 immunoreactivity (red) in the endothelium of the greater and the lesser curvature of aortic arches from LPS treated (serotype 055:B5, 4mg/kg; i.p for 30 minutes) (A) WT mice (C57BL/6J, bred in-house) (B) COX-1^{-/-} mice and (C) COX-2^{-/-} mice (3-4 month old male and female). The aortic arches were fixed according to the transcatheter perfusion fixation procedure. Nuclei visualised with DAPI (blue) and endothelial cells outlined with CD31 (green). Merge represent DAPI (blue), CD31 (green) and NF- κ B/p65 (red) channels merged. Red and green represents NF- κ B (red) and CD31 (green) merged. Red and blue channels were merged in order to identify nuclear location of red NF- κ B/p65 in the blue DAPI stained nuclei.

Figure 5.7 Non-specific background immunoreactivity from the secondary antibody goat-anti-rabbit Alexa Fluor 568 in the descending aorta from a WT mouse

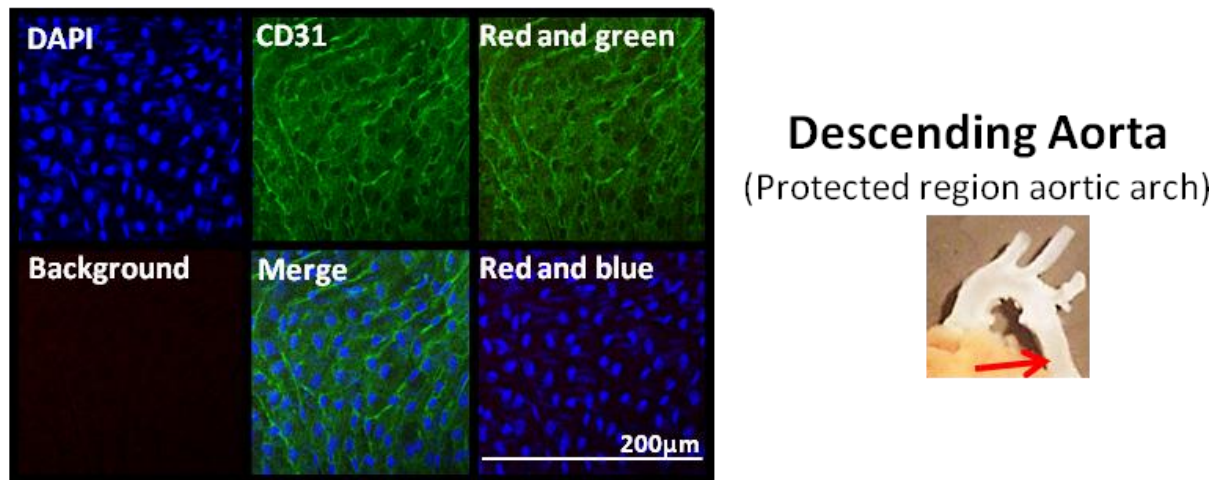


Figure 5.7 Non-specific background immunoreactivity from the secondary antibody goat anti-rabbit Alexa Fluor 568 in the descending aorta from a WT mouse. Representative cropped images (200x200µm) from a descending aorta from a WT mouse that has only been incubated with the secondary antibody goat anti-rabbit Alexa Fluor 568 (Invitrogen, A11026) used to detect the rabbit polyclonal NF-κB/p65 primary antibody (Santa Cruz SC-372). Background mean fluorescence of WT descending aorta (incubated solely with secondary control antibody) was subtracted from each image of the NF-κB data set before values were included in the analysis.

Figure 5.8 Effect of LPS treatment to **total and nuclear NF- κ B immunoreactivity in the greater and lesser curvature of aortic arches from WT, COX-1^{-/-} and COX-2^{-/-} mice**

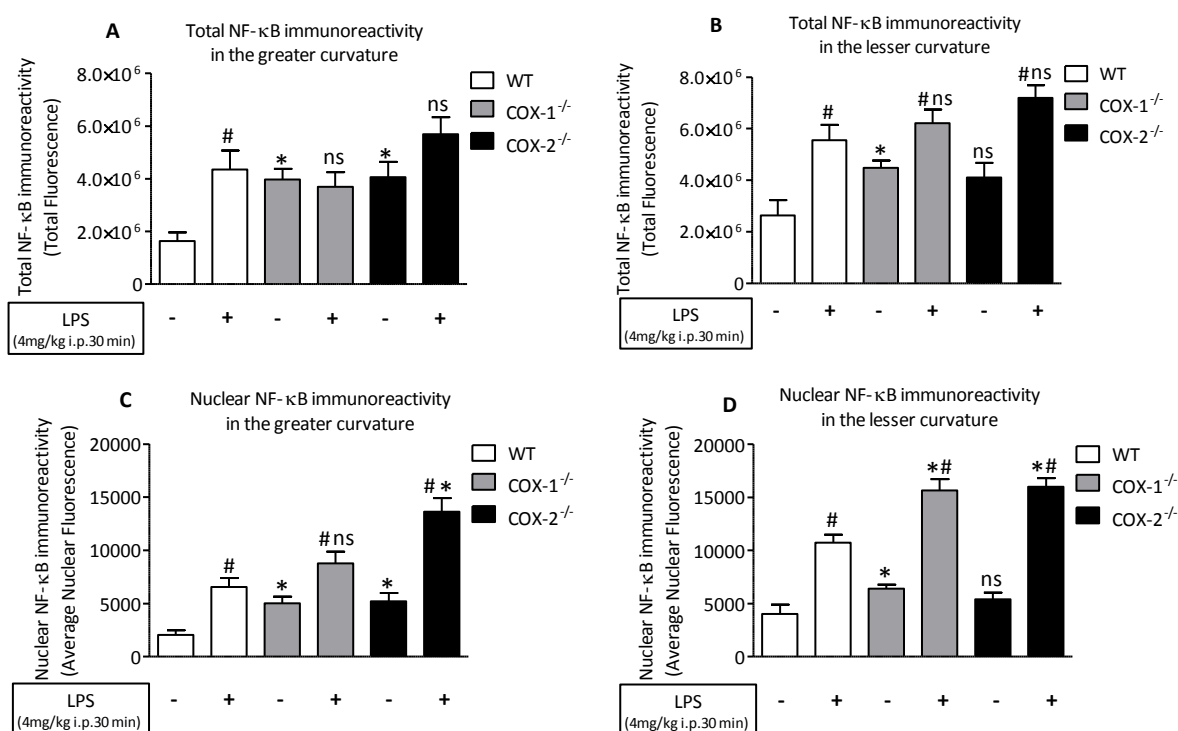


Figure 5.8 Effect of LPS treatment to **total and nuclear NF- κ B immunoreactivity in the greater and lesser curvature of aortic arches from WT, COX-1^{-/-} and COX-2^{-/-} mice**. Pooled data quantified using Velocity 5 software for NF- κ B/p65 immunoreactivity as total red fluorescence and average nuclear fluorescence in the **(A+C)** greater curvature and in the **(B+D)** the lesser curvature in aortic arches from untreated versus LPS treated (serotype 055:B5, 4mg/kg; i.p for 30 minutes) WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice. Secondary control background was subtracted from each image before analysis. Data is the mean $\pm$ S.E.M of $n=13-15$ untreated and $n=11-15$ LPS treated (3 separate images per region) from $n=5$ 3 month old male ($n=3$) and female ($n=2$) mice. Within group analysis (untreated versus LPS treated) was performed using unpaired Student's t-test and $p < 0.05$ was taken as significant and denoted by #. Between groups analysis was analysed using one-way ANOVA with Dunnett's multiple comparison post-test where the WT data from each region was set as the control column. $P < 0.05$ taken as significant and denoted by * and $p > 0.05$ was taken as non-significant and denoted by ns.

Table 5.9 Regional differences in total (global) NF-κB immunoreactivity in the greater versus the lesser curvature of untreated or LPS treated WT, COX-1^{-/-} and COX-2^{-/-} mice

Total NF-κB	A) Untreated		B) LPS treated	
	Greater curvature	Lesser curvature	Greater curvature	Lesser curvature
WT	1.6 ± 0.3 E ⁶	2.6 ± 0.6 E ⁶ (ns)	4.4 ± 0.7 E ⁶	5.6 ± 0.6 E ⁶ (#)
COX-1^{-/-}	4.0 ± 0.4 E ⁶	4.5 ± 0.3 E ⁶ (ns)	3.7 ± 0.6 E ⁶	6.2 ± 0.5 E ⁶ (#)
COX-2^{-/-}	4.1 ± 0.6 E ⁶	4.1 ± 0.6 E ⁶ (ns)	5.7 ± 0.7 E ⁶	7.2 ± 0.5 E ⁶ (#)

Table 5.9 Regional differences in total red (global) NF-κB immunoreactivity in the greater versus the lesser curvature of untreated or LPS treated mice of different genotypes. Pooled data quantified using Velocity 5 software for total (global) NF-κB/p65 immunoreactivity in the greater and lesser curvature in aortic arches from (A) untreated and (B) LPS treated (serotype 055:B5, 4mg/kg; i.p for 30 minutes) WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice. Secondary control background was subtracted from each image before analysis. The data is the mean ± SEM for n=11-15 images using tissue from 5 mice for each genotype. Data was analysed using Student's paired T-test and p<0.05 was taken as significant and denoted by (#) and p>0.05 was taken as non-significant and denoted by (ns).

Table 5.10 Regional differences in nuclear NF-κB immunoreactivity in the greater versus the lesser curvature of untreated or LPS treated WT, COX-1^{-/-} and COX-2^{-/-} mice

Nuclear NF-κB	A) Untreated		B) LPS treated	
	Greater curvature	Lesser curvature	Greater curvature	Lesser curvature
WT	2024 ± 451	4031 ± 862 (#)	6564 ± 836	10731 ± 766 (#)
COX-1^{-/-}	5020 ± 602	6411 ± 369 (#)	8764 ± 1094	15634 ± 1067 (#)
COX-2^{-/-}	5207 ± 773	5398 ± 625 (ns)	13643 ± 1285	15988 ± 823 (#)

Table 5.10 Regional differences in nuclear NF-κB immunoreactivity in the greater versus the lesser curvature of untreated or LPS treated mice of different genotypes. Pooled data quantified using Velocity 5 software for nuclear NF-κB/p65 immunoreactivity in the greater and lesser curvature in aortic arches from **(A)** untreated and **(B)** LPS treated (serotype 055:B5, 4mg/kg; i.p for 30 minutes) WT (C57BL/6J, bred in-house), COX-1^{-/-} and COX-2^{-/-} mice. Secondary control background was subtracted from each image before analysis. The data is the mean ± SEM for n=11-15 images using tissue from 5 mice for each genotype. Data was analysed using Student's paired T-test and p<0.05 was taken as significant and denoted by (#) and p>0.05 was taken as non-significant and denoted by (ns).

Figure 5.11 CD41 immunoreactivity in blood smears from untreated WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

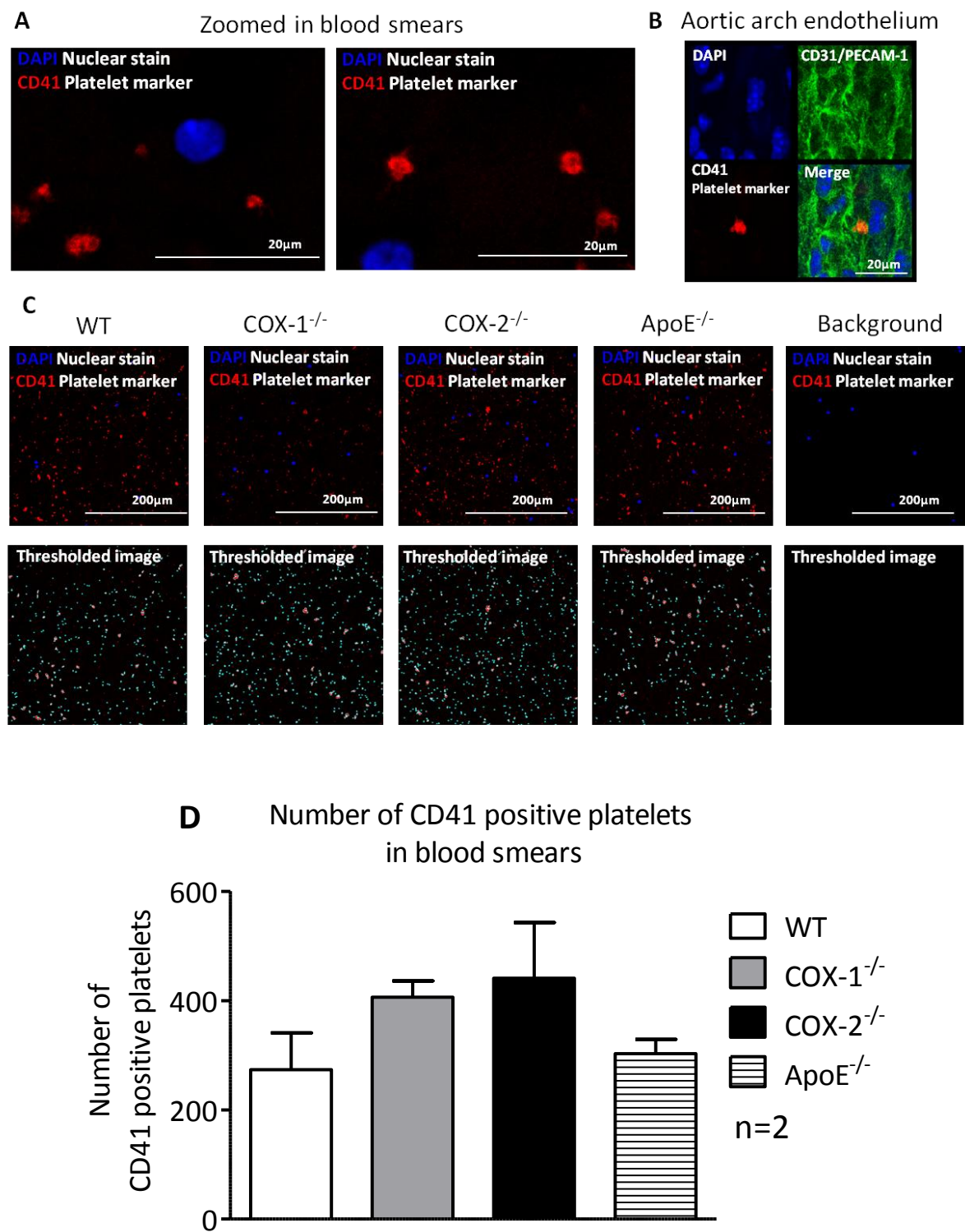


Figure 5.11 Figure legend located on the next page.

Figure 5.11 CD41 immunoreactivity in blood smears from untreated WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Representative images of CD41 (also referred to as platelet glycoprotein IIb or integrin alpha 2b) immunoreactivity in **(A)** zoomed in blood smears from a WT mouse **(B)** in aortic arch endothelium from a male, 3 month old WT mouse (C57BL/6J bred in-house). The aortic arch was fixed according to the transcardial perfusion fixation procedure. **(C)** Blood smears from WT (C57BL/6J, bred in-house), COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. All the blood smears were subject to the same staining protocol parallel to the aortic arch en face imaging study. Nuclei visualised with DAPI (**blue**) endothelial cells outlined with CD31 or Platelet Endothelial Adhesion Cell Molecule- 1 (PECAM-1, which is another name for CD31) (**green**) and platelets were visualised by the platelet marker CD41 (**red**). Blood smears were thresholded in Image J using the default criteria and CD41 positive bodies in the size range of 1µm to infinity were selected for analysis. At this setting the background fluorescence from the secondary antibody (Alexa Fluor 594 goat anti-rat Invitrogen, A-11007) was zero (image in C lower panel far right). **(D)** Pooled quantified data from blood smears using Image J software. CD41 positive bodies (larger than 1µm²) were selected for analysis and all of the images were thresholded using the default criteria in Image J. At this setting, the secondary control non-specific immunoreactivity was zero. Data is the mean ± S.E.M of n=2 mice (1 image per blood smear).

Figure 5.12 CD41 positive platelets adhered to the endothelium of the **greater curvature** of aortic arches from untreated WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

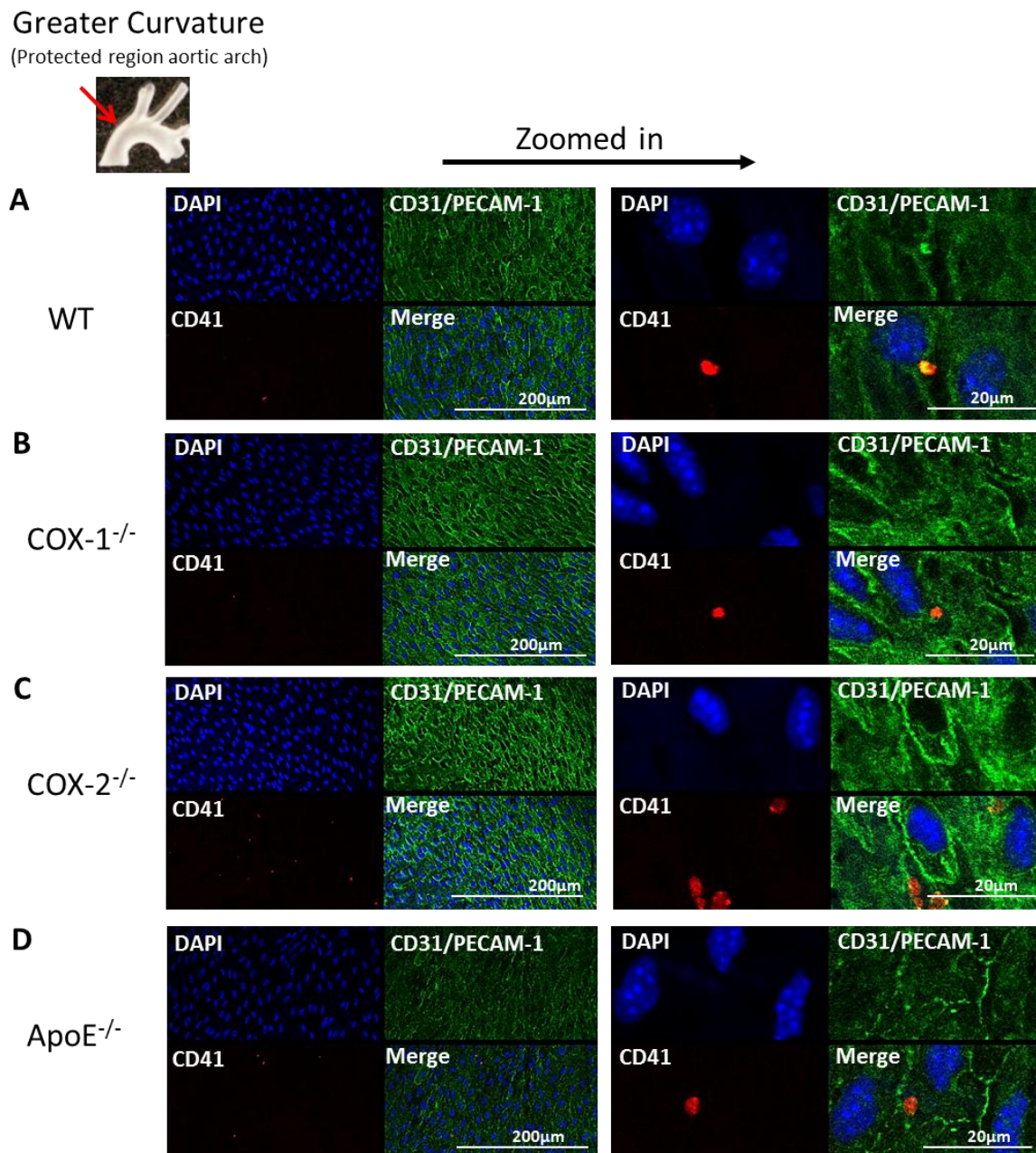


Figure 5.12 CD41 positive platelets adhered to the endothelium of the **greater curvature** of aortic arches from untreated WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Representative zoomed in images of CD41 positive platelets in endothelium from the greater curvature in aortic arches from 3 month old male and female (A) WT mice (C57BL/6J, bred in-house) (B) COX-1^{-/-} mice (C) COX-2^{-/-} mice and (D) ApoE^{-/-} mice (fed a normal chow diet). The aortic arches were fixed according to the transcardial perfusion fixation procedure. Nuclei visualised with DAPI (**blue**) endothelial cells outlined with CD31 or Platelet Endothelial Adhesion Cell Molecule-1 (PECAM-1) (**green**) and platelets were visualised by the platelet marker CD41 (platelet glycoprotein IIb or integrin alpha 2b) (**red**).

Figure 5.13 CD41 positive platelets adhered to the endothelium of the **lesser curvature** of aortic arches from untreated WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

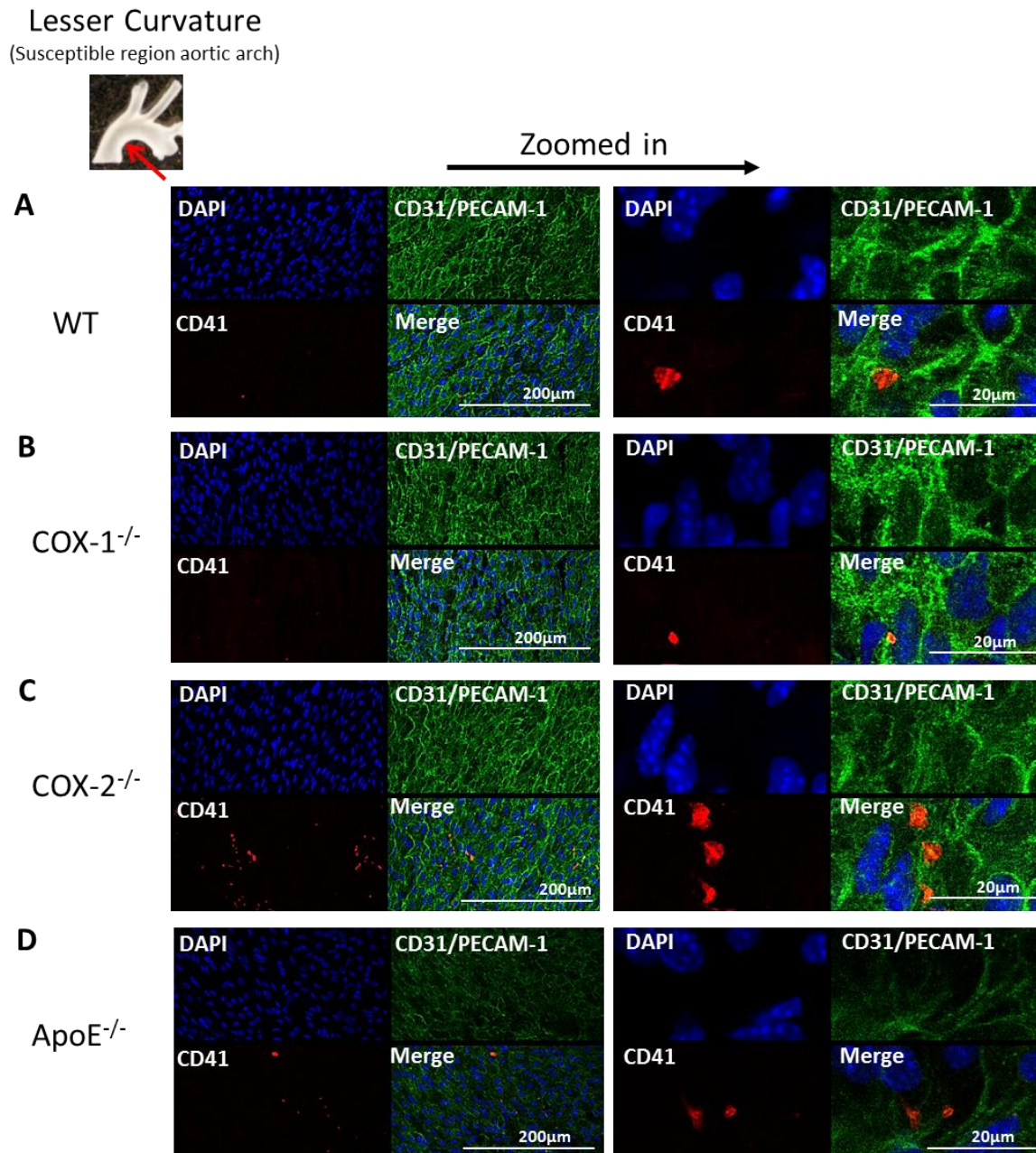


Figure 5.13 CD41 positive platelets adhered to the endothelium of the **lesser curvature** of aortic arches from untreated WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Representative zoomed in images of CD41 positive platelets in endothelium from the lesser curvature in aortic arches from 3 month old male and female **(A)** WT mice (C57BL/6J, bred in-house) **(B)** COX-1^{-/-} mice **(C)** COX-2^{-/-} mice and **(D)** ApoE^{-/-} mice (fed a normal chow diet). The aortic arches were fixed according to the transcardial perfusion fixation procedure. Nuclei visualised with DAPI (**blue**) endothelial cells outlined with CD31 or Platelet Endothelial Adhesion Cell Molecule- 1 (PECAM-1) (**green**) and platelets were visualised by the platelet marker CD41 (platelet glycoprotein IIb or integrin alpha 2b) (**red**).

Figure 5.14 Average number of CD41 positive platelets adhered to the endothelium of the greater and lesser curvature of aortic arches from WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice quantified by Image J or by manual blind scoring

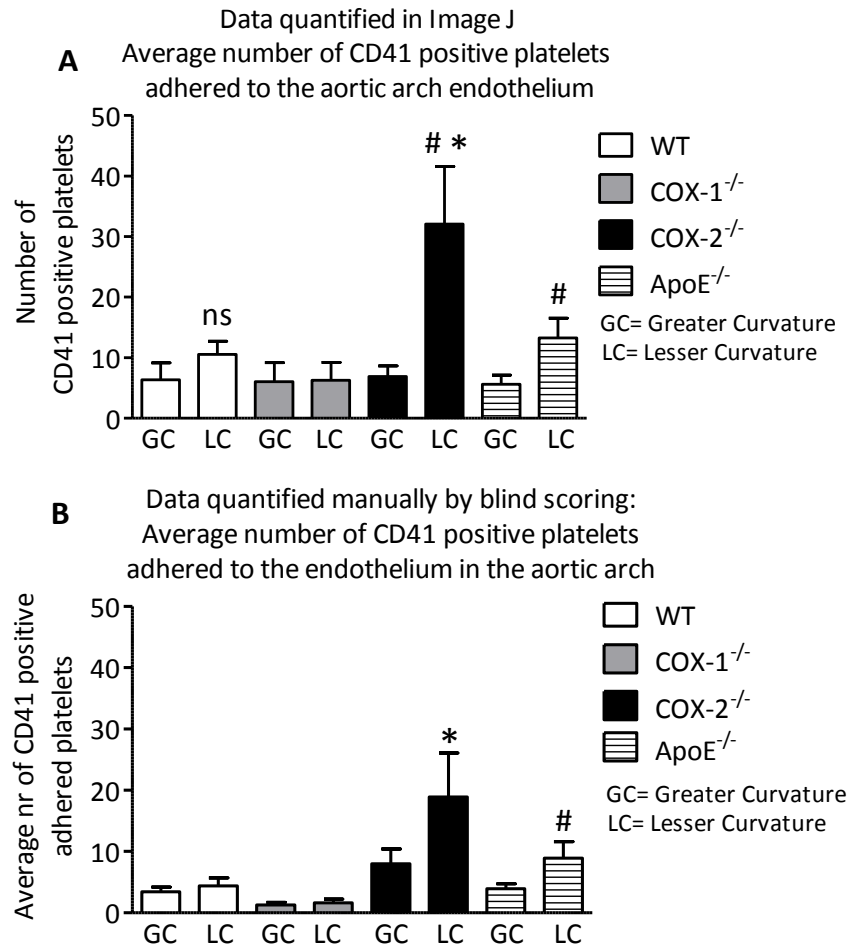


Figure 5.14 Average number of CD41 positive platelets adhered to the endothelium of the greater and lesser curvature of aortic arches from WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice quantified by Image J or by manual blind scoring. (A) Pooled quantified data from manual blind scoring (data represent the average result from two blind scorers) (B) or by Image J software program (from thresholded images, excluding values with an area lower than 1 μm^2), showing average number of CD41 positive platelets per aortic arch region. The aortic arches were fixed according to the transcardial perfusion fixation procedure. Secondary control background was zero for both measurements from both regions. Data is the mean $\pm$ S.E.M of $n=12$ separate images from $n=6$ male and female, 3 month old mice. Within group analysis (greater curvature versus the lesser curvature) was performed using paired Student's t -test and $p<0.05$ was taken as significant and denoted by #. Between groups analysis was analysed using one-way ANOVA with Dunnett's multiple comparison post-test where the WT data from each region was set as the control column. $P<0.05$ taken as significant and denoted by * and $p>0.05$ was taken as non-significant and denoted by ns.

Figure 5.15 Average size in diameter of CD41 positive platelets in blood smears versus CD41 positive platelets adhered to the endothelium of the greater and the lesser curvature of aortic arches from WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

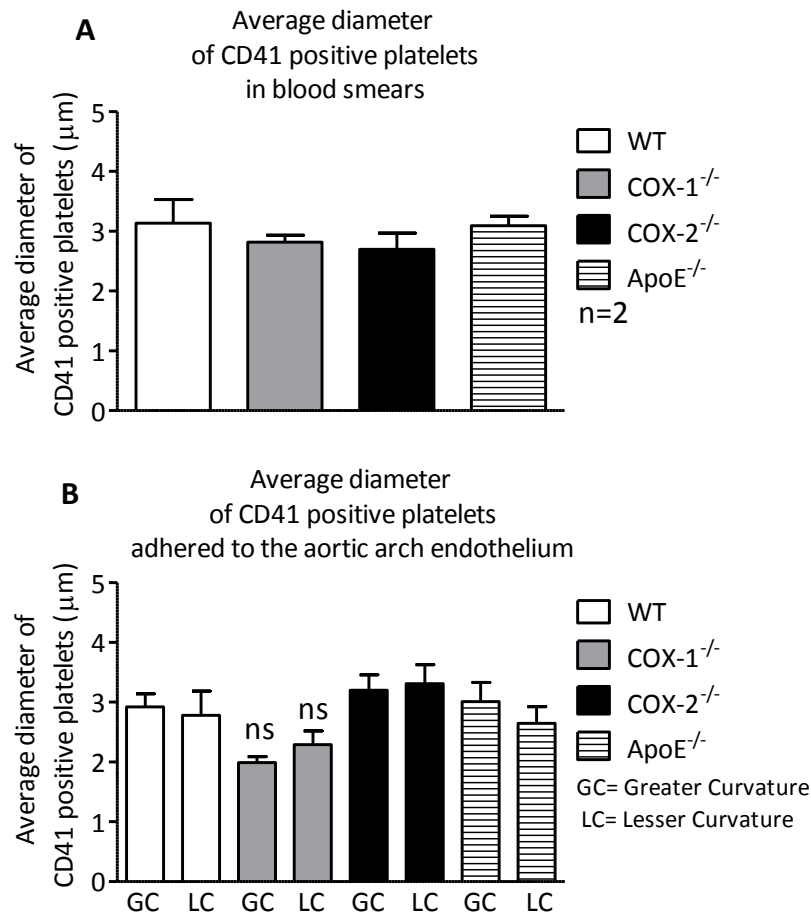


Figure 5.15 Average size in diameter of CD41 positive platelets in blood smears versus CD41 positive platelets adhered to the endothelium of the greater and the lesser curvature of aortic arches from WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice. Thresholded images quantified in Image J showing (A) average diameter of CD41 positive platelets in blood smears from n=2 WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice and (B) average diameter of CD41 positive platelets adhered to the endothelium in the greater and the lesser curvature of aortic arches from n=12 separate images from n=6 WT versus COX-1^{-/-}, COX-2^{-/-} and chow fed ApoE^{-/-} mice. The aortic arches were fixed according to the transcardial perfusion fixation procedure. Secondary control background was zero for both the blood smears and for the aortic arches from both regions. Data is the mean $\pm$ S.E.M. Within group analysis (greater curvature versus the lesser curvature) was performed using paired Student's t-test and $p < 0.05$ was taken as significant and denoted by #. Between groups analysis was analysed using one-way ANOVA with Dunnett's multiple comparison post-test where the WT data from each region was set as the control column. $P < 0.05$ taken as significant and denoted by * and $p > 0.05$ was taken as non-significant and denoted by ns.

Figure 5.16 Manually made tile composite of 3x4 images showing CD41 immunoreactivity in a female $ApoE^{-/-}$ mouse fed an atherogenic diet

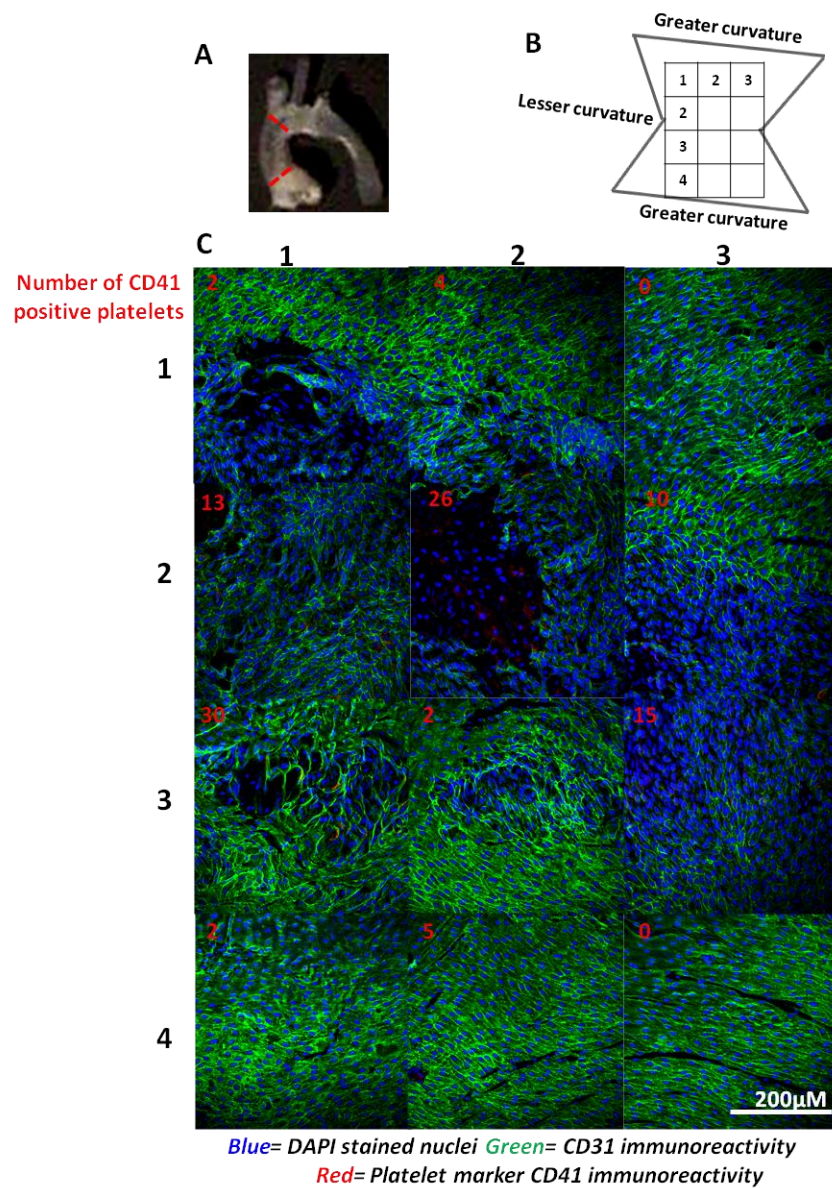


Figure 5.16 Manually made tile composite of 3x4 images showing CD41 immunoreactivity in a female $ApoE^{-/-}$ mouse fed an atherogenic diet. Images from a perfusion fixed (according to the transcardial perfusion fixation procedure) aortic arch dissected from a female $ApoE^{-/-}$ mouse. The tile was manually created by moving the field of view to the next field along and then manually puzzled together as a tile. (A) Red dotted line, mark dissection cutting points. (B) A schematic of the mounted (opened and flattened) aortic arch, marking the location of the greater and the lesser curvature as well as the position of the tile composite (3x4 images, coordinates 1.1-4.3). (C) The entire tile of 3x4 images. DAPI stained nuclei (blue) and CD31 immunoreactivity (green) and CD41 platelet marker (red). Number of CD41 positive platelets in each frame/image is indicated by a red number in the top left corner of each individual frame/image. All of the images have been enhanced in brightness (+20) and contrast (+40) for clarity of viewing purposes.

Figure 5.17 Manually made tile composite of 3x4 images showing thresholded DAPI positive nuclei in a female $ApoE^{-/-}$ mouse fed an atherogenic diet

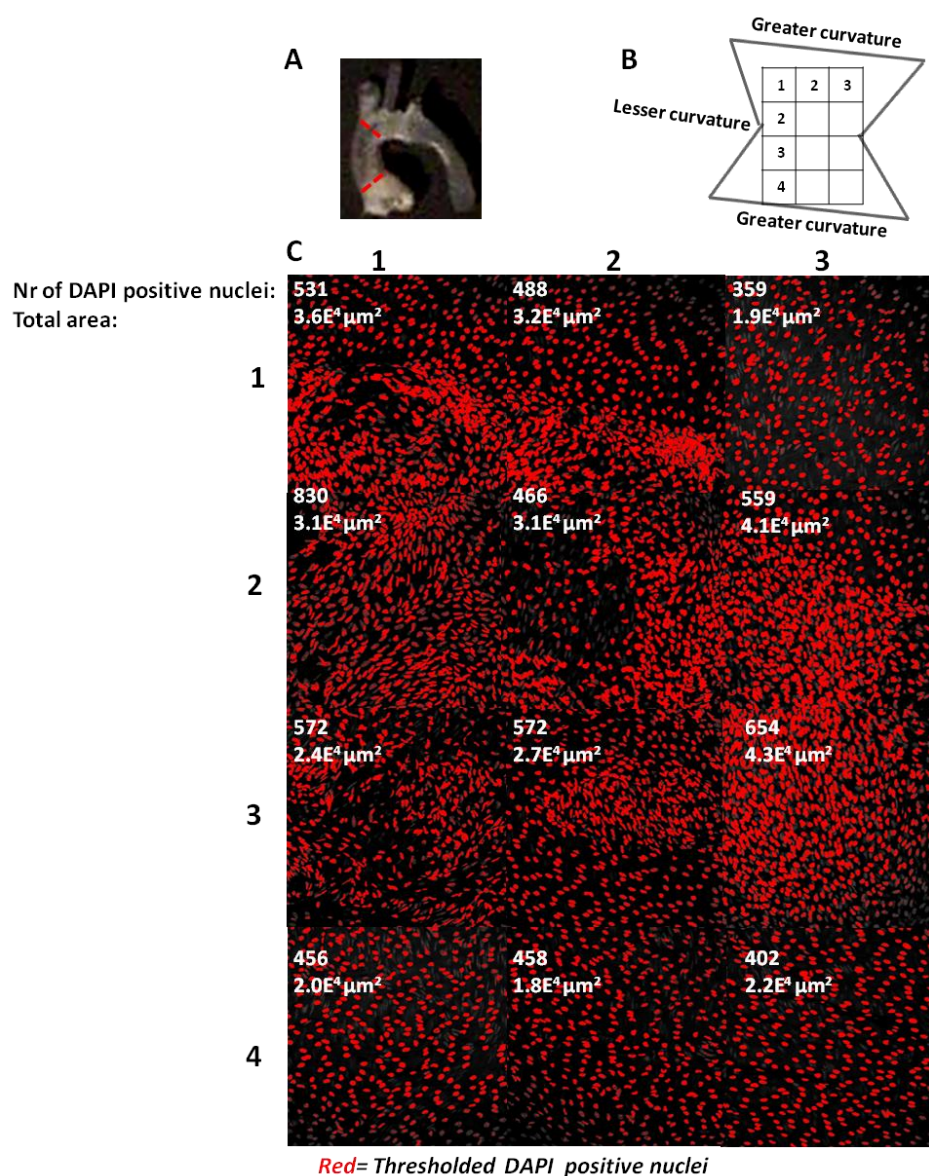


Figure 5.17 Manually made tile composite of 3x4 images showing thresholded DAPI positive nuclei in a female $ApoE^{-/-}$ mouse fed an atherogenic diet. Images from a perfusion fixed (according to the transcatheter perfusion fixation procedure) aortic arch dissected from a female $ApoE^{-/-}$ mouse. The tile was manually created by moving the field of view to the next field along and then manually puzzled together as a tile. (A) Red dotted line, mark dissection cutting points. (B) A schematic of the mounted (opened and flattened) aortic arch, marking the location of the greater and the lesser curvature as well as the position of the tile composite (3x4 images, coordinates 1.1-4.3). (C) The entire tile of 3x4 images. DAPI positive nuclei were thresholded according to the 'default function' in Image J and shown in red. The total number of thresholded DAPI positive nuclei (larger than $2\mu\text{m}^2$) was calculated for each individual image and indicated in white text in the upper left corner of each image alongside the total nuclear density (total nuclear area μm^2) for each individual image.

Figure 5.18 Zoomed in images of CD41 immunoreactivity in different layers of a plaque like structure in a female $ApoE^{-/-}$ mouse fed an atherogenic diet

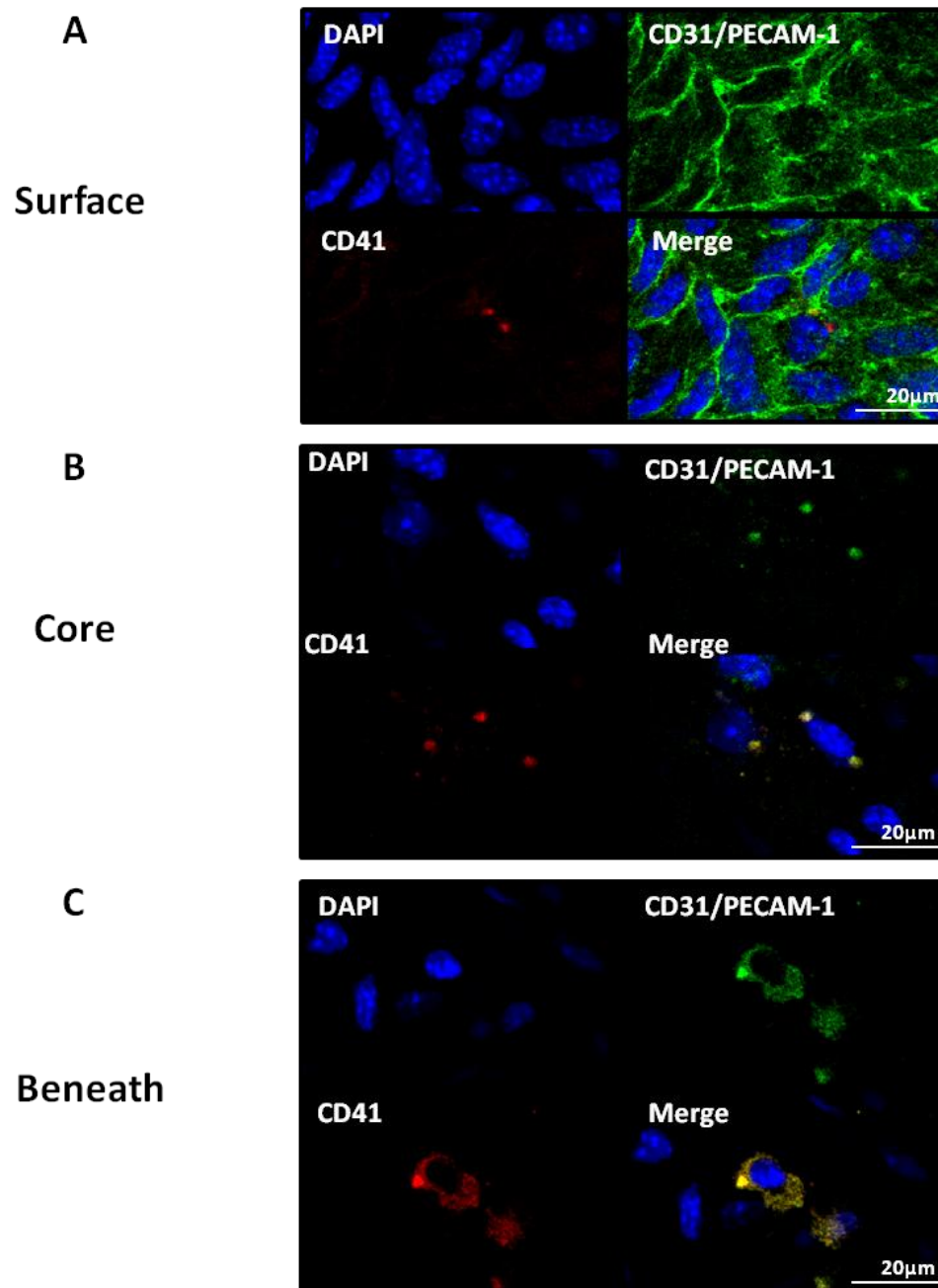


Figure 5.18 Zoomed in images of CD41 immunoreactivity in different layers of a plaque like structure in a female $ApoE^{-/-}$ mouse fed an atherogenic diet. Zoomed in images from a perfusion fixed (according to the transcardial perfusion fixation procedure) aortic arch dissected from a female $ApoE^{-/-}$ mouse. DAPI stained nuclei (**blue**) and CD31/Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) immunoreactivity (**green**) and CD41 platelet marker immunoreactivity (**red**) in the (A) CD31 positive endothelial surface layer of the plaque, (B) core or inside the plaque and (C) beneath the superficial and core levels of the plaque. All of the images have been enhanced in brightness (+20) and contrast (+40) for clarity of viewing purposes.

Summary of Chapter 5

The aim of this chapter was primarily to study the functional consequences of COX-1 and COX-2 gene deletion in aortic arch endothelium. However, for some of the experiments ApoE^{-/-} mice was included in the study as well.

The main findings from this chapter can be summarised as follows:

CD31 immunoreactivity and DAPI positive nuclear density in the greater and lesser curvature of aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

- CD31 positive endothelium from all of the mice displayed the typified differences in morphology associated with the greater and the lesser curvature regions as reported in WT mice in Chapter 3.
- There was abundant CD31 immunoreactivity in both the greater and the lesser curvature all of the mice studied and there was no significant difference in CD31 immunoreactivity between the groups of mice.
- There was no difference in CD31 immunoreactivity between the greater and the lesser curvature of the mice (except for in the COX-2^{-/-} mice).
- Nuclear density (quantified as the total area of the DAPI positive thresholded nuclei) and number of DAPI positive nuclei was significantly higher in the lesser curvature compared to the greater curvature in all the types of mice studied, consistent with the findings in WT mice in Chapter 3.
- The nuclear density or number of nuclei in either region did not significantly vary between the types of mice.
- The average size (μm^2) of the thresholded DAPI positive nuclei was also significantly larger in the lesser curvature compared to the greater curvature in all mice tested (except for in the normal chow fed ApoE^{-/-} mice). This measurement is confounded by the difficulty to separate the densely packed nuclei in the lesser curvature compared to the greater curvature where they are evenly separated/spaced out and may therefore be a measure of 'clumped together' nuclei as supposed to individually larger nuclei or alternatively a combination of both.

Overall, the genetically modified mice had similar endothelial morphology as the WT mice.

NF-κB immunoreactivity in WT versus COX-1^{-/-} and COX-2^{-/-} mice

- Nuclear levels of NF-κB expression were significantly higher in the lesser curvature region compared to the greater curvature region in untreated WT mice as well as in LPS treated animals. These results are in line with previous findings suggesting that NF-κB is 'primed for activation' in this region of the mouse aortic arch, which has been linked to the increased susceptibility for atherosclerosis in this area (Hajra *et al.*, 2000).
- LPS treatment resulted in significantly higher levels of both total and nuclear NF-κB immunoreactivity in both regions of all the mice studied.
- In untreated (control) conditions, both the global/total and nuclear levels of NF-κB immunoreactivity were increased in both regions of aortic arches from COX-1^{-/-} mice, compared to levels detected in WT control animals. A similar pattern was seen in the greater curvature of untreated COX-2^{-/-} mice.
- Following LPS stimulation, there was a marked increase of nuclear NF-κB in the both regions of aortic arches from COX-2^{-/-} mice compared to WT mice. Nuclear NF-κB in the greater curvature of COX-1^{-/-} mice was however not different from WT mice. Both the COX-1^{-/-} and the COX-2^{-/-} mice had significantly more nuclear NF-κB levels in the lesser curvature compared to WT mice following LPS treatment.

The results in this chapter indicates that COX-1^{-/-} and COX-2^{-/-} mice have elevated immunoreactivity levels of NF-κB, both in terms of general (total/global) expression and nuclear 'primed' or activated NF-κB compared to WT controls, in the endothelium of both protected (greater curvature) and susceptible (lesser curvature) regions of the aortic arch in both untreated (control) and LPS treated (inflammatory) conditions.

CD41 positive platelet adhesion to the aortic arch endothelium in WT versus COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice

- The selectivity of the CD41 antibody for platelet detection was validated in blood smears where DAPI negative, non-nucleated CD41 positive platelets were clearly visible whereas nucleated cells (blood leukocytes) did not stain with CD41.
- There was a trend for more CD41 positive platelets in the blood smears from the COX-1^{-/-} and COX-2^{-/-} mice compared to the WT mice.
- There were no CD41 positive platelets detected in the secondary control blood smears or in aortic arches stained only with secondary control.
- Non-nucleated, CD41 positive, CD31 positive platelet-like bodies, that seemed adhered to the endothelium (in between or close to nucleated endothelial cells) were observed in both regions of the aortic arch from all of the mice studied.
- There were significantly more CD41 positive platelets adhered to the endothelium in the lesser curvature of COX-2^{-/-} mice compared to WT mice.
- This observation was independent of quantification method (manual counting or automated quantification by Image J).
- The number of CD41 positive platelets did not vary between regions except for in the COX-2^{-/-} and in the normal chow fed ApoE^{-/-} mice where there was a significant increase in the lesser curvature compared to the greater curvature.
- The average size (in diameter) of the CD41 positive platelets in the aortic arch were around the same size as the platelets recorded from the blood smears from these mice (around 3µm). This is in line with the reported diameter for mouse platelets (1-3µm) (Levin *et al.*, 1994).

According to the validation protocols, the CD41 positive objects detected appears to be platelets adhered to the aortic arch endothelium. More CD41 positive platelets were detected adhered to the lesser curvature of aortic arches from COX-2^{-/-} mice compared to WT mice, indicating that the endothelium in this inflammation and atherosclerosis susceptible area have reduced anti-platelet properties in the COX-2^{-/-} mice.

Pilot study of CD41 positive platelets adhered to the endothelium of the aortic arch in an ApoE^{-/-} mice fed an atherogenic diet

- When the CD41 protocol was repeated in a pilot study using a high cholesterol chow fed ApoE^{-/-} mouse, there were more CD41 positive platelets adhering to areas of the aortic arch (mainly the lesser curvature) enriched in a plaque-like structure compared to 'normal looking' adjacent endothelium located mainly in the greater curvature.
- The plaque enriched areas was also associated with an increased nuclear density (quantified by number of thresholded DAPI positive nuclei and the total area of these nuclei).
- There was also a CD41 positive (and CD31 positive) platelet detected inside the core of the plaque and in the deeper layers underneath; where the CD41 immunoreactivity was found associated with a nucleated cell. This may have been a leukocyte that has taken up a CD41 positive platelet since it has been shown that activated platelets can interact with circulating leukocytes/monocytes which has been linked to exacerbate atherosclerosis (Huo *et al.*, 2003).

The preliminary findings from this pilot study indicate that plaques are enriched with CD41 positive platelets compared to surrounding plaque-free areas.

In summary, the genetically modified COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice had similar endothelial morphology (in terms of nuclear density or CD31 immunoreactivity) as the WT mice. Both the untreated and the LPS treated COX-1^{-/-} and COX-2^{-/-} mice had elevated levels of nuclear or 'primed' NF-κB in their aortic arch endothelium compared to WT controls. More CD41 positive platelets were detected adhered to the susceptible lesser curvature of aortic arches from COX-2^{-/-} mice compared to WT mice. The implications of these observations are discussed in detail in my General Discussion chapter (Chapter 6).

Chapter 6

General Discussion

It is without question that COX is a critical regulator of cardiovascular homeostasis. Nevertheless, we still do not fully understand how COX isoforms and their activity regulate vascular health and disease. It is, however, well established that platelets are enriched in COX-1 which drives thromboxane production crucial for platelet activation and aggregation. The overriding question of my thesis has been to investigate the relative expression of COX-1 and COX-2 in endothelial cells and importantly their contribution to cardioprotective prostacyclin production from vascular endothelium. This is an area of controversy.

Traditionally, COX-1 has long been considered the ubiquitous and constitutively expressed isoform present in almost every tissue of the body (including the vascular endothelium), responsible for the basal production of prostanoids. On the contrary, COX-2 has generally been viewed as the inducible isoform upregulated following inflammation; central to driving the increased prostanoid production during an inflammatory response. Hence, the general idea was that COX-1 was the constitutively expressed, predominant COX isoform in the vasculature whereas COX-2 was the inducible isoform, expressed at sites of vascular inflammation, injury or atherosclerosis.

This notion was turned on its head after the Vioxx withdrawal, where COX-2 selective inhibitors were shown to have unexpected cardiovascular side effects. One of the early explanations for these adverse effects was that perhaps COX-2 is (contrary to the traditional view) constitutively and ubiquitously expressed within vascular endothelium and that when this COX-2 is inhibited with COX-2 selective inhibitors (or traditional NSAIDs), there is a reduction in anti-thrombotic endothelial prostacyclin which may increase arterial thrombotic risk.

I set out to investigate this in my thesis with the hypothesis:

‘COX-2 is present in vascular endothelium and regulates prostacyclin release
and functional responses in health and disease’

During the course of this PhD I have made every effort to investigate objectively whether COX-2 is constitutively expressed in the endothelium of blood vessels and/or if the expression and activity pattern changes with different cardiovascular paradigms such as aging, chronic heart failure or atherosclerosis. Given that COX-2 has been shown to be a shear stress regulated gene, I studied COX-1 and COX-2 expression and activity in the aortic arch since this is a complex vascular structure where the hemodynamic effect of different kinds of shear stress patterns on endothelial form and function can be studied within the same tissue. Additionally, since the lesser curvature of the aortic arch has been shown to be an atherosclerosis susceptible region where NF- κ B is 'primed for activation', this was an area of the vasculature that I was particularly interested in examining since COX-2 is a NF- κ B regulated gene.

The en face aortic arch mouse model

Throughout my thesis, I have been struck by how consistent the endothelial morphology have been in the greater and the lesser curvatures of the aortic arches that I have studied. The endothelial cells lining the greater curvature were typically elongated, evenly spread out and aligned with the direction of the blood flow whereas those cells lining the lesser curvature were more randomly orientated with polygonal shapes. The most striking distinction between the two regions was the increased nuclear density in the endothelial layer of the lesser curvature. These morphological differences indicate that each region has its own separate hemodynamic environment resulting in unique endothelial phenotypes.

The characteristic endothelial morphology in the two regions has been described previously (Hajra *et al.*, 2000; Jongstra-Bilen *et al.*, 2006) in isolated, zoomed-in images showing propidium iodide stained nuclei and silver nitrate stained endothelial junctions. However, the tiles presented in this thesis are the first to provide an overview of the full range of endothelial phenotypes exhibited in the aortic arch including the 'interface region' between the greater and the lesser curvature, which has not been described previously. This 'interface region' had both aligned and randomly orientated endothelial cells; which appear to be a mixture of flow-responsive endothelial phenotypes with similar morphology to endothelial cells in both the greater and the lesser curvature of the mouse aortic arch. There was a progressive increase in nuclear density (quantified by total nuclear area) from

the greater curvature via the interface region to the lesser curvature. Since there was no difference in nuclear density of the underlying smooth muscle between the two regions, this observation appears to be restricted to the endothelial layer.

The increased nuclear density could reflect either a higher endothelial cell turnover in the lesser curvature and/or a higher number of infiltrated nucleated cells into the intimal layer of this region. It has been proposed that the increased nuclear density can be accounted for by infiltrated intimal leukocytes (Jongstra-Bilen *et al.*, 2006). As I have used the blue fluorescent nucleic acid stain DAPI, which preferentially stains AT-rich double stranded DNA in the minor groove (Kubista *et al.*, 1987) to label the nuclei, it does not allow for discrimination of different nucleated cell types that may be present in the aortic intima of my images. Furthermore, even if anti-CD31 antibodies are typically used to identify endothelial cells in immunohistological preparations, CD31 is not only expressed on endothelial cells and hence does not facilitate for discrimination between endothelial cells and other CD31 positive cell types potentially resident in the intimal wall.

For example, platelets have been shown to express approximately 5000 CD31 molecules per cell, leukocytes around 85000 molecules but to put this in context; endothelial cells have nearly 1 million CD31 molecules expressed on their surface (Newman, 1994). Accordingly, although CD31 is not specific to endothelial cells, their surface is highly enriched in this membrane glycoprotein. Therefore, the abundant CD31 immunoreactivity, along with endothelial-like morphological characteristics in both regions of the aortic arch suggest that most of the nucleated cells detected in these regions of the aortic intima are endothelial cells. The exact ratio of endothelial cells to other cell types potentially present in the nuclear dense lesser curvature remains to be determined, perhaps by scanning electron microscopy.

The distinct difference in nuclear density between the greater and the lesser curvature is important to bear in mind when comparing immunohistology from the two regions. In some cases, elevated levels of immunoreactivity in the lesser curvature compared to the greater curvature, could simply be due to the increased number of nucleated cells/nuclear material in this region. Importantly, however, there was no difference in the nuclear density or CD31 immunoreactivity between the WT, COX-1^{-/-}, COX-2^{-/-} or normal chow fed ApoE^{-/-} animals studied in Chapter 5 and is therefore less likely to be a confounding factor when comparing

immunohistological results between these animals. This also confirms that the images were comparable in terms of endothelial integrity with intact CD31 positive endothelium in both regions of the aortic arch from all the different types of mice studied.

In Chapter 3, there was a significant difference in CD31 immunoreactivity between the greater and the lesser curvature in WT mice, but this was not the case when the protocol was repeated in more WT mice in Chapter 5. There was, however, a significant difference in CD31 immunoreactivity between the two regions in the COX-2^{-/-} mice, but on the whole, these levels did not differ from WT mice.

It is important to keep in mind that CD31 is abundantly expressed in every region of the aortic endothelium and that although the CD31 staining pattern varies between the two regions, the overall mean fluorescence values come out very similar. The CD31 immunoreactivity tends to be distributed throughout the cytoplasm as well as outlining the intracellular junctions between the endothelial cells in the greater curvature, whereas it seems more localised to the intracellular junctions in the lesser curvature. This distinct distribution may be due to the hemodynamic differences between the two regions, since CD31 has been identified to play an important role in how endothelial cells sense and respond to shear stress (mechanoreception and mechanotransduction) through interactions with the cytoskeleton and subsequent cytoskeletal rearrangements (Newman *et al.*, 2003). Interestingly, CD31 knockout mice have impaired NF- κ B responses in aortic arch endothelium (Tzima *et al.*, 2005) and CD31^{-/-}ApoE^{-/-} mice have reduced atherogenic indices, which suggest that CD31 is a critical mediator of atherosclerosis (Stevens *et al.*, 2008).

Overall, the distinct endothelial morphology in the lesser and greater curvature of the mouse aortic arch is likely to reflect the hemodynamic environment that these cells are constantly exposed to. Flow measurements in the mouse aortic arch have revealed that there are clear differences in shear stress magnitude and directionality between the two regions of the aortic arch: unidirectional shear stress of approximately 600 dynes/cm² in the greater curvature compared to oscillatory or multidirectional shear stress of around 150 dynes/cm² in the lesser curvature (Suo *et al.*, 2007). Although this is 20-fold higher in magnitude compared to the shear stress estimated in the human aortic arch (Weinberg *et al.*, 2007), the relative difference in shear stress magnitude and the different patterns of

shear stress directionality in the greater and the lesser curvature of the aortic arch seems to be consistent between mice and men (Suo *et al.*, 2007).

Towards the end of my PhD I had the rare opportunity to obtain a human aortic arch post mortem from one donor (the same female donor that I imaged the pulmonary artery from in Chapter 3). It was fascinating to handle something so much larger than the mouse and rat tissues that I had become used to. When I cut the aortic arch open to examine the intima, it made a brittle, crunchy sound due to the calcified and ulcerated atherosclerotic lesions present and located mainly around the carotid branches. It was, however, interesting to note that plaque like structures were located also in the lesser curvature of the human aortic arch (although at a position slightly higher up compared to the mouse aortic arch), whereas the greater curvature seemed relatively free from plaques (Figure 6.1).

Figure 6.1 Photos showing atherosclerotic lesions in a human aortic arch

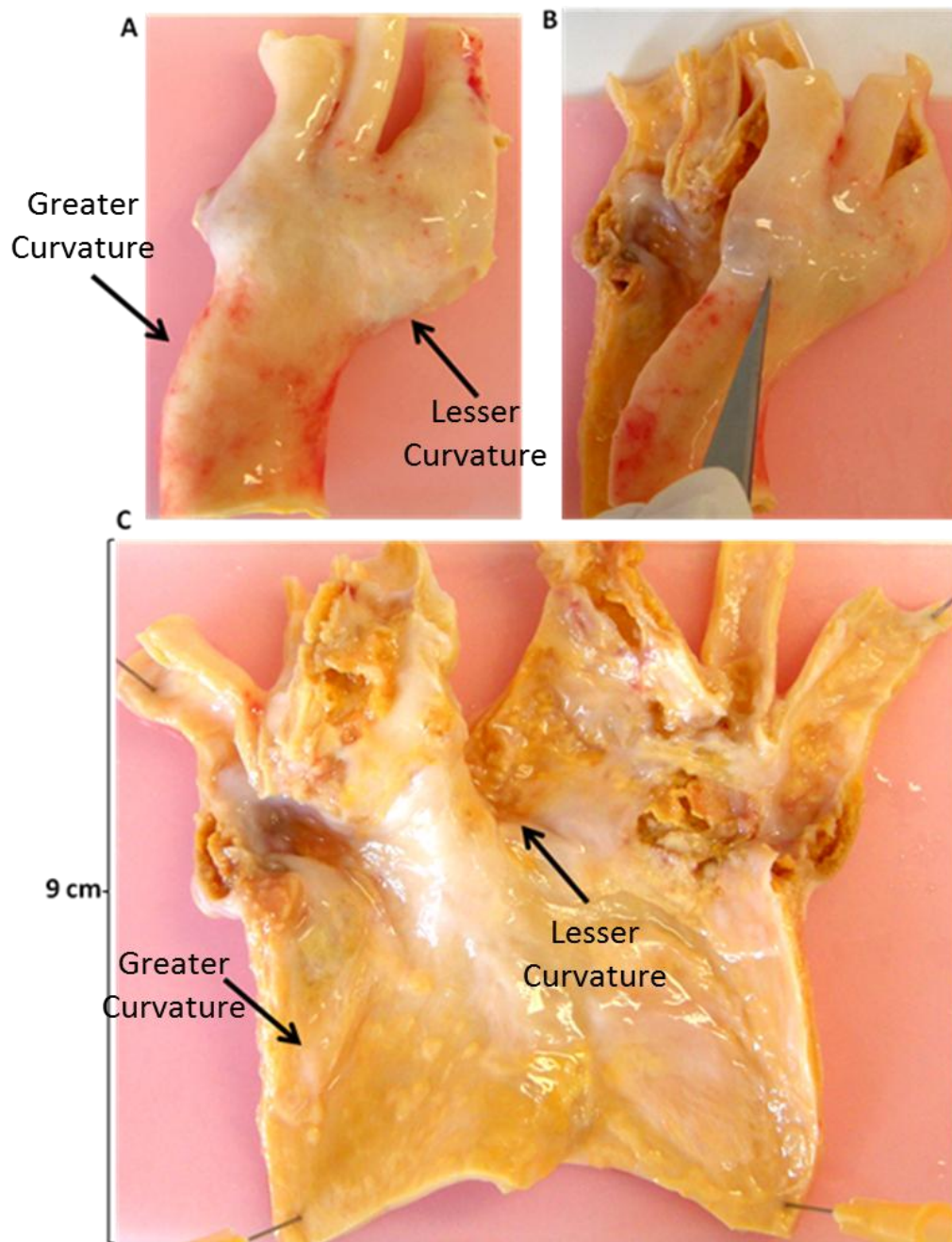


Figure 6.1 Photos showing atherosclerotic lesions in a human aortic arch. This aortic arch comes from the same female donor from whom the pulmonary artery was dissected from (imaged for COX-1 and COX-2 in Chapter 3). The aortic arch was fixed in 2% formaldehyde once it reached our lab from a biobank in the US ~36 hours after time of death. **(A)** Photo of the aortic arch before it was cut open. **(B)** Photo illustrating how the aortic arch was cut open. **(C)** The opened and pinned out aortic arch. Black arrows indicate plaque-like structures in the lesser curvature and plaque free areas in the greater curvature. This donor was a 67 year old Caucasian female smoker (1 pack a day for 49 years) with a known medical history of COPD, essential hypertension and diabetes mellitus. Cause of death was spontaneous intracerebral haemorrhage.

Even if no firm conclusions can be drawn from just this one specimen, large population studies also suggest that atherosclerosis does feature in the lesser curvature and carotid branch points in human aortic arches (Figure 6.2) which can be a source of cerebral atheroemboli and is thereby regarded as a risk factor for ischemic strokes (Amarenco *et al.*, 1994). Atherosclerosis in the aortic arch is considered to be an indicator of generalised atherosclerosis and thereby increased morbidity and mortality (Dávila-Román *et al.*, 1999). There is a strong correlation between increasing age and hypertension and ulcerated aortic arch plaques. In one autopsy study including 500 patients, 31% of the patients who were 60 years or older that had suffered an ischemic stroke had ulcerated plaques in their aortic arch compared to only 1% of the patients under 60 years of age (Amarenco *et al.*, 1992).

Figure 6.2 Multidetector computed tomographic (CT) angiography of atheroma in a human aortic arch



Figure 6.2 Multidetector computed tomographic (CT) angiography of atheroma in an aortic arch. Sagittal reconstruction of an aortic arch from an 84 year old man with a 5mm thick atheroma in the lesser curvature of the aortic arch (indicated by black arrows). Figure modified from (Boussel *et al.*, 2011).

In conclusion, despite obvious species differences between mice and men, the aortic arch appears to have surprisingly similar features in terms of shear stress patterns and susceptibility to atherosclerosis, which hopefully (to some extent) gives the mouse aortic arch model translational value when interpreting how results from this model may relate to the human situation.

Once I was confident with the mouse *en face* aortic arch model and when I had overcome the initial technical difficulties of careful micro-dissection to avoid inflicting damage to the very fragile endothelial monolayer, it was time to interrogate the question of relative COX-1 and COX-2 expression in the polarised endothelial phenotypes represented in this complex vascular structure.

COX expression in the aortic arch: which COX isoform predominate(s) in 'normal'/healthy vascular endothelium?

In the first results chapter of this thesis (Chapter 3), my main objective was to investigate which COX isoform predominates in healthy aortic arch endothelium. Since it has been suggested that COX-2 may have rapid offset kinetics (Funk *et al.*, 2007; Grosser *et al.*, 2006), it was essential to perform immediate perfusion fixation of these aortic arches without post-mortem delay, in order to eliminate the risk of protein decay and loss of any constitutive COX-2 during the experimental procedure.

The difference in immunoreactivity between the two isoforms was striking; in every aortic arch that I imaged, abundant COX-1 immunoreactivity greatly predominated over very sparse COX-2 immunoreactivity.

The intracellular staining pattern of the COX-1 immunoreactivity appeared diffused cytoplasmic and intense perinuclear. This is similar to what has been described previously (Morita *et al.*, 1995) and immunoelectron studies have demonstrated that COX-1 mainly locates with the endoplasmic reticulum (ER) and the nuclear envelope; this is where the arachidonic acid is converted to PGG₂ and PGH₂ (Rollins *et al.*, 1980).

Although COX-1 immunoreactivity predominated over COX-2 immunoreactivity in all areas of the aortic arch, modest levels of punctate COX-2 immunoreactivity were consistently detected 'enriched' in the endothelium lining the lesser curvature of the aortic arch compared to the greater curvature where COX-2 immunoreactivity was nearly always absent.

Considering that COX-2 is a NF- κ B regulated gene and NF- κ B has been shown to be 'primed for activation' and probably to various degrees 'constitutively' active in the lesser curvature of the mouse aortic arch (Hajra *et al.*, 2000), low (but nevertheless potentially important) levels of constitutive COX-2 expression localised to this inflammation and atherosclerosis susceptible region was perhaps to be expected. Consistent with this finding, a gene expression study of the lesser curvature of porcine aortic arches indicated that *Ptgs2* (COX-2) gene expression was elevated in this micro-domain of the vasculature compared to straight segments of the descending aorta (Passerini *et al.*, 2004). It has been suggested that in these focal regions, COX-2 is likely to contribute to cardioprotective prostacyclin production (Funk *et al.*, 2007).

Following LPS driven COX-2 induction (after LPS injection), the COX-2 staining pattern in the mouse aortic arch endothelium was more perinuclear. This is consistent with reports about COX-2 (as an immediate early gene) being localised to the inner membrane of the nuclear envelope where COX-2 activity is often associated with nuclear activities such as cell differentiation or replication (Morita *et al.*, 1995).

There was no difference in COX-1 immunoreactivity between the greater and the lesser curvature in untreated WT mice, but there was however, somewhat surprisingly, increased COX-1 immunoreactivity in the lesser curvature following LPS treatment. This could reflect an increased number of infiltrating COX-1 positive leukocytes to this susceptible area considering that aortic tissues were harvested 4 hours after LPS injection. The same could of course also be the case for the increased COX-2 immunoreactivity, and different more specific cell markers and/or scanning electron microscopy could be used to evaluate the ratio of endothelial versus non-endothelial cell types present in the lesser curvature following LPS treatment. However, as stated in earlier sections, it is still most likely that the majority of cells present in the intima of both regions of the mouse aortic arch are endothelial cells.

COX expression in the endothelial layer versus the smooth muscle layer

Both the COX-1 and COX-2 immunoreactivity detected in my experiments seemed localised to the endothelial layer when compared to the underlying smooth muscle layer. However, since I did not included a positive control (or smooth muscle marker) for detection of the

smooth muscle cell layer, other than the small and penetrable stain DAPI, it cannot be certain that the COX-1 or COX-2 antibodies and/or their fluorescent tag (AlexaFluor594) really penetrated through the endothelial layer down to the underlying smooth muscle layer of these tissues.

There was, however, clear COX-1 and COX-2 immunoreactivity in the deeper smooth muscle layers of the plaque-like structures detected in the ApoE^{-/-} mice fed an atherogenic diet (Chapter 4). This still does not fully serve as a positive control for smooth muscle staining, since it is possible that the endothelium on top of plaque lesions may be easier to penetrate for the COX-1 or COX-2 antibodies and/or their fluorescent tag (AlexaFluor594) compared to 'normal' endothelium.

After all, the endothelial layer has been the focus of my investigation as it has been reported that both COX immunoreactivity and prostacyclin synthesis is more than 20 times higher in endothelial cells compared to smooth muscle cells, despite equal levels of prostacyclin synthase expression (DeWitt *et al.*, 1983), clearly indicating that the endothelium is the predominant source of prostacyclin synthesis in vascular tissue. This is the main reason why I have focused on the role of COX-1 and COX-2 in the endothelial layer and not the smooth muscle layer in the tissues studied.

COX expression in female mice and mature adult (aged) mice

In order to consider any gender differences in COX expression, the COX staining protocol was repeated in tissue from female mice (Chapter 4). The same pattern was evident in the female mice (as in the male mice studied in Chapter 3); COX-1 immunoreactivity predominated over very sparse COX-2 immunoreactivity in the endothelium of all the areas of the aortic arch and the lesser curvature was again significantly enriched in COX-2 immunoreactivity compared to the greater curvature.

This was also the case in mature (12 month old) mice, and although these animals were not studied head to head in the same study, there was no apparent difference compared to the younger (3 month old) mice (Chapter 4).

COX expression in human endothelium

Towards the end of my thesis I was able to repeat the COX *en face* protocol in a validation experiment using human pulmonary artery to investigate relative COX-1 and COX-2 immunoreactivity in human endothelium. Even if I was only able to perform one experiment it was still interesting to note the similarities in COX-1 and COX-2 immunoreactivity and staining pattern between the human and mouse vessel.

I tried to repeat the *en face* protocol in aortic arch tissue from the same donor (photos shown in Figure 6.1), but unfortunately, technical mounting difficulties due to the thickness of the human aortic arch tissue prevented it so I was not able to examine if the observations of constitutive COX-2 in the lesser curvature of the mouse aortic arch would be similar in the human aortic arch. A specialised fixing and mounting protocol for thick human tissue would need to be developed but theoretically it should be possible to image human aortic arch tissue with *en face* confocal microscopy.

The pilot data obtained from the successful imaging of human pulmonary artery endothelium in this thesis is consistent with findings reported in the literature where COX-1 and COX-2 expression have been examined in other types of human blood vessels:

- ❖ Human internal mammary artery from 4 patients undergoing coronary bypass surgery. Endothelial COX-1 immunoreactivity was shown to greatly predominate over COX-2 at all time points (immediately after surgery and after 6 or 24 hours in culture). COX-2 immunoreactivity was only detected after culture and following induction with inflammatory stimuli (Foudi *et al.*, 2009).
- ❖ Saphenous vein, internal mammary artery and radial artery removed after coronary bypass surgery and immediately snap frozen. COX-1 protein was shown to greatly predominate over COX-2 protein (Mitchell *et al.*, 2006a).
- ❖ Aorta and carotid artery from 5 patients with non-atherosclerotic vessels had immunostainable COX-1 but no or little COX-2 (Schönbeck *et al.*, 1999).

- ❖ Extensive study of large arteries from 20 supposedly healthy people who died in accidents where the post-mortem delay was 24 hours or less. Abundant endothelial COX-1 immunoreactivity was detected in the vascular endothelium in every subject (with little variation between cases) whereas COX-2 was found only in occasional endothelial cells in large arteries and this immunoreactivity varied more between subjects (Zidar *et al.*, 2008).
- ❖ Aorta from post-mortem study of 5 young individuals without gross or microscopic evidence of atherosclerosis. COX-1 mRNA was detected but no COX-2 mRNA (Belton *et al.*, 2000).
- ❖ Aorta from post-mortem study of 3 females, where the tissue was fixed within 6 hours of fatality. Intense co-expression of COX-1 and prostacyclin synthase were observed throughout the aortic endothelium without detecting any COX-2 (Kawka *et al.*, 2007).

These studies provide convincing evidence for COX-1 being the predominant isoform expressed under normal conditions in human endothelium. However, it does not rule out a role for localised COX-2 in susceptible regions of the vasculature that may be more difficult to detect, unless the focus is to specifically study these micro-domain areas. Furthermore, all of these studies presented COX immunoreactivity data from sectioning, which offers limited study of a particular transverse section at any one point in the tissue compared to the *en face* imaging which gives a better overview over a larger area of endothelium where susceptible areas can be studied in parallel to protected areas.

However, minor foci of COX-2 immunoreactivity have been reported in carotid artery endothelium (at the branch point with the aortic arch) in tissue from dogs (Kawka *et al.*, 2007). It would therefore be interesting to specifically focus on studying COX-2 expression (and activity) in the endothelium lining the lesser curvature (and/or branch points) of the human aortic arch to see if the findings in mice and dogs translate to human tissue and what relevance any constitutive COX-2 may have in this area of the human vasculature.

In vitro modelling of the effects of shear stress on COX-1 and COX-2 expression

During my PhD I have also been involved in one of my colleagues Ms Claire Potter's PhD project of setting up an orbital shaker shear stress model to study if the endothelial morphology and COX expression results from the aortic arch could be reproduced *in vitro* (Potter *et al.*, 2011). Other *in vitro* shear stress models have previously examined COX expression after relatively short time points (between 6-24 hours) (Di Francesco *et al.*, 2009; Okahara *et al.*, 1998; Topper *et al.*, 1996). However, we were concerned that applying shear stress to statically cultured endothelial cells could initially be conceived as an inflammatory insult and that perhaps 7 days on the orbital shaker would better reflect the effects of chronic shear stress. After 7 days, porcine aortic endothelial cells (cultured on filters) were randomly orientated in the middle of the well where the shear stress was multidirectional with low pulsatility (theoretically similar to the lesser curvature in the mouse aortic arch), but aligned and elongated nearer the periphery of the well where the shear stress was unidirectional with high pulsatility (theoretically similar to the greater curvature of the mouse aortic arch) (Potter *et al.*, 2011).

The COX-1 immunoreactivity was again shown to greatly predominate over COX-2 in all areas of the well (Potter *et al.*, 2011). Interestingly, there was no increase in COX-2 immunoreactivity in the centre of the well (the area that theoretically was exposed to a similar shear stress pattern as the lesser curvature of the aortic arch). It is of course difficult to accurately model the complex hemodynamic environment that endothelial cells are chronically exposed to in the aortic arch *in vitro*, however it raises questions about the COX-2 that I found to be constitutively expressed in the lesser curvature of the aortic arch:

- Is COX-2 chronically induced by a particular shear stress profile?
- Is COX-2 constitutively expressed due to 'primed' NF- κ B in this region?
- Is the detectable COX-2 immunoreactivity actually from COX-2 expressed on leukocytes that have adhered to this susceptible area of the aortic arch due to the altered adhesion molecule expression reported in this area (Hajra *et al.*, 2000; Iiyama *et al.*, 1999; Suo *et al.*, 2007)?

Whatever the answer may be, one of the main findings with my thesis is that COX-2 seems to be constitutively expressed in the lesser curvature of the mouse aortic arch and the final results chapter of my thesis (Chapter 5) was dedicated to investigating what functional role this localised COX-2 might have.

Validation studies

Throughout the thesis, I have performed numerous validation experiments to test the robustness of the COX-1 and COX-2 immunoreactivity data reported above. When the COX-2 immunoreactivity turned out to be very modest and sparse in untreated healthy mice, I injected some mice with LPS in order to purposely upregulate COX-2 for immunodetection and this resulted in clear COX-2 immunoreactivity in both regions of the aortic arch, hence serving as a positive control for the COX-2 antibody (Chapter 3).

Negative controls included secondary antibody control (which was always low without any specific pattern) and the use of blocking peptides which significantly reduced or 'blocked' the COX-1 and COX-2 immunoreactivity detected (Chapter 3).

I also examined COX-1 and COX-2 immunoreactivity in the COX-1^{-/-} and COX-2^{-/-} mice respectively. COX-1 immunoreactivity in the normal cytoplasmic and perinuclear staining pattern was detected in all types of mice (including the COX-1^{-/-} mice, both young and mature, Chapter 3 and 4). This is possibly due to that the COX-1 antibody was raised against an amino acid sequence that comes before the neomycin insert disruption and is therefore likely to pick up a truncated version of the COX-1 protein that may be present in the aortic arch endothelium of these mice.

However, since the COX-1 active site has been disrupted in the COX-1^{-/-} mice, it is highly unlikely that any truncated protein has functional COX-1 enzymatic activity. This was confirmed by the lack of prostacyclin production capacity in COX-1^{-/-} vessels (from both young and mature mice, Chapter 3 and 4).

COX-2 immunoreactivity was however significantly reduced and without the typical LPS induced nuclear envelope staining pattern in the COX-2^{-/-} mice compared to the WT or the COX-1^{-/-} mice. This was expected considering that the COX-2 antibody was raised against an

amino acid sequence after the genetic disruption, and is therefore less likely to pick up any truncated version of COX-2 in the COX-2^{-/-} animals (Chapter 3).

One validation study that I have not performed (that would potentially be important to consider) is examining any masking effects that the formaldehyde fixing protocol might have on the COX-1 or COX-2 antigens and what effect any antigen retrieval methods would have.

I found a paper that reported that in their protocol, the COX-1 immunoreactivity did not depend on antigen retrieval methods, whereas the COX-2 immunoreactivity on the other hand to some extent did (Zidar *et al.*, 2008).

As my focus was on the relative immunoreactivity of COX-1 and COX-2, I wanted to keep the immunohistology protocol identical between the two and I feared that introducing different antigen retrieval methods would potentially introduce new unknown errors which could make it more difficult to draw any firm conclusions.

Any masking effects that the fixing procedure might have on COX-2 detection may therefore be a potential limitation with my results, but it also serves as an important reminder that immunoreactivity is far from an absolute measure of protein expression and needs to be backed up with bioactivity data to investigate the physiological role of a protein. Importantly, the COX-1 and COX-2 immunohistochemistry detected in the aortic arch endothelium, does not predict the amount of prostacyclin that is produced by each isoform.

Prostacyclin bioactivity assays: which COX isoform drives endothelial prostacyclin production in healthy blood vessels?

In order to be able to measure meaningful amounts of prostacyclin, blood vessels need to be stimulated with calcium ionophore in order to activate cPLA₂ which liberates arachidonic acid from the membrane phospholipids and starts the COX and prostacyclin synthase driven production of prostacyclin. Basal release (before calcium ionophore stimulation) is typically very low or undetectable, unless extensive handling during dissection has mechanically triggered the release.

In every bioassay that I have performed during my thesis, thoracic or aortic arch tissue from WT or COX-2^{-/-} mice have always released comparable levels of prostacyclin following stimulation with calcium ionophore. This release was readily inhibited by the NSAID

diclofenac, which validated that this represents a COX driven prostacyclin production in the vessel during the bioassay (rather than any carryover of prostacyclin from a non-endothelial source). Vessels from COX-1^{-/-} mice however, never released any prostacyclin other than a very low level similar to the unstimulated baseline, despite stimulation with calcium ionophore. This pattern was the same no matter if the vessels came from male or female, young adult (3 month old) or mature adult (12 month old) mice (Chapter 3 and 4).

In order to consider the possibility that any constitutively expressed COX-2 may have rapid offset kinetics, the ~2 hour equilibrated protocol was modified to ~30 minute total incubation so that acutely stimulated prostacyclin release could be measured (without any post-mortem delay). Again the pattern was the same; prostacyclin was robustly released from stimulated aortic tissue from WT and COX-2^{-/-} mice, but not from COX-1^{-/-} mice. It also made no difference if the 6-ketoPGF_{1α} was measured by RIA or EIA (Chapter 3 and 4).

In conclusion, according to this bioassay model of stimulated prostacyclin release; COX-1 appears to drive the vast majority of the production of prostacyclin in both the thoracic aorta and the aortic arch, in male and female, young and mature mice.

Reports from the literature about urinary markers of prostacyclin and the use of inhibitors

Controversy surrounds the area of which COX isoform drives vascular prostacyclin production. The most relevant papers are listed below:

- ❖ The urinary marker that is thought to reflect 'systemic' prostacyclin (PGI-M) is reported to be greatly reduced in COX-2^{-/-} mice compared to WT mice, but unaltered in COX-1^{-/-} mice (knockouts or knockdowns) (Cheng *et al.*, 2006). Similarly, urinary PGI-M was also reported to be significantly decreased in WT mice treated with celecoxib or COX-2^{Y385F/-} mice (where the COX site is inactivated, but not the POX site) (Cheng *et al.*, 2006). Urinary markers of thromboxane (TX-M) was however unaltered in both types of COX-2^{-/-} mice and celecoxib treated mice, but significantly reduced in both types of COX-1^{-/-} mice (Cheng *et al.*, 2006).

- ❖ As described in the general introduction (Chapter 1); COX-2 selective inhibitors (celecoxib and rofecoxib) (and) NSAIDs depress urinary PGI-M substantially (but incompletely) in healthy volunteers (Catella-Lawson *et al.*, 1999; McAdam *et al.*, 1999).
- ❖ On the other hand, when the prostacyclin breakdown product 6-keto PGF_{1α} was measured in the aorta of rats treated with a COX-1 selective inhibitor, the levels were significantly reduced compared to aorta from rats left untreated or treated with a COX-2 selective inhibitor (Kawka *et al.*, 2007).
- ❖ Furthermore, when forearm blood flow was measured in healthy volunteers, there was no change in endothelial vasodilator responses following 7 days of treatment with rofecoxib (Verma *et al.*, 2001).
- ❖ Another study of microvascular injury in healthy volunteers, concluded that rofecoxib did not lead to reduced prostacyclin generation in healthy humans and that COX-1 and not COX-2 appears to be the source of prostacyclin in the human microvasculature (Tuleja *et al.*, 2003).

Thus, the results from my thesis are in conflict with some reports and in support of others. A major area of debate surrounds the validity of the urinary markers of prostacyclin (PGI-M) and thromboxane (TX-M). It has been argued that it represents a non-invasive sampling technique to measure the endogenous generation of prostacyclin and thromboxane (FitzGerald *et al.*, 1981) and that urinary markers of 6-keto PGF_{1α} is thought to mainly reflect renal prostacyclin whereas urinary markers of PGI-M supposedly represent an index of endogenous systemic (extra-renal) prostacyclin generation (FitzGerald *et al.*, 1983).

This assumption has been criticised, however, because the exact origin of the PGI-M cannot be determined considering that prostacyclin is first rapidly (within minutes) hydrolyzed to 6-keto PGF_{1α}, and then metabolised by β -oxidation in the liver. Furthermore, it has been argued that the kidney also possesses β -oxidation capabilities (since isolated rabbit kidney perfused with prostacyclin yields PGI-M) and that PGI-M may therefore reflect both systemic and renal prostacyclin (Flavahan, 2007; Van Hecken *et al.*, 2000). It could therefore be possible that the PGI-M levels detected in COX-1^{-/-} mice (Cheng *et al.*, 2006) could

potentially be derived from a non-endothelial source, for instance COX-2 derived prostacyclin in the kidneys of these mice.

Yet it is difficult to think of an alternative accurate measurement of thromboxane and prostacyclin in human (or animal) biological fluids due to their short half-lives, the pattern of their synthesis; platelet thromboxane is generated in dramatic bursts whilst endothelial prostacyclin is produced more tonically (Mitchell *et al.*, 2006a) and artifacts due to invasive sampling techniques (FitzGerald *et al.*, 1983).

Moreover, prostacyclin is unlike many other prostanoids not extensively metabolised by the lung and it was first suggested that prostacyclin was a circulating platelet inhibitory (short-lived) hormone. This was, however, updated with experiments showing that the endogenous secretion rate of prostacyclin in humans is very low and that plasma levels of the breakdown (hydrolysis) product 6-keto PGF_{1α} is in pico grams range, indicating that prostacyclin may be more important for prevention of local platelet-vessel wall interactions than as a systemic circulating anti-platelet agent (FitzGerald *et al.*, 1981).

Local levels of prostacyclin may in fact be a more relevant measure than systemic levels. This was why I focused on measuring stimulated prostacyclin (as 6-keto PGF_{1α}) released from aortic vessels (parallel to the vessels that I imaged) rather than measuring urinary PGI-M from these animals. It is, however, important to remember that the bioassay I used in this thesis is more likely to reflect the overall prostacyclin producing capacity in these vessels *ex vivo* rather than actual endogenous production rates *in vivo*, since a potent stimulus with calcium ionophore was used to maximise the prostacyclin production in each tissue. In this model, COX-1 appears to drive the overall prostacyclin production. Yet, there is a possibility that localised and relatively low but still bioactive levels of prostacyclin is produced by the constitutive COX-2 detected in the lesser curvature. Theoretically, this COX-2 derived prostacyclin may be too low and/or too localised to detect in the protocols that I have used with the whole vessel, where COX-1 activity may be superseding and hiding any potential contribution from COX-2.

For instance, COX-2 has been shown to have a lower threshold for hydroperoxide activation (required for the priming event from the POX site) than COX-1 (Murakami *et al.*, 1999), which could be an advantage in the endothelium where the hydroperoxide tone is low (Boutaud *et al.*, 2002). It would be interesting to measure if the hydroperoxide tone differs between the greater and the lesser curvature and if so, the predominant COX-1 detected in the lesser curvature may be less relevant than the much lower levels of COX-2 in terms of local prostanoid production in this area.

Lipid peroxide tone has also been shown to influence the pharmacology of several NSAIDs which appear to inhibit endothelial prostacyclin production more potently than platelet thromboxane production (*in vitro*), attributed to a lower endogenous peroxide tone in endothelial cells compared to platelets (Mitchell *et al.*, 2006a). This may be relevant to how these drugs can create an increased thrombotic environment, inhibiting endothelial COX-1 (and local levels of COX-2) more strongly than platelet COX-1.

Since I have always stained for COX-1 and COX-2 in separate pieces of tissues (and never studied colocalisation), it cannot be ruled out that COX-1 and COX-2 may form heterodimers (Yu *et al.*, 2006), which may have implications for their respective stereochemistry and bioactivity.

Overall, the functional role of the localised constitutive COX-2 that I have detected in the lesser curvature of the aortic arch needs to be evaluated. Considering that prostacyclin synthase is the predominant prostanoid synthase in vascular endothelium, it would be fair to assume that prostacyclin may be the local COX-2 product in this region, but it could equally be PGE₂ or PGD₂ or any other prostanoid. Perhaps the bioactivity profile of this COX-2 could be studied more easily by isolating the lesser curvature region of the aortic arch, and inhibiting overriding COX-1 activity with a COX-1 selective inhibitor (i.e. SC-560) (Wallace *et al.*, 2000).

COX expression and bioactivity profile during cardiovascular disease; animal models of heart failure and atherosclerosis

In the final year of my PhD an opportunity arose for me to take part in a tissue share of rats with post-infarction chronic heart failure, to examine if the relative COX expression or bioactivity profile changes following cardiovascular disease (Chapter 4). This was also an opportunity for me to see if my observations in mice could be extended to another species (rats).

The post-infarction rats were clearly compromised with distinct manifestations of chronic heart failure such as ventricular hypertrophy and impaired contractile function (Lyon *et al.*, 2011; Lyon *et al.*, 2009). In the aged matched control rats, abundant COX-1 immunoreactivity alongside modest COX-2 immunoreactivity (in similar perinuclear staining patterns as in the mice) was detected in aortic arch tissue and in carotid artery branches. Overnight incubation with LPS resulted in intense COX-2 immunoreactivity which served as a positive control that the COX-2 antibody could detect rat COX-2. Both the COX-1 and the COX-2 immunoreactivity were readily blocked by the addition of blocking peptide. Following the bioassay protocol of stimulated prostanoid release, prostacyclin was most abundantly released from the thoracic aorta (compared to modest levels of PGE₂ and thromboxane).

I was surprised to see that there was no difference in either COX-1 or COX-2 immunoreactivity or prostanoid profile between the control rats and the rats with chronic heart failure. This indicates that although these animals had clear signs of gross cardiovascular disease, maybe it was not the correct model to use in terms of vascular inflammation, since even at strain, COX-2 was not upregulated on a chronic basis.

I then returned to earlier theories about the aortic arch and decided to apply the question about the effects of cardiovascular disease/vascular inflammation to an atherosclerotic model where plaques were clearly present in the vascular endothelium (Chapter 4 and 5). Even without specific markers for the plaque-like structures, they were strikingly nuclear dense and morphologically clearly different to the surrounding endothelium. The plaque-like structures were spanning most of the lesser curvature (consistent with that this region has an increased susceptibility to atherosclerotic plaques) and continued along the lesser curvature into the mid arch and the descending arch.

Due to the bulkiness of plaques, atherosclerotic tissues are often imaged by sectioning rather than examined *en face*. It was exciting to see that it was possible to repeat the *en face* method also in aortic arches from ApoE^{-/-} mice that had been fed an atherogenic diet and that had clear advanced plaques. Viewing the atherosclerotic aortic tissue *en face* provided a better overview over a larger area of atherosclerotic endothelium, allowed for investigation of the inside layers of the plaque and enabled study of the surrounding and surprisingly 'normal looking' endothelium. Intense COX-2 immunoreactivity was detected, clearly localised to the atherosclerotic plaque-like structure. This could have been endothelial in origin and/or expressed on infiltrating leukocytes (for instance monocytes/macrophages). As I did not have any other cell markers than CD31 and DAPI, the exact origin and/or distribution of COX-2 immunoreactivity between different cell types could not be established.

Considering that it is already well recognised that COX-2 is expressed in atherosclerotic plaques (Schönbeck *et al.*, 1999), these findings serve more as an additional positive control (other than LPS induction) for what positive COX-2 immunoreactivity can look like in a mouse vessel when imaged *en face*. Interestingly, the surrounding 'normal looking' endothelium was still clear of COX-2 immunoreactivity but importantly, predominant COX-1 immunoreactivity was still clearly present, abundantly expressed on the plaque-like structure and elsewhere.

A colleague of mine (Dr Nick Kirkby) furthered the investigation about the role of COX-2 in atherosclerosis by creating double knockout ApoE^{-/-} COX-2^{-/-} mice (where both ApoE and COX-2 have been knocked out). It is worth noting that there were significantly more plaques in the lesser curvature of aortic arches from ApoE^{-/-} COX-2^{-/-} mice compared to ApoE^{-/-} COX-2^{+/+} mice fed an atherogenic diet. This data support an athero-protective role for the localised COX-2 in the lesser curvature.

Furthermore, there were also differences in systemic cholesterol levels. Similar results were reported by another group in a different study that showed that after 3 weeks on an atherogenic diet, ('normal' ApoE^{+/+}) COX-2^{-/-} mice had increased lipid deposition in their aorta (Narasimha *et al.*, 2007). Serum levels of cholesterol indicated that the COX-2^{-/-} mice were hyperlipidemic compared to WT mice and after 4 weeks, 10 out of 15 of the fat fed

COX-2^{-/-} mice had died (Narasimha *et al.*, 2007). These results indicate that COX-2 might have an important role in cholesterol metabolism (in the liver or elsewhere) and that deletion of this COX-2 (or potentially through pharmacological inhibition with NSAIDs or COX-2 selective inhibitors) could (independently or in conjunction with any alterations in global or local prostacyclin levels) contribute to an increased atherogenic risk.

This could be highly relevant considering extended treatment with celecoxib or rofecoxib was associated with a risk transformation, where the cardiovascular hazard evolved slowly over time in 1-2% of patients, which may be linked to an acceleration of atherogenesis (Bresalier *et al.*, 2005; Fries *et al.*, 2006; Solomon *et al.*, 2005).

Keeping these observations about an athero-protective role of COX-2 in mind, this takes us to the final part of my thesis which concerned the physiological consequences of COX-1 or COX-2 deletion in the aortic arch.

COX-1 or COX-2 deletion in the aortic arch: consequences for NF-κB activity

In the final results chapter of my thesis (Chapter 5), I set out to investigate NF-κB activity in the aortic endothelium of the COX-1^{-/-} or COX-2^{-/-} mice. The rational was that the globally diminished prostacyclin (in the COX-1^{-/-} mice) or locally reduced prostacyclin and/or other COX products and/or altered cholesterol metabolism in the COX-2^{-/-} mice, may influence NF-κB activity in these animals. This is particularly relevant to study since activated NF-κB is thought to play a key role in the development of vascular inflammation and atherosclerosis (Collins *et al.*, 2001).

The NF-κB protocol was validated with LPS treatment that consistently resulted in nuclear translocation of NF-κB from the cytosol to the nucleus in the endothelium of both the greater curvature and the lesser curvature in all the animals. Nuclear levels of NF-κB immunoreactivity were significantly higher in the lesser curvature region compared to the greater curvature region in both untreated and LPS treated WT mice. This is consistent with reports that NF-κB is 'primed for activation' in the lesser curvature of the aortic arch which may have relevance to the increased susceptibility of atherosclerotic plaques forming in this area (Hajra *et al.*, 2000).

Despite finding very low levels of COX-2 in the greater curvature of the aortic arch, levels of 'primed' or nuclear NF- κ B immunoreactivity were significantly higher in this 'protected' region from untreated COX-2^{-/-} mice compared to WT mice. The same was true for COX-1^{-/-} mice. Following LPS stimulation, nuclear NF- κ B was again higher in the greater curvature in COX-2^{-/-} mice compared to WT mice (but were not different in COX-1^{-/-} mice). Both COX-1^{-/-} and COX-2^{-/-} mice had significantly more nuclear NF- κ B levels in the lesser curvature compared to WT mice following LPS treatment. Therefore, the aortic endothelium of both COX-1^{-/-} and COX-2^{-/-} mice appears to have primed (nuclear) and potentially more active NF- κ B compared to WT mice.

One can speculate that the 'primed' NF- κ B in the COX-1^{-/-} mice may be due to the lack of prostacyclin restraint on NF- κ B translocation (through negative feedback inhibition), as has been shown in other cell types with prostacyclin analogues (Raychaudhuri *et al.*, 2002; Zhou *et al.*, 2007a; Zhou *et al.*, 2007b). In fact, the primed NF- κ B in the COX-2^{-/-} tissue was somewhat more surprising, considering that on a gross level, COX-2 did not appear to contribute to prostacyclin release from these tissues. Still, as mentioned in earlier sections, it is possible that the activity of the highly localised COX-2 in the lesser curvature that I may have failed to detect in my bioassays of gross sections of tissue, could in fact modulate local NF- κ B activity. Alternatively, loss of COX-2 in a remote site (e.g. the kidney or the liver) may predispose the circulation of COX-2^{-/-} mice to inflammation due to indirect phenotypic manifestations (such as altered cholesterol metabolism described in earlier sections).

Either way, both the cause(s) and any consequence(s) of the 'primed' NF- κ B in the COX-1^{-/-} and COX-2^{-/-} mice still remain to be established. Furthermore, the 'primed' NF- κ B in the COX-2^{-/-} mice may contribute to the increased susceptibility to plaques in the lesser curvature of the ApoE^{-/-} COX-2^{-/-} mice (as investigated by my colleague Dr Nick Kirkby). The 'primed' NF- κ B in the protected greater curvature of the COX-2^{-/-} mice did not however result in any more plaques forming in this 'protected' area of the ApoE^{-/-} COX-2^{-/-} mice.

It is worth mentioning that I did performed a pilot study in WT mice In order to investigate if pharmacological treatment of with an NSAID (diclofenac administered via the drinking water for consecutive 3 days) could mimic the elevation in NF- κ B seen in the COX-1^{-/-} and COX-2^{-/-} mice. This data was not included in this thesis, but initial results indicated that there was no

obvious difference in nuclear NF- κ B immunoreactivity in diclofenac treated animals compared to untreated controls (in either untreated or LPS treated conditions). Either this pilot study failed to achieve sufficient inhibition of COX-1 (and COX-2) and/or longer more chronic dosing is required and/or it might suggest that this is a phenotypic effect in these animals (due to developmental compensations or any other indirect consequences of the chronic lack of COX-1 or COX-2) that may or may not be relevant to scenarios of 'normal' dosing of NSAIDs humans.

Nevertheless, the 'primed' NF- κ B in the aortic endothelium of COX-1^{-/-} and COX-2^{-/-} mice is still an interesting observation which is important to bear in mind when studying aortic endothelium from these mice, however, further investigation is warranted into the causes and consequences of the 'primed' NF- κ B.

Thrombotic potential in the COX-1^{-/-} and COX-2^{-/-} mice: platelet adherence to aortic arch endothelium

Since platelets and endothelial cells share many cell surface markers it was initially challenging to think of a platelet selective marker that could be used in the mouse aortic arch *en face* model. It was therefore a welcome finding when the platelet marker CD41 specifically stained platelet-like objects but not the endothelium (Chapter 5). These CD41 positive platelet-like objects were of the same size and shape as the CD41 positive platelets detected in the blood smears which validated that the CD41 positive objects detected adhered to the endothelium in the aortic arch were platelets.

After this validation, it was time for *en face* investigation of the number of CD41 positive platelets adhered to aortic arches from WT, COX-1^{-/-}, COX-2^{-/-} and normal chow fed ApoE^{-/-} mice.

The whole experiment and quantification was performed blinded and just to be sure that the manual scoring was accurate, I re-calculated the results (still blinded) through automated quantification in Image J.

The result came out the same either way; there was significantly more CD41 positive platelets adhered to the lesser curvature in aortic arches from COX-2^{-/-} mice than WT mice (or any other mice).

This was a fascinating result that gave rise to a whole new set of research questions; firstly, how long had the CD41 positive platelets been adhered to this susceptible region of the aortic arch?

It is difficult to draw any firm conclusions about this, but at least at the point in time of the perfusion fixation, they were clearly there. It is however probably important to examine what effect different perfusion methods might have on this type of platelet adhesion, to test if there is a difference between no perfusion at all versus manually maintained pressure perfusion (as was performed in this thesis) versus regulated pressure perfusion (i.e. ~100mmhg maintained with a perfusion rig set up).

Perhaps it would have been more ideal if the mice had been perfused with a perfusion rig set up (consistently at ~100mmhg). Still, even though the animals used in this study were perfused manually (via a needle inserted through the heart, flushed through with PBS and fixative at high pressure) all the aortic arches were completely clear of blood and the procedure was performed by randomising the order of animal type perfused.

Furthermore, a single ApoE^{-/-} mouse that had been fed an atherogenic diet was included in the study as a positive control to see if more platelets would adhere to plaque enriched areas compared to 'normal' areas of adjacent endothelium as reported previously (Davies *et al.*, 1988; Huo *et al.*, 2003). In this pilot study, there were more CD41 positive platelets detected, mainly localised to the plaque enriched areas which serves as an initial (positive control) validation for this model of platelet adhesion.

It was also interesting to note that despite elevated levels of 'primed' NF-κB in the aortic arch endothelium of COX-1^{-/-} mice (alongside greatly diminished prostacyclin synthesis in these vessels), there was no difference in the number of CD41 positive platelets adhered to the aortic arch endothelium in these mice compared to WT mice. This is probably due to a lack of both COX-1 driven platelet thromboxane production and COX-1 driven endothelial prostacyclin in COX-1^{-/-} mice. It has been shown that vascular injury-induced platelet

activation was increased in $IP^{-/-}$ mice, decreased in $TP^{-/-}$ mice, and the augmented platelet response seen in the $IP^{-/-}$ mice was abolished in mice deficient in both receptors ($IP^{-/-}TP^{-/-}$ mice) (Cheng *et al.*, 2002).

The final figure in Chapter 5 (5.18 that shows images of CD41 positive platelets on the different layers of the plaque-like structure in the $ApoE^{-/-}$ mouse fed an atherogenic diet), shows in panel C a nucleated cell in complex with a CD41 positive platelet. This unexpected finding highlighted the possibility that the CD41 positive platelets may have been adhered on their own and/or alternatively in a complex with a leukocyte that may have adhered to the (nuclear dense) lesser curvature endothelium. It has been suggested that circulating platelet-leukocyte aggregates promote atherosclerotic lesion formation (Huo *et al.*, 2003). With that said, there was no significant difference in overall nuclear density in the lesser curvature between the $COX-2^{-/-}$ and WT mice used in this study. Hence, the number of potential resident leukocytes in the lesser curvature is probably similar between these animals, which indicate that it is more likely to be an increased number of individual platelets that have adhered to susceptible endothelium in the $COX-2^{-/-}$ mice rather than platelet-leukocyte complexes.

In conclusion, despite normal prostacyclin production capacity in aortic arches from $COX-2^{-/-}$ mice, it seems like the aortic endothelium in the lesser curvature of $COX-2^{-/-}$ mice is more susceptible to platelet adhesion. This may either be due that the platelets are more reactive in the $COX-2^{-/-}$ mice and/or alternatively due to a more 'sticky' or activated endothelium in this particular region of these animals. Findings from another study support the latter, where it was suggested that selective COX-2 inhibition may diminish anti-platelet properties of the endothelium (Buerkle *et al.*, 2004).

Speculatively, the increased number of CD41 positive platelets adhered to the lesser curvature endothelium of $COX-2^{-/-}$ mice (and the augmented susceptibility for atherosclerotic plaques in the lesser curvature of $ApoE^{-/-}COX-2^{-/-}$ mice) may be due to the lack of localised COX-2 that may have important anti-thrombotic and anti-atherogenic effects in this particular region of the aortic arch.

Summary

In every protocol that I have used, I have ubiquitously found abundant COX-1 immunoreactivity predominating over very sparse COX-2 immunoreactivity in both regions of the mouse aortic arch. This pattern has been very similar in all the blood vessels investigated in this thesis. Moreover, in every bioassay that I have performed, stimulated prostacyclin release has been abundant in aortic vessels from WT and COX-2^{-/-} mice, but more or less absent in vessels from COX-1^{-/-} mice.

Therefore, it seems very clear that in these protocols COX-1 appears to be the overriding COX isoform for vascular prostacyclin production. With this in mind, it would be all too easy to conclude that COX-2 has no role in cardiovascular health and disease, at least in terms of endothelial prostacyclin production from large vessels, especially when considering that the majority of research in this area concludes that COX-2 inhibition has deleterious vascular effects.

The question remains exactly how the vasculature responds to COX-2 inhibition? It was surprising to note that despite finding minimal contribution of COX-2 to vascular prostacyclin production (when studied in large vessels); loss of COX-2 had profound effects on NF- κ B activation as well as increased platelet adhesion to susceptible areas of endothelium in the mouse aortic arch.

Conclusion

In conclusion to the hypothesis of this thesis:

‘COX-2 is present in vascular endothelium and regulates prostacyclin release and functional responses in health and disease’

This hypothesis needs to be updated, since COX-1 was undeniably the predominant endothelial COX isoform found in every blood vessel that was examined and COX-1 also appears to be responsible for the majority of prostacyclin production in vascular endothelium.

With that said, seemingly modest levels of localised, constitutively expressed COX-2 in micro-domain ‘hot spot’ areas of the vasculature (such as the atherosclerosis and inflammation susceptible lesser curvature of the aortic arch) may still contribute to prostacyclin production and/or to the production of another prostanoid, on a local level, to limit inflammation, atherosclerosis and platelet adhesion.

The results presented in this thesis provide a lead for further investigation into the clinical relevance of these findings, to see if, alongside other cardiovascular risk factors; chronic use of NSAIDs or COX-2 selective inhibitors may inhibit the activity of anti-thrombotic and athero-protective, constitutively expressed COX-2 localised to susceptible areas of the vasculature, increasing the risk of arterial thrombotic outcomes such as myocardial infarction or stroke.

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