Heme and metformin coordinate human and murine macrophage heme oxygenase 1 expression with foam cell resistance partly via AMPK-ATF1

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Abstract (229)

Background: Intraplaque hemorrhage (IPH) is a key driver of the progression of atherosclerotic plaques. We recently characterised Mhem as a novel macrophage phenotype that limits the atherogenicity of IPH. Mhem are directed by activating transcription factor 1 (ATF1), which is activated by phosphorylation. A better understanding of the counter-atherogenic ATF1-Mhem pathway may facilitate anti-atherosclerotic therapies.

Methods: We tested the hypothesis that heme in pathologically relevant concentrations activates the ATF1-Mhem pathway via 5'-AMP-activated protein kinase (AMPK) in primary human monocyte-derived macrophages and mouse bone marrow macrophages.

Results: We found that heme (10 μ M) activates AMPK, and downstream ATF1 mediated co-induction of HO-1 and LXR that characterise Mhem. Heme increased macrophage phospho-AMPK, phospho-ATF1 and its target genes, and these effects were inhibited by the AMPK antagonist dorsomorphin, or by AMPK-knockdown with siRNA. The AMPK-activating oral hypoglycemic agent metformin also induced and phosphorylated ATF1 at a clinically relevant concentration (10 μ M). Functional effects of heme and metformin were inhibited by AMPK-knockdown and included suppression of macrophage oxidative stress; increased cholesterol export; protection from foam cell formation; and suppression of macrophage inflammatory activation (HLA-DR expression).

Conclusions: Our data indicate that heme activates the ATF1 pathway in human macrophages via AMPK, and that a similar response occurs following treatment of cells with metformin. Our results suggest an *in vitro* mechanism that may explain the clinical evidence that metformin has vascular protective effects beyond its role in treating hyperglycemia.

Introduction

Intraplaque hemorrhage

Intraplaque hemorrhage (IPH) drives atherosclerosis partly via the dual metabolic insult of cholesterol-enriched erythrocyte membranes and pro-oxidant heme/iron¹. Indeed, IPH may acutely destabilise coronary lesions causing myocardial ischemia². We have recently shown that IPH generates a distinct adaptive macrophage state (Mhem) in human plaques³. We went on to identify activating transcription factor 1 (ATF1) as the key transcription factor (TF) mediating adaptive responses to heme at 10 μ M, a pathophysiologically relevant concentration⁴. Others have independently designated a similar macrophage phenotype as M(Hb)⁵. Mhem exhibit coordinated properties including increased heme oxygenase (HO)-1 expression, reduced oxidative stress, suppressed inflammatory activation, increased lipid export and resistance to foam cell formation and increased levels of the anti-inflammatory cytokine interleukin-10³. Taken together, these may inhibit atherogenesis if they also apply *in vivo*. Co-induction of the genes for HO-1 and LXR is a key component of ATF1 transcriptional network driving macrophages to become Mhem⁴.

ATF1

ATF1 is the closest homologue of cyclic-AMP (cAMP) response element binding protein 1 (CREB1). Like CREB1, ATF1 was named for its capacity to activate gene expression in response to cAMP⁷. ATF1 corresponds to a GWAS locus associated with sudden cardiac death in advanced coronary artery disease, consistent with a role in responding to IPH *in vivo*. Both CREB1 and ATF1 are phosphorylated on homologous motifs by cAMP-dependent protein kinase (protein kinase A, PKA) and 5'-AMPK-activated protein kinase (AMPK)^{8,9}. We have previously demonstrated that heme induces ATF1 at both mRNA and protein levels, and causes its activation by Ser63 phosphorylation⁴. The coregulation of HO-1 and LXR β by phospho-ATF1 allows ATF1 to play a pivotal role in linking storage of iron with export of

lipid. Since ATF1 directs these two key protective systems to work in concert, it holds enormous therapeutic promise^{10, 11}. We therefore asked which kinases phosphorylate (and thereby activate) ATF1 in response to heme.

AMPK

AMPK is a key mediator of cellular energy homeostasis, and responses to metabolic stress^{12, 13}. When activated by low energy levels (low ATP / AMP ratio), it promotes ATP-creating catabolic pathways over ATP-expending anabolic pathways^{12, 13}. It is also activated by oxidative or genotoxic stress¹². Structurally, AMPK is composed of three chains (α, β, γ), each with up to three isoforms¹⁴. AMPK may modify transcription via phosphorylation of a large and growing number of transcription factors and chromatin proteins^{13, 15-21}. Critically, these include ATF1⁹. Purified AMPK phosphorylated recombinant purified ATF1 in an *in vitro* kinase assay in a cell-free system, in a paper defining AMPK-mediated phosphorylation of CREB family proteins *in vivo* in skeletal muscle during exercise²². Moreover, AMPK activation in macrophages induces an anti-inflammatory response, with release of IL-10²³. Thus AMPK is a strong candidate to mediate ATF1 phosphorylation and transcription in response to heme following IPH and erythrophagocytosis.

Metformin

Metformin is effective in Type-II diabetes^{24, 25}, without causing hypoglycaemia. Indeed, metformin has proven sufficiently safe over some 50 years that it is now prescribed for debilitating but non-life threatening endocrine disease and metabolic syndrome^{26, 27}. In two independent recent large scale clinical trials, metformin has exhibited a significantly greater reduction of cardiovascular events (which are mainly due to atherosclerosis), than would be expected from the improved glycemic control alone²⁸. This suggests that metformin may have a direct vascular protective effect, possibly located in the vessel wall.

AMPK is also known to be activated by metformin ²⁶. However, although metformin clearly can activate AMPK, it is not clear how much of its clinical effect critically requires AMPK versus other pathways ^{29, 30}. Interestingly, there is some evidence that AMPK may be anti-inflammatory in classical T-lymphocyte / macrophage mediated inflammation models such as extrinsic allergic encephalomyelitis ³¹. This raises the possibility that metformin may have wider potential as an anti-inflammatory agent in classical inflammatory diseases such as transplant rejection and autoimmunity, as well as in atherosclerotic inflammation ³¹.

Hypothesis and summary

We tested the hypothesis that an AMPK-ATF1 pathway mediates heme-induced HO-1 and LXR β and examined the implications for potential mechanisms of action of metformin. We show here that heme at a pathophysiologically relevant concentration (10 μ M) ⁴ and metformin at a pharmacologically relevant concentration (also 10 μ M) ³⁷ increase ATF1 transcripts, ATF1 protein and activated (phospho-Ser63) ATF1, via AMPK, and that thereby metformin induces ATF1-dependent pathways including HO-1 and LXR β . Thus AMPK is a key initiator of ATF1 signaling evoked by IPH, and AMPK $\rightarrow$ ATF1 may be therapeutically activated by metformin.

Methods

We cultured human blood-derived macrophages from normal human volunteers, with Ethical approval and informed consent; and monocyte purification was as we previously described³. We used established transfection methods for siRNA and plasmids in order to obtain molecular specificity, supplemented with pharmacological reagents. Mouse bone marrow derived macrophages (BMM) were cultured as widely established. Cell culture, RNA analysis, western blotting were carried out as before⁴.

Results

Heme activates AMPK in human blood-derived and plaque macrophages

AMPK has a catalytic α subunit that can be activated by autophosphorylation, with $\alpha 1$ being the main α chain in human macrophages. We probed lysates of human macrophages by immunoblotting with anti-phospho-AMPK- $\alpha 1$. We found that heme (10 μ M) increased (activated) phospho-AMPK, in a biphasic pattern, with an initial peak within minutes and a later one at 2-4h coincident with the p-ATF1 (Figure 1A). Heme at this pathologically relevant concentration induced ATF1 protein progressively over 4h; phospho-ATF1 over 0.125-4h; and HO-1 target protein at 4h (Figure 1A). Thus, the timing of AMPK activation by heme was sufficiently early to be consistent with an initiating event. We next assessed the *in vivo* relevance of AMPK by carrying out confocal microscopy of human plaques (Figure 1B). We found that phospho-AMPK was expressed by Mhem macrophages in areas of IPH, but not control foam cell areas, identified by comparison with serial sections that had been immunostained for p-ATF1, HO-1, LXR, ABCA-1, 8-oxo-G, CD163 and HLA-DR in previous publications (^{3,4} and not shown again). We have previously shown that p-ATF1 is a key functional and immunohistochemical identifier of Mhem, since it directs the majority of heme-induced genes and is found selectively in Mhem cells in plaques ⁴. The sections were therefore also double-stained for p-ATF1 and p-AMPK, which indeed showed colocalisation in the same cells (Figure 1B). As might be expected, p-AMPK was predominantly in the cytoplasm and p-ATF1 predominantly in the nucleus (Figure 1B).

Pharmacological inhibition of AMPK inhibits induction of phospho-ATF1, ATF1, HO-1 and LXR β by heme and metformin

Since heme (10 μ M) activated AMPK and ATF1, we next determined whether there was a causal connection, initially using a simple pharmacological strategy and protein quantification by western analysis (representative blots in Figure 2A, densitometry in Figure

2B). We found that metformin at 10 μ M (a known AMPK activator) induced phospho-ATF1, and its target genes HO-1 and LXR; and that these activation events in response to either heme (10 μ M) or metformin (10 μ M) were antagonised by 1 μ M compound-C (dorsomorphin), a known inhibitor of AMPK (Figure 2A, B).

We then assessed whether the induction of target proteins by metformin was via mRNA (*i.e.* transcriptional) (Supplemental Figure S-I). We found that metformin induced mRNA for ATF1 (Supplemental Figure S-IA,B), followed by that for HO-1 (Supplemental Figure S-IC). Heme (10 μ M) and metformin (10 μ M) induced mRNA for ATF1, HO-1 and LXR β , and induction of each was inhibited by the specific AMPK-inhibitor compound-C (dorsomorphin) at 1 μ M (Supplemental Figure S-IB,D,E). Thus heme and metformin induce ATF1, HO-1 and LXR β via an AMPK-dependent transcriptional pathway.

AMPK-knockdown inhibits induction of phospho-ATF1, HO-1 and LXR by heme and metformin

To show that heme and metformin specifically activated ATF1 via AMPK, we next corroborated the pharmacological inhibition with an siRNA approach, using transfection protocols that we have validated for human blood-derived macrophages (Figure 3)³². Heme (10 μ M) induced phospho-ATF1, and this was suppressed by AMPK- α 1 knock-down by siRNA, with oligos from Qiagen (Figure 3A,C). These data were extended and corroborated with siRNA oligos from an independent supplier (namely Dharmacon) individually targeting each subunit of AMPK that had been identified as expressed in macrophages by microarray⁴ (Supplemental Figure S-II). These experiments showed that the stimulants heme (10 μ M) (Supplemental Figure S-II) and metformin (10 μ M) (Supplemental Figure S-II) each induced phospho-ATF1, and in each case phospho-ATF1 induction was inhibited by AMPK-knockdown, to variable degrees depending on which individual AMPK subunit was suppressed. We then examined the effects of suppressing each of the AMPK subunits, and of

all of the AMPK subunits together, again using siRNA transfection in primary human macrophages (Supplemental Figure S-III). This combined AMPK-knockdown suppressed AMPK, which inhibited the capacity of heme (10 μ M) to increase phospho-ATF1, HO-1 and LXR β proteins (Supplemental Figure S-III, quantified by densitometry in Supplemental Figure S-III).

We then carried out essentially the same procedure for metformin (Figure 3B,D). Metformin (10 μ M) induced each of p-ATF1, p-AMPK, HO-1 and LXR β , and the induction of each of these was suppressed by AMPK-knockdown (Figure 3B,D).

We then examined the AMPK-dependence of key heme-modulated macrophage functions that were potentially therapeutic ⁴. We found that AMPK-knockdown caused heme (10 μ M) to become pro-oxidant (Figure 4A); prevented the cholesterol export induced by heme (10 μ M) (Figure 4B); and reversed suppression of cholesterol accumulation caused by heme (10 μ M) (Figure 4C); and prevented heme from inducing IL-10 secretion (Figure 4D). Whilst heme suppresses foam cell formation, heme in fact promoted uptake of di-O-labelled OxLDL indicating that it does not prevent phagocytic clearance of extracellular OxLDL (Figure 4E). AMPK-knockdown and ATF1-knockdown inhibited this effect (Figure 4E).

Beta (β)-oxidation pathway of cellular lipid disposal is activated by heme via AMPK and ATF1

Our microarray data on heme-stimulated macrophages identified induction of multiple β -oxidation genes, one of the principal pathways for fatty acid degradation ⁴. AMPK classically activates β -oxidation pathways (fatty acid degradation) by a non-genomic mechanism involving phosphorylation of acetyl-CoA-carboxylase (ACC) ³³. While LXR directs cholesterol export, its ability to combat lipid overload is blunted by co-induction of the fatty acid synthase (FASN) gene ^{34, 35}. Thus, coordinated activation of both fatty acid oxidation

(regulated by AMPK directly) and cholesterol export (regulated via LXR) is likely to be complementary for foam cell protection.

We therefore assessed the role of heme in β -oxidation by accepted methodology, using decay of a fluorescently-tagged fatty acid BODIPY-C11 as in Supplemental Figure S-IV. We found that heme (10 μ M) induces the degradation of this fluorescent fatty acid tracer in a time-dependent manner, and this was blocked by etomoxir, a specific inhibitor of mitochondrial β -oxidation (Supplemental Figure S-IVA)³⁶. This heme-induced β -oxidation was blocked by ATF1-siRNA (Supplemental Figure S-IVB) and by AMPK-knockdown by combined siRNA (Supplemental Figure S-IVC). This indicated that it is dependent on both AMPK and ATF1 consistent with an AMPK-ATF1 pathway. As expected for a known AMPK activator, metformin (10 μ M) also induced β -oxidation, which was also blocked by AMPK-knockdown by combined-siRNA (Supplemental Figure S-IV D).

The AMPK activator metformin reproduces ATF1 signaling in Mhem

In normal human volunteers, the peak plasma metformin concentration after oral absorption of a standard prescribed tablet (CP_{max}) is 10 μ M³⁷. Figure 5A shows a concentration-effect curve of metformin on the release of highly reactive oxygen species (hROS) from macrophages derived from normal human volunteers, with the peak plasma concentration highlighted. In contrast to simple dihydro-dyes for redox potential, hROS specifically detect the most pathological species (peroxynitrite, hypochlorite and hydroxyl radicals) via electrophilic adduction of the amino group of the dye aminophenylfluorescein (APF)³⁸. Notably, substantial antioxidant protection (25-50%) was identified at the CP_{max} (10 μ M) and at considerably *lower* metformin concentrations than the CP_{max} (100nM-1 μ M).

The suppression of macrophage hROS by metformin at CP_{max} (10 μ M) was reversed by AMPK-knockdown by combined-siRNA (Figure 5B). We had previously found that heme

(10 μ M) suppresses the macrophage antigen presentation molecule and activation marker HLA-DR via an ATF1 pathway. We therefore examined the effects of metformin on macrophage HLA-DR, finding that 10 μ M metformin substantially suppressed surface HLA-DR on macrophages (Figure 5C).

Next, since we had shown that 10 μ M metformin also induced LXR β mRNA and protein, we assessed whether this had resultant beneficial effects on lipid metabolism. We found that metformin prevented foam cell formation due to incubation with oxidised LDL (OxLDL) in five separate macrophage cultures from normal volunteers (Figure 5D), as previously published with heme (10 μ M) ⁴. We next analysed the mechanism of this effect with the AMPK-knockdown by combined-siRNA protocols used in preceding experiments, quantifying foam cell formation as published (Figure 5E). Both 10 μ M heme and 10 μ M metformin suppressed OxLDL-induced foam cell formation; and both effects were prevented with AMPK-knockdown by combined-siRNA (Figure 5E). We then corroborated this effect by analysing cellular cholesterol, and showed that this effect of metformin was AMPK-dependent since it was abolished by AMPK-knockdown by combined-siRNA (Figure 5F). We have previously validated an ApoA1-dependent export assay that measures the LXR / ABCA1 pathway ⁴. Macrophage cholesterol export to ApoA1 in the culture medium was stimulated by metformin in macrophage from five normal human volunteers and prevented by AMPK-knockdown by combined-siRNA (Figure 5G). Thus metformin reproduces multiple potentially therapeutic features of the effect of heme on macrophages, and both do so in an AMPK-dependent and ATF1-dependent pathway.

Heme and metformin induce less p-ATF1, HO-1 and LXR in AMPK-KO compared to wild type murine bone marrow macrophages

In human blood-derived macrophages, which are highly relevant, AMPK-knockdown reduced the ability of heme and metformin to activate p-ATF1, HO-1 and LXR β and promote linked antioxidant and foam cell protective effects. However, AMPK was only knocked down and not out in these experiments, it was difficult to be definitively identify the contribution of AMPK-dependent and AMPK-independent pathways. We therefore assessed responses to heme and metformin in bone marrow macrophages (BMMs) from AMPK-knockout (AMPK-KO) and matched wild-type (WT) mice (Figure 6).

In WT BMMs, heme (10 μ M) activated pThr172-AMPK, with subsequent induction of pSer63-ATF1, t-ATF1, HO-1 and LXR β (n= 10 mice) (Figure 6A). This was less apparent in BMMs from AMPK-KO mice (Figure 6A). As controls, AMPK was absent in AMPK-KO mice, as was AMPK activation (Figure 6A). In WT BMMs, metformin (100 μ M) activated pT172-AMPK, with subsequent activation of pSer63-ATF1, t-ATF1, HO-1 and LXR β (n= 10 mice) (Figure 6B). This was less apparent in BMMs from AMPK-KO mice (Figure 6B). Thus in murine BMMs, heme and metformin activate the same ATF1/HO-1/LXR cascade that they do in human, and the activation is dependent on AMPK.

The functional consequences of this activation were next tested (Figure 6C-F). In WT BMMs, incubation with heme (10 μ M) for 48 h suppressed oxidative stress as measured by APF, which detects highly reactive oxygen species (Figure 6C). In contrast, in BMMs from AMPK-KO mice, heme and metformin had the opposite effect, increasing hROS (Figure 6C). A similar mechanism was found with metformin (Figure 6D). In WT BMMs, incubation with metformin (10 μ M) for 48 h suppressed oxidative stress as measured by APF, which

detects highly reactive oxygen species (Figure 6D). In contrast, in BMMs from AMPK-KO mice, heme and metformin had the opposite effect, increasing hROS (Figure 6D).

Next the effects of heme and metformin on survival of WT and AMPK-KO BMMs were assessed, using the XTT vital dye. This revealed an effect converse to that on oxidative stress, that is heme or metformin increased survival in WT cells, but reduced survival in AMPK-KO cells (Figure 6E).

We then studied the resistance of WT and AMPK-KO murine BMMs to lipid accumulation (foam cell formation) (Figure 6E). Oxidatively modified LDL evoked formation of foam cells (as with human macrophages) (Figure 6F). Heme (10 μ M) and metformin (10 μ M) also suppressed foam cell formation (Figure 6F) (as with human macrophages). Protection from foam cell formation by either heme or metformin was lost in AMPK-KO BMMs (akin to AMPK-knockdown in human macrophages) (Figure 6F).

Thus, we have previously shown that the transcription factor ATF1 is a key mediator of development of the Mhem phenotype in human atherosclerotic plaques ⁶. We here show here that heme and metformin activate ATF1 via AMPK in both human and murine cells *in vitro*, with transcriptional induction of HO-1 and LXR that allows interlinking of antioxidant activity and resistance to foam cell formation.

Discussion

Summary

We show here that ATF1, which we previously established is the key driver of Mhem macrophages, and is activated by heme (10 μ M) via AMPK-dependent phosphorylation. We have defined a clinical application of this pathway, in which metformin, a common safe oral hypoglycemic agent, may coregulate anti-inflammatory, antioxidant and foam-cell protective mechanisms in macrophages. The data is therefore highly significant for clinical treatment of atherosclerotic risk, and potentially inflammation in general.

AMPK and metabolic stress

While AMPK is primarily a responder to low fuel ³⁹⁻⁴¹, it is also a wider stress-activated kinase, being also activated by oxidative stress, osmotic stress ⁴², ionizing radiation ⁴³ and hypoxia ⁴⁴, and may therefore play a role in signalling akin to classical stress-activated protein kinases such as p38MAPK and JNK. We suggest that the metabolic stress of erythrophagocytosis parallels that of energy deprivation, since both are associated with oxidative stress and require activation of catabolic pathways. Indeed, the term ‘heme stress’ may be appropriate.

AMPK and macrophage phenotype.

It was previously shown that transfecting a mouse macrophage cell line with expression plasmids for constitutively active AMPK modified macrophage signalling, increasing phospho-CREB, increasing phospho-PI3K and suppressing phospho-I κ B α ; together with induction of IL-10 and suppression of TNF- α ²³. The converse was obtained with dominant negative AMPK ²³. Moreover, a classical anti-inflammatory stimulus promoted AMPK phosphorylation and induced AMPK, whereas LPS (a classical inflammatory stimulus) had

the reverse effect ²³. We now extend these data and translate them to metformin pharmacology.

ATF1 and stress kinases other than AMPK

ATF1 is phosphorylated by several stress kinases including AMPK in skeletal muscle during exercise ²², p38MAPK in leptin signalling ⁴⁵, mitogen stimulated kinase (MSK) in UV-induced stress ^{46, 47}, and by protein kinase D ⁴⁸. Tyrosine phosphorylation of ATF1 by HIPK2 has also been described in response to genotoxic stress ⁴⁹. Understanding the full role of AMPK / ATF1 in the context of other upstream kinases regulation in the differentiation of Mhem is a major priority for further work. A combinatorial role of alternative kinases seems possible, since there were subtle mismatches between increases in phospho-ATF1 and in the target genes.

AMPK and TFs other than ATF1

Although AMPK was first, and best, characterised as a post-translational regulator of metabolic enzymes, recent work reveals that it has important and numerous transcriptional effects (ie mediated by phosphorylation of transcription factors) ^{14, 18}. Indeed, there is a described AICAR-response element and corresponding AICAR-response element binding protein, which is a zinc-finger protein ⁵⁰. AMPK mediates *in vitro* and *in vivo* phosphorylation of CREB-family proteins in skeletal muscle in exercise, and in the same paper was shown to direct phosphorylation of purified ATF1 by purified AMPK in a cell-free system ²². The functional consequences are potentially profound since microarray-validated target genes for AMPK, modulated in skeletal muscle in AMPK-KO mice *in vivo* include: PEPCK (the rate limiting enzyme for gluconeogenesis), fatty acid synthase (the rate limiting enzyme for fatty acyl / triglyceride production), eNOS (the endothelial cytoprotective vasodilator), ferritin (which safely stores iron), d-amino-levulinic acid synthase (d-ALA-

synthetase, ALAS, the enzyme for the rate limiting step of heme biosynthesis)⁵¹. Thus, AMPK regulate genes controlling vascular tone, iron storage, porphyrin / heme biosynthesis in addition to its traditional roles in glucose metabolism⁵¹. Moreover, AMPK may inactivate NFκB⁵²; and suppresses oxidative stress via upregulation of thioredoxin⁵³. Given the anti-inflammatory effects of AMPK, and its direction of a plethora of protective transcriptional effects, it is plausible that AMPK might initiate commitment to Mhem. Of interest, we found by systems biology approaches that the heme-induced geneset was enriched in ChIP-Seq validated binding sites for approximately 7 TFs, which in turn contained conserved AMPK-phosphorylation motifs (not shown).

Metformin and AMPK-dependence

The mechanism of action of metformin is still in the process of elucidation²⁶. Some data, using the small molecule AMPK inhibitor dorsomorphin, indicated that the hypoglycemic effects of metformin may be largely AMPK-mediated⁵⁴. Metformin mediated activation was also found to be dependent on the tumour-suppressor gene liver kinase B (LKB), which is often taken as an exclusive upstream kinase of AMPK⁵⁵. More recently, doubt was thrown on the specificity of metformin for AMPK since many of its hepatic hypoglycemic effects are identical in AMPK-knockout mice³⁰. In this paper, we carefully showed AMPK-dependence of the effects of metformin in human primary macrophages *in vitro*, using corroborating siRNA transfection and pharmacological strategies.

Possible clinical applications

Metformin is relatively safe oral hypoglycaemic agent that, unlike sulphonylureas, does not provoke hypoglycemia, even in normoglycemic subjects or in overdose²⁷. This is thought to be related to its activation of cellular energy homeostasis²⁷. In two recent large-scale clinical trials of cardiovascular endpoints in diabetes, metformin had a greater reduction in

cardiovascular events than was predicted from its effects on glycemic control alone²⁸. This suggested the possibility of a putative direct effect on vessels⁵⁶. Other studies have suggested variable but favourable effects on lipid profile, with reduced LDL in diabetes^{28, 57} or enhanced HDL in polycystic ovarian syndrome^{28, 58}. These findings provide a potential mechanism for these clinical effects. Namely, metformin may direct a major pathogenic cell type in atherosclerotic lesions to initiate joint anti-oxidant, anti-inflammatory and cytoprotection, with promotion of cholesterol efflux to HDL. Metformin may therefore be an agent that could suppress atherosclerotic plaque inflammation useful in a wider range of risk factors than in solely overt diabetes. Moreover, since there is evidence that metformin is also cardioprotective by improving myocardial remodelling and myocardial protection in ischemia-reperfusion²⁸, this may reflect commitment to Mhem macrophages in damaged myocardium. Our work is yet limited by being restricted to *in vitro* macrophage – albeit human monocyte derived macrophages from multiple normal volunteers. Taking the work further will require progression to *in vivo* model such as LDR^{-/-}, ATF1^{-/-} and AMPK^{-/-} mice. Moreover, pigs readily develop vascular and cardiometabolic disease in response to diet – like humans – and would make a representative model. Finally, analysing peripheral blood monocytes in patients before and after metformin commencement would yield insights into the clinical relevance. *In vivo* and transcriptomic studies are now urgently required to define potential therapeutic implications and these are planned.

Conclusions

We here show here that heme and metformin activate ATF1 via AMPK in both human and murine cells *in vitro*. The AMPK-ATF1 pathway drives transcriptional co-induction of HO-1 and LXR that allows interlinking of antioxidant activity, resistance to foam cell formation and macrophage deactivation. We have previously identified that ATF1 initiates a coordinated transcriptional response and macrophage phenotype that we termed Mhem. Since

metformin is an important antidiabetic drug, these data yield a new mechanistic insight into recent clinical trial data indicating an enhanced suppression of diabetic macrovascular disease by metformin. Whether this pathway protects against vascular disease in vivo is under active investigation.

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Disclosures

None

Figure Legends

Figure 1 Human Mhem have activated AMPK *in vitro* and *in vivo*

- (A) **Early time course of human macrophage response to heme** Human monocyte derived macrophages were purified from peripheral blood and cultured for 24h. Then they were stimulated with heme or vehicle control and protein lysates were made at the indicated time points (0, 0.125, 0.25, 0.4, 1, 2 and 4h). Lysates were run on SDS-PAGE, electrotransferred to PVDF membranes which were then immunoblotted with the indicated antibodies. Bands are at the appropriate Mr (indicated).
- (B) **Mhem macrophages in coronary plaques with intraplaque hemorrhage contain increased activated AMPK relative to plaque foam cells** Confocal micrographs: **upper image pair** shows anti-phospho-AMPK (activated AMPK) immunofluorescence (Alexa568, red) or DNA-binding dye (TOPRO, blue) as indicated. Scalebars as indicated. Cells were in the matching areas of serial sections to those previously characterised ^{3, 32}. Lu = Lumen; CC = cholesterol cleft. LNC = lipid necrotic core. Mhem and FC areas of the same plaque were imaged at the same settings and were identified from p-ATF1, CD163, HLA-DR, HO-1, LXR β , ABCA1, ApoE, 8-oxo-G immunostaining of serial sections. **Lower image pair:** dual immunostaining with anti-phospho-AMPK (activated AMPK, green) and anti-phospho-ATF1 (red).

Figure 2 Heme and metformin induce, and pharmacological AMPK inhibition suppresses, phospho-ATF1, HO-1 and LXR β in human macrophages

Human monocyte derived macrophages were purified from peripheral blood and rested for 24h. Then they were cultured with heme (10 μ M), metformin (10 μ M), the AMPK-inhibitor compound-C (Dorsomorphin 1 μ M) and protein lysates made at 4h. Lysates were run on SDS-PAGE, electrotransferred to PVDF membranes, which were immunoblotted with antibodies to phospho-ATF1, total ATF1, phospho-ACC, HO-1 and LXR β as indicated (Methods). Representative blots (n=5 donors) showing bands at the appropriate Mr (indicated) are shown in (A). (B) Densitometry for the bands as before; expressed as mean $\pm$ SE for n=5 donors; * $<p<0.05$, paired Wilcoxon test, n=5 donors.

Figure 3 Heme and metformin induce AMPK activation and induce phospho-ATF1, ATF1, HO-1 and LXR β dependent on AMPK

Human blood-derived macrophages were transfected with matched control-siRNA or Qiagen AMPK- α 1 siRNA oligos 0.1nmol/10⁵ cells for 24h as indicated; and then cells were stimulated with heme 10 μ M for 4h as indicated and stimulated with either heme (**A**, **C**) or metformin (**B**, **D**), then lysed at 4h and western blotted for the proteins and phospho-proteins indicated. **A**, **B**, represent n=6 experiments each. **C**, **D**, densitometry, mean $\pm$ SE, n=6 experiments. * $<p<0.05$, paired Wilcoxon test, n=5 donors.

Figure 4 Heme induces potentially therapeutic macrophage functional responses dependent on AMPK

- (A) **AMPK-knockdown renders heme pro-oxidant.** Human blood derived macrophages were cultured with a combined-AMPK-siRNA protocol (Methods). Then heme (10 μ M) was added for a further 24h and highly reactive oxygen species (peroxynitrite, hypochlorite, hydroxyl radicals) were measured using APF dye as before⁶. * $p < 0.05$, Student's t-test, AMPK-knockdown by combined-siRNA reverses heme-mediated hROS suppression [note in the absence of double-stranded RNA, heme is normally outright anti-oxidant].
- (B) **AMPK-dependence of induction of cholesterol export by heme.** Human blood-derived macrophages were cultured with a combined-AMPK-siRNA protocol (Methods). Then heme (10 μ M) was added for a further 24h and the medium was changed and then cholesterol export to supernatant containing lipoprotein-free fetal calf serum and 10 μ g.mL⁻¹ ApoA1 (both Sigma-Aldrich), using an ultrasensitive enzymatic assay based on cholesterol oxidase and Amplex Red as before⁶. * $p < 0.05$, Student's t-test, AMPK-siRNA reverses heme-induced cholesterol export, n=5 donors.
- (C) **AMPK-dependence suppression of cholesterol accumulation by heme.** Human blood-derived macrophages were cultured with a combined-AMPK-siRNA protocol (Methods). Then heme (10 μ M) was added to the culture medium, along with 30 μ g.mL⁻¹ OxLDL. Supernatant and cell-associated cholesterol were measured with an ultrasensitive enzymatic assay based on cholesterol oxidase and Amplex Red as before⁶. * $p < 0.05$, Student's t-test, heme suppresses cholesterol accumulation, strongly reversed by AMPK-knockdown by combined-siRNA, n=5 donors. In macrophages with AMPK-knockdown, heme causes an increase in cell cholesterol.

(D) IL-10 secretion in response to heme is AMPK-dependent. Human blood-derived macrophages were cultured with the combined-AMPK-siRNA protocol (Methods). Macrophages were cultured as before in 96-well plates, supernatants were collected and assayed for IL-10 using a DuoSet (R&D Systems). Data are mean $\pm$ SE, n=5 donors. * $p < 0.05$, Student's t-test.

(E) Heme increases OxLDL uptake in an AMPK-dependent and ATF1-dependent manner. Human blood-derived macrophages were cultured with the combined-AMPK-siRNA protocol (Methods). Data are representative flow cytometric histograms n=3 donors. Treatments are as indicated.

Figure 5 Modulation of macrophage functions by metformin

Human blood derived macrophages were cultured in the presence or absence of metformin and key functions relevant to atherosclerosis were measured, in the presence or absence of combined knockdown of all the AMPK chains where indicated AMPK- $\alpha\beta\gamma$. Human blood-derived macrophages were transfected with pooled control-siRNA or Dharmacon combined siRNA oligos targetting all major AMPK chains (AMPK- $\alpha\beta\gamma$) at 0.01nmol/10⁴ cells for 24h, as indicated.

(A) Metformin suppresses oxidative burst in human blood-derived macrophages

at clinically relevant concentrations. Human blood-derived macrophages were cultured with increasing concentrations of metformin and oxidative stress was measured as highly reactive oxygen species after 48h (peroxynitrite, hypochlorite, hydroxyl radicals) with aminophenylfluorescein (APF) as before⁶. Red box = peak plasma concentration after oral dose (10 μ M). Data are mean $\pm$ SE. * $<p<0.05$, Student's t-test, n=5 independent experiments.

(B) AMPK-dependence of suppression of oxidative stress by metformin.

Human blood-derived macrophages were transfected with control-siRNA oligos or combined-AMPK-siRNA oligos as indicated; using the protocol validated in Figure 3. Then the cells were cultured in the presence or absence of metformin for a further 24h and oxidative stress measured using APF as in Figure 6A. Data are mean $\pm$ SE. * $<p<0.05$, Student's t-test, n=5 independent experiments.

(C) Metformin suppresses activation marker HLA-DR in human blood-derived

macrophages at clinically relevant concentrations. Human blood-derived macrophages were cultured in the presence or absence of 10 μ M metformin as indicated; macrophages were harvested after 4 days of culture, and fluorescently

stained for HLA-DR and analysed by flow cytometry. Representative of n=3 donors.

(D) Metformin suppresses foam cell formation in human blood-derived macrophages at clinically relevant concentrations. Human blood-derived macrophages were cultured in the presence or absence of 10 μ M metformin (bottom panels) and 30 μ g.mL⁻¹ OxLDL (right hand panels) for 1 week and then fluorescently stained for lipid with Nile Red and counterstained with a nuclear counterstain. Metformin prevents OxLDL-induced foam cell formation. Image representative of n=5 donors. [green = completely neutral lipid eg triglyceride, yellow = mildly polar lipids eg phospholipid, red = TOPRO nuclear dye].

(E) Metformin suppresses foam cell formation in human blood-derived macrophages via AMPK. Human blood-derived macrophages (n=5 donors) were cultured in the presence or absence of 10 μ M metformin, 10 μ M heme, and 30 μ g.mL⁻¹ OxLDL for 1 week, as in (D), and also in the presence of AMPK-knockdown by combined AMPK- $\alpha\beta\gamma$ siRNA or matched pooled control-siRNA as indicated. Cells were fluorescently stained for lipid as in (D) and analysed by counting foam cells with a confocal microscope as before⁶. *p<0.05, ANOVA, metformin and heme suppress OxLDL-induced foam cell formation prevented by AMPK-knockdown by combined-siRNA. Note, heme had no significant effect on foam cell formation in the absence of OxLDL feeding, a control that is already published and omitted in the interests of space and duplication⁶. Data are mean $\pm$ SE, n=9 donors. *<p<0.05, Student's t-test.

(F) Metformin increases cholesterol export to HDL via AMPK. Human blood-derived macrophages were cultured in the presence or absence of 10 μ M metformin, the medium was changed and then cholesterol export to supernatant

containing lipoprotein-free fetal calf serum and $10\mu\text{g.mL}^{-1}$ ApoA1 (both Sigma-Aldrich). Cholesterol in the cells and supernatant were compared using an ultrasensitive enzymatic assay based on cholesterol oxidase and Amplex Red as before ⁶. * $p<0.05$, metformin induces total cell cholesterol export to ApoA1 (forming HDL), prevented AMPK-knockdown by combined-siRNA.

(G) Metformin decreases human blood-derived macrophage cholesterol content via AMPK. Human blood-derived macrophages were cultured in the presence or absence of $10\mu\text{M}$ metformin and $30\mu\text{g.mL}^{-1}$ OxLDL for 1 week, in the presence of AMPK-knockdown by AMPK- $\alpha\beta\gamma$ siRNA or matched control-siRNA as indicated. Then cholesterol was assessed in lysates using an ultrasensitive enzymatic assay based on cholesterol oxidase and Amplex Red as before ⁴. Metformin reduces total cell cholesterol accumulation, prevented by AMPK-knockdown by combined-siRNA. Data are mean $\pm$ SE. * $p<0.05$, Student's t-test.

Figure 6 Heme and metformin induce p-ATF1, HO-1 and LXR- β less in AMPK-KO than in WT mice

(A, B) are representative western blots of bone marrow macrophages (BMMs) from WT or AMPK-KO mice as indicated. BMMs were treated with heme (10 μ M) (A) or metformin (100 μ M) (B) over the indicated time course. Bands are at appropriate Mr (as stated). Representative of n = 6 WT and n = 6 KO mice. (C, D) generation of hROS (OCl, OH, HONOO) by BMMs from WT or AMPK-KO mice as indicated, measured with APF dye, expressed in arbitrary fluorescence units (Methods). Y-axes, APF-related fluorescence, X-axis, gene status. (C) hROS in BMM treated in presence or absence of heme (10 μ M); *p<0.05, Student's t-test, Data are mean $\pm$ SE, n=5 mice. (D) hROS in BMM from WT or AMPK-KO mice, treated in presence or absence of metformin (10 μ M); *p<0.05, Student's t-test, Data are mean $\pm$ SE, n=5 mice. (E) Y-axis, cell survival measured with XTT dye, expressed in arbitrary fluorescence units. X-axis, genotype. Treatment with heme or metformin are as indicated on Figure. Data are mean $\pm$ SE, n=5 mice. *p<0.05, Student's t-test. (F) Resistance to foam cell formation (lipid accumulation) in macrophages from WT or AMPK-KO mice. Y-axis, foam cell formation measured as before. X-axis, treatments of cultures as indicated. Open and filled bars, knockout status as indicated. *p<0.05, Student's t-test, Data are mean SE, n=5 mice.

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Significance

Several groups, including Virmani's, have identified a novel macrophage subset that responds to intraplaque hemorrhage and has properties that may limit its atherogenic effects. We have termed these Mhem and previously implicated the transcription factor ATF1. This work shows that ATF1-mediated activation is initiated by AMPK in both human and murine macrophages responding to heme. This leads to coordinated induction of HO-1 and LXR, which link antioxidant protection with protection from lipid accumulation. The drug metformin initiates a closely similar pathway, which may explain its increased effect on preventing diabetic macrovascular disease. In vivo and human translational studies are warranted to follow these findings up.

Figure 1

A

B

Figure 2

Figure 3

A

C

B

D

Figure 4

Figure 5

Figure 6