High levels of circulating epinephrine trigger apical cardiodepression in a β_2-adrenoceptor/Gi-dependent manner: a new model of Takotsubo Cardiomyopathy

Short title: Paur: A physiological mechanism of Takotsubo Cardiomyopathy

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Abstract

Background- Takotsubo cardiomyopathy is an acute heart failure syndrome characterized by myocardial hypocontractility from the mid left ventricle to apex. It is precipitated by extreme stress and can be triggered by intravenous catecholamine administration, particularly epinephrine. Despite its grave presentation, Takotsubo cardiomyopathy is rapidly reversible with generally good prognosis. We hypothesised that this represents switching of epinephrine signalling through the pleiotropic β_2-adrenoceptor (β_2AR) from canonical Gs-activated cardiostimulant to Gi-activated cardiodepressant pathways.

Methods and Results- We describe an *in vivo* rat model in which a high intravenous epinephrine, but not norepinephrine, bolus produces the characteristic reversible apical depression of myocardial contraction coupled with basal hypercontractility. The effect is prevented via Gi inactivation by pertussis toxin pretreatment. β_2AR number and functional responses were greater in isolated apical cardiomyocytes compared to basal cardiomyocytes, confirming higher apical sensitivity and response to circulating epinephrine. *In vitro* studies demonstrated high dose epinephrine can induce direct cardiomyocyte cardiodepression and cardioprotection in a β_2AR-Gi dependent manner. Preventing epinephrine-Gi effects increased mortality in the Takotsubo model, while β-blockers which activate β_2AR-Gi exacerbated the epinephrine-dependent negative inotropic effects without further deaths. In contrast levosimendan rescued the acute cardiac dysfunction without increased mortality.

Conclusions- We suggest that biased agonism of epinephrine for β_2AR-Gs at low and Gi at high concentrations underpins the acute apical cardiodepression observed in Takotsubo cardiomyopathy, with an apical-basal gradient in β_2ARs explaining the differential regional

responses. We suggest this epinephrine-specific β_2AR-Gi signalling may have evolved as a cardioprotective strategy to limit catecholamine-induced myocardial toxicity during acute stress.

Introduction

There has been a rapid increase in the recognition of a syndrome of acute and severe, but reversible, heart failure called Takotsubo or Stress cardiomyopathy,[1-3] also known as 'Broken Heart Syndrome', which usually follows within hours of an identifiable emotional, psychological or physical stress. Takotsubo cardiomyopathy mimics symptoms of acute myocardial infarction (MI), but is distinguished by the lack of coronary occlusion and by characteristic regional wall motion abnormalities, classically a virtual apical ballooning appearance due to a hypercontractile base of the heart relative to hypo- or akinetic apical and mid left ventricular myocardium, the latter extending beyond a single coronary artery territory.[1, 2] Initial recognition in earthquake survivors in Japan, plus the characteristic ventricular shape, led to the 'Takotsubo' (meaning octopus-pot) label.[3, 4] It has become apparent that ~1-2% of all presentations with suspected acute coronary syndrome cases are finally diagnosed as Takotsubo cardiomyopathy.[3]

The pathophysiological mechanisms for this increasingly recognised syndrome are not known. Evidence points to epinephrine as the precipitating factor. Physical or psychological stress is a frequent precipitant, and serum catecholamine levels in Takotsubo patients 1-2 days after presentation are higher than those in patients with myocardial infarction with pulmonary oedema: epinephrine falls back to MI levels only after 7-9 days.[1] Catecholamine storms, more associated with epinephrine-secreting phaeochromocytomas than norepinephrine- and dopamine-secreting phaeochromocytomas,[5] can also precipitate Takotsubo cardiomyopathy.[6] Particularly, the reproduction of the signs of Takotsubo by accidental administration of epinephrine (including single intramuscular 1mg doses from an 'Epi-pen') is most indicative of its central role.[7] Although there is a significant mortality in

the early period (1-1.5%) there is also a characteristic rapid (days to weeks) recovery of patients surviving the acute period of profound depression in left ventricular contractile function,[1] with excellent prognosis and absent, or minimal, residual cardiac impairment. This striking difference from the normal prognosis of heart failure has led us to propose previously that the cardiodepression has elements derived from a beneficial physiological protective adaptation.[8] Thus, the syndrome has interest for the cardiologist over and above the design of optimal treatment for the individual Takotsubo patient.

We have previously proposed a mechanism based upon two overarching principles for which there is prior evidence. Firstly the mammalian left ventricle contains apical-basal gradients of βARs and sympathetic innervation, with the apex characterised by highest βAR and lowest sympathetic nerve density.[8] Rat, feline, rabbit and dog left ventricles show increased apical responses to global high dose isoproterenol challenges,[9-12] with increased apical versus basal βAR levels measured directly in the dog ventricle.[10] This pattern results in increased apical responsiveness to circulating catecholamines, predominantly epinephrine from the adrenal glands, as a compensatory mechanism for the sparse apical sympathetic innervation, to ensure optimal ventricular ejection during times of stress. Conversely the sympathetic innervation is highest in the basal myocardium, and lowest in the apex, and therefore cannot explain the localised apical dysfunction. This is also true of human left ventricle,[13] whereas presence of a ventricular cardiomyocyte βAR gradient in the human heart remains to be determined.

Secondly epinephrine, at high levels, can act as a negative inotrope via ligand mediated trafficking of the β_2AR from Gs to Gi subcellular signalling pathways. The β_2AR is widely

reported as pleiotropic, having the potential to couple through Gs-AC-cAMP (like the β_1AR) but also though Gi_α, $G_{\beta\gamma}$ and non-G-protein pathways.[14, 15] β_2AR-Gi-mediated depression of contraction was initially demonstrated using transgenic mice over expressing the β_2AR (TGβ_2).[16, 17] At high epinephrine concentrations, the β_2AR switches its coupling from Gs protein to an inhibitory Gi protein,[16] a process described as ligand or stimulus directed-trafficking or biased agonism. This switch would be favoured in conditions of high catecholamine stress, since it depends upon β_2AR phosphorylation by both protein kinase A (PKA)[18] and G-protein receptor-coupled kinases (GRKs).[19] This is particularly relevant given the increased frequency of the L41Q GRK5 polymorphism, known to increase cardiac GRK5 activity and βAR phosphorylation, in recent study genotyping Takotsubo cardiomyopathy patients.[20] The negative inotropic effect through Gi [21, 22] has contributions both from inhibition of Gs-cAMP production and through other pathways such as p38 mitogen-activated protein kinase (MAPK) alteration of myofilament sensitivity.[23] No such role for β_1ARs in this Gs-Gi trafficking switch has been documented, and the phenomenon is epinephrine specific. Norepinephrine has 20-fold lower affinity for the β_2AR compared to the β_1AR, and much weaker trafficking of β_2AR stimulus trafficking to the Gi pathway.[16] While this negative inotropy is detrimental from a mechanical perspective, the Gs to Gi switch is potentially both antiapoptotic and antiarrhythmic,[24, 25] and may represent a cardioprotective mechanism against β_1AR-catecholamine cardiotoxicity. Both p38 MAPK and PI3K/Akt pathways have been implicated in β_2AR-Gi mediated antiapoptotic effects in the adult cardiac myocytes,[26, 27] and evidence for increased PI3K and Akt activation has been reported in myocardial biopsies from Takotsubo cardiomyopathy patients during the acute phase.[28] Interestingly, direct negative inotropic effects of some β-blockers in human

ventricular cardiomyocytes have been shown to depend on β_2AR-Gi signalling,[29] an observation which may have implications for their use in Takotsubo Cardiomyopathy.

In this study we have developed an epinephrine-induced *in vivo* model of Takotsubo cardiomyopathy which reproduces both the apically located negative inotropism and the reversible nature of this cardiodepression. We have used this to explore the role of β_2AR apical-basal gradients; the involvement of Gi signalling and the cardioprotective nature of this condition. It has been supplemented by an *in vitro* model of acute epinephrine exposure to explore underlying cellular mechanisms. Potential pharmacological agents have been assessed in terms of treatment of the established Takotsubo cardiomyopathy, with the intention to mitigate the cardiodepression without disrupting any cardioprotective elements of the syndrome.

Methods

All studies complied with the United Kingdom Home Office Regulation Governing the Care and use of Laboratory Animals and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

In vivo Takotsubo cardiomyopathy model. Adult male Sprague-Dawley rats (250-350g) were anaesthetised and injected with $4.28x10^{-8}$ moles.$100g^{-1}$ epinephrine or $1.43x10^{-7}$moles.$100g^{-1}$ norepinephrine via the right jugular vein as a bolus injection. Regional left ventricular responses were recorded using 2D echocardiography (Visualsonics Vevo 770) in the parasternal long axis. Baseline scans were performed before catecholamine administration. Preventative studies: a subgroup of animals were pretreated with the Gi protein inhibitor pertussis toxin (PTX) (25µg.Kg-1), the p38MAP kinase antagonist SB203580 (0.1-10mg.Kg^{-1}) or the β_2AR selective antagonist ICI 118,551 (1mg.Kg^{-1}) followed by intravenous epinephrine bolus. A separate cohort of cases had continuous aortic blood pressure recording during the protocol using a 1.9F pressure-volume catheter (Scisense Inc, Ontario, Canada). Rescue strategies: a subgroup of animals were treated with intravenous propranolol ($1.43x10^{-11}$ moles.$100g^{-1}$), carvedilol ($1.43x10^{-11}$ moles.$100g^{-1}$), or levosimendan infusion (4.7µg/kg/min) fifteen minutes post epinephrine injection.

Rat cardiomyocyte isolation and β_2AR overexpression studies. Myocytes were isolated from adult male Sprague-Dawley rats (Harlan, Bicester,UK; weight 250-350 grams) using the standard enzymatic technique as described previously.[30] Isolated rat cardiomyocytes were

plated in culture medium at a field density of 10,000 cells/well and infected with either Ad.β_2AR.GFP, β_2AR with mutations at the PKA phosphorylation sites 261, 262, 345, 346 S/A (β_2AR-PKA-KO) (Ad.β_2AR-PKA-KO) or Ad.GFP (control) at a multiplicity of infection (MOI) of 500 for 48 hours. For pertussis toxin (PTX) treatment, Ad.β_2AR.GFP infected rat ventricular myocytes were cultured in the presence or absence of PTX (1.5 μg/ml) for 48 hours. Survival in culture was shown as a percentage of rod-shaped myocytes at the time of plating: >100 cells per well were counted, with triplicates for each condition. β_2AR-specific contractile responses were measured on separately isolated apical and basal ventricular cardiomyocytes using isoproterenol (100nM) plus the β_1AR selective antagonist CGP20712A (300nM) (see supplementary methods).[21, 29]

In vitro Takotsubo cardiomyopathy model. Freshly isolated rat ventricular cardiomyocytes were perfused with epinephrine (1μM) for 20mins followed by washout (10min). A subgroup of cells was preincubated with PTX for 3h at 35°C (1.5 $\mu g.ml^{-1}$).

βAR radioligand binding assay. Cell membranes, prepared from apical and basal-derived adult rat cardiomyocytes, were incubated for 2 hours at RT in assay buffer (50mM Tris, 5mM $MgCl_2$) (pH 7.4), with 0.1-10nM of the non-selective β-AR radioligand, [^{125}I]-cyanopindolol ([^{125}I]-CYP) (Amersham, Freiburg, Germany), and increasing concentrations of the selective β_2AR antagonist, ICI-118,551 ($1x10^{-11}$M to $1x10^{-2}$M). Non-specific binding was determined in the presence of 10μM of the non-selective β-AR antagonist, propranolol.

FRET-mediated cAMP assay. FRET studies in EPAC-cAMPS expressing apical and basal ventricular cardiomyocytes were performed as previously described.[31] Whole cell epinephrine-stimulated β_2AR-mediated cAMP transients were recorded. A subgroup of cells was preincubated with PTX for 3h at 35°C (1.5 $\mu g.ml^{-1}$).

Human tissue samples and cardiomyocyte isolation. Left or right ventricular tissues were obtained from failing human hearts at the time of heart transplantation; procedures for collecting human heart tissues conformed to the Ethics Committee requirements of the Royal Brompton and Harefield Hospital, UK. Written informed consent was provided by all patients. The investigation conforms to the principles outlined in the Declaration of Helsinki. Single human ventricular myocytes were isolated from explanted failing human hearts using a standard enzymatic technique as described before.[32]

Cardiomyocyte contractility studies – see online supplementary methods

Results

High dose epinephrine injection recapitulates Takotsubo Cardiomyopathy. High serum epinephrine levels are a common feature in Takotsubo Cardiomyopathy patients suggesting a mechanistic link. We developed a model of Takotsubo Cardiomyopathy in which an anaesthetised rat receives an intravenous (jugular) bolus of $4.3x10^{-8}$moles.$100g^{-1}$ epinephrine (equivalent to ~5mg in an adult human). Intravenous bolus delivery was selected to mimic the human physiological response to sudden high stress. Initial dose-response curves determined the highest catecholamine dose without excessive mortality (Figure S1). Epinephrine bolus triggered a rapid hypertensive response with reflex bradycardia within seconds of administration, which stabilised back to normotension after several minutes (Figure S2), and was associated with an initial global increase in left ventricular contractility (Figure 1). However, this dropped away to give a marked decrease in cardiac contraction, initiating at 15 min and reaching a nadir between 20 and 25 min. Contraction normalised within an hour. One defining characteristic of Takotsubo Cardiomyopathy is the apical and mid-ventricular localisation of dysfunction, and this was clearly reproduced in our model (Figure 1), and was confirmed by cardiac MRI (Figure S3 and Supplementary movie).

Apical hypokinesia is epinephrine-specific. We and others have previously reported that epinephrine or isoproterenol at high concentrations can switch the β_2AR from positively inotropic Gs to negatively inotropic Gi coupling,[17, 22, 33] while norepinephrine cannot.[16] We found that equivalent high dose intravenous norepinephrine did not generate the negative effect observed after epinephrine bolus (Figure 1A-C), and concentration-response curves (Figure S1) confirmed that no concentration of norepinephrine was negatively inotropic.

Changes in heart rate and systemic arterial blood pressure did not differ between epinephrine and norepinephrine, indicating appropriate matching of effective concentrations (Figure S2). Lack of negative effect of norepinephrine additionally eliminates either myocardial β_1AR, or α_1AR-mediated vasoconstriction as the principal mediator of the epinephrine-stimulated negative inotropic effect.

Epinephrine-induced apical hypokinesia is Gi dependent. We used pertussis toxin (PTX) to inhibit Gi by *in vivo* pre-treatment of the rats three days before the intravenous epinephrine challenge. *In vitro* challenge of isolated cardiomyocytes from these hearts with carbachol (after βAR stimulation) was used to verify inhibition of Gi effects (not shown). The negative effect of epinephrine was completely abolished by PTX (Figure 2A-C), providing strong evidence for a Gi-dependent mechanism of action. Apical and mid-LV switched completely to give an increase in contraction, and even basal hypercontractility was significantly enhanced. PTX pretreatment did not alter baseline function, the systemic arterial pressure response to epinephrine (Figure S3), or time-matched responses following control saline bolus (Figure S4-5). PTX pretreatment reduced the vagally-mediated reflex bradycardia during the first minutes after epinephrine injection (Figure S2E). However systemic vagal blockade with atropine pretreatment failed to prevent epinephrine-induced hypokinesia as observed with PTX pretreatment, and significantly increased mortality from cardiogenic shock (Figure S6). This excluded systemic vagal inhibition as the explanation for the PTX-mediated prevention of apical hypokinesia.

We also developed an *in vitro* model, in which isolated rat ventricular cardiomyocytes were treated for 20 min with epinephrine. These cells showed a decreased positive inotropic response to a subsequent β_2AR challenge (Figures 3A and S7). Maximum responses to high

calcium were unchanged, indicating that overall cellular and contractile function was not compromised (Figure S8). The depression of β_2AR response after epinephrine pretreatment observed in this *in vitro* model was completely prevented (and the response became higher than control) after PTX treatment (Figures 3A and S7). Notably, measurement of cAMP under the same conditions showed much less marked changes: PTX treatment increased contraction 11-fold without a significant increase in cAMP levels (Figure 3B). This implies a parallel negative inotropic pathway activated through Gi. Since p38 MAPK has been shown to be both Gi-dependent and negatively inotropic in rat ventricular myocytes we compared treatment with PTX and a p38 MAPK inhibitor (Figure 3C). Both were able to increase β_2AR responses to a similar degree, and the effects of the two were not additive.

Apical ventricular cardiomyocytes have higher β_2AR density and β_2AR-mediated contractile responses compared to basal cardiomyocytes. We have hypothesized that the increased apical sensitivity observed in Takotsubo cardiomyopathy patients and our model is due to a greater proportion of β_2ARs relative to β_1ARs in the apex,[8] since the greater concentration of sympathetic innervation in the base of the heart[34] is counterbalanced by increased apical βAR functional responses to circulating catecholamines.[9-12] Using a radioligand binding-displacement assay to directly quantify the β_2:β_1AR ratio, we found that apical cardiomyocytes demonstrated an increased β_2:β_1AR ratio (Figure 3D). The functional consequences of a higher β_2:β_1AR ratio was studied and confirmed greater β_2AR-specific contractile responses in apical ventricular cardiomyocytes compared to paired basal cardiomyocytes isolated from the same heart (Figure 3E). β_2AR-dependent and maximal cAMP responses demonstrated no difference between apical and basal cardiomyocytes (Figure S9), and therefore could not explain the observed gradient and contractile response.

Epinephrine-induced β_2AR-Gi signalling is cardioprotective. Since β_2AR-Gi is widely reported to be antiapoptotic and cardioprotective,[35-37] we hypothesized that blocking β_2AR-Gi signalling might increase the cardiotoxic effects of high epinephrine levels via uninhibited β_1AR-Gs and β_2AR-Gs signalling. In the rat Takotsubo model *in vivo*, epinephrine-induced mortality was significantly increased by prior selective β_2AR-blockade with ICI 118,551 (at concentrations insufficient to activate Gi) or p38MAPK inhibition with SB203580 (Figure 4A). Death often occurred within 5-10 min, and was due to cardiogenic shock and hypokinesia rather than primary ventricular fibrillation. *In vitro*, isoproterenol increased cell death in cultured myocytes, an effect largely inhibited by β_1AR blockade (Figure 4B) while overexpression of the β_2AR (Figure 4B) or Gi (Figure 4C) protected against catecholamine-induced cell death. β_2AR switching from Gs to Gi coupling is thought to be enhanced after strong βAR-Gs activation, mediated by cAMP-dependent protein kinase (PKA).[18] Overexpression of a β_2AR construct in which PKA sites had been mutated to prevent phosphorylation, not only failed to protect but produced β_1AR-independent cell death (Figure 4B). This mutant was also unable to support β_2AR-dependent negative inotropism, in contrast to wild-type β_2AR (Figure S10).

β-blockers which activate β_2AR-Gi do not rescue, and may worsen, established apical hypokinesia. In the previous section we note that pretreatment with a specific β_2AR blocker before the epinephrine bolus did not appear to be a therapeutically useful manoeuvre. We also predicted that clinically used β-blockers which activate β_2AR-Gi might exacerbate the epinephrine-induced negative inotropic effect. The Gi-dependent negative effect of β-

blockers is most readily seen in myocytes from failing human hearts (where Gi is increased [29]): we selected compounds that had either strong (propranolol) or modest (carvedilol) effects on these cells (Figure 5A). Figures 5B-C show the effect of the two blockers added 15 min after epinephrine in the *in vivo* model, when peak negative responses are developing. Propranolol, with higher β_2AR-Gi agonism, significantly enhanced and prolonged the negative effects of epinephrine at both apex and base (Figure 5B), while carvedilol, with less pronounced β_2AR-Gi agonism, had little effect on apex but converted the base from positive to significant negative responses (Figure 5C). In contrast, the β_1AR-selective blocker bisoprolol reduced the positive effect of epinephrine at the base but did not convert it to significant negative response: there was no effect on the apical epinephrine response (Figure S11). These data support our hypothesis of synergistic effects of epinephrine with propranolol (and to a minor extent carvedilol) upon β_2AR-Gi signalling. While the negative inotropic of epinephrine was enhanced, there was no increase in mortality with the addition of propranolol or carvedilol (Figure 4A).

Levosimendan reverses epinephrine-induced apical dysfunction without increased mortality. Levosimendan was selected for comparison as it is an inotrope with a cAMP-independent mechanism of action, increasing myofilament calcium sensitivity.[38] Global cardiac contraction in untreated hearts was increased with infusion of this compound (not shown). In contrast to other agents, application of levosimendan at the point where epinephrine negative effects were beginning, was effective in preventing further decline in cardiac function (Figure 6). This contractile benefit and rescue occurred with no deaths in the epinephrine-treated group (Figure 4A).

Discussion

Takotsubo cardiomyopathy is an increasingly recognised acute cardiac syndrome in the modern era of early access to diagnostic coronary angiography.[1-3] As a cardiac response to extreme stress levels it carries a relatively good prognosis, but has the intriguing feature of regional (apical) hypokinesia, which is counterintuitive in relation to the systemic nature of the trigger and the evolutionary drive for increased cardiac output during 'flight or fight' responses. We have developed a rat model mimicking the clinical features with acute, reversible apical and mid-ventricular myocardial hypokinesia, but preserved or enhanced basal contractility (Figure 1). Rapid high dose intravenous epinephrine bolus, designed to mimic the serum catecholamine response to acute stress compared with the traditional infusion protocols, recapitulated the classical clinical findings, whereas the equivalent norepinephrine bolus did not (Figure 1). This implied the mechanism is epinephrine-specific, and confirms the observation that dysfunction is not typically observed in the region with the highest density of norepinephrine-releasing sympathetic nerve terminals.[13]

We have further investigated this concept of apical-basal gradients of catecholamine responsiveness to βAR subtype, and demonstrate that apical ventricular cardiomyocytes have a higher β_2AR density and a greater β_2AR-induced sensitivity compared with basal cardiomyocytes isolated from the same heart (Figure 3D-E). The inability of norepinephrine at equivalent (and higher) doses to initiate acute apical dysfunction excludes coronary vasospasm or β_1AR-mediated signalling as a primary effector (Figure 1). This agrees with clinical observations that the apical dysfunction in Takotsubo cardiomyopathy extends beyond the territory of a single coronary bed.[1-3, 8] Supporting the predominance of a cardiomyocyte-based explanation rather than a vascular one is also the ability of our *in vitro*

cardiomyocyte model used here to reproduce a number of the key *in vivo* observations (Figure 3), as well as the matched responses of heart rate and blood pressure between epinephrine and norepinephrine cohorts.

Norepinephrine also differs in that it does not couple β_1ARs or β_2ARs to Gi signalling, while epinephrine at high concentrations produces a β_2AR-Gs to Gi switch. β_2AR-Gi coupling has been reported in a number of experimental models including β_2AR and Gi overexpression, and importantly in chronic heart failure, where Gi levels are increased.[29] β_2AR-Gi coupling occurs via a process termed stimulus/ligand-directed trafficking or biased agonism. Other agonists such as high dose isoproterenol also produce this switch, and we note a study in which isoproterenol infusion over 2 weeks also produced a specific apical contraction defect.[9] The key role of Gi in the cardiodepression was shown by the ability of PTX to convert apical responses to epinephrine from negative to positive (Figure 2). It should be noted that the response of basal myocardium was also increased, implying that β_2AR-Gi was operational even in this region despite the β_1AR predominance. Non-classical examples of Takotsubo Cardiomyopathy have been observed where base or mid-LV is affected,[39] and this may reflect individual patterns of β_2AR expression. The *in vivo* observations were supported by those in isolated cells. In untreated apical myocytes, positive inotropic responses to β_2AR stimulation were enhanced by PTX (Figures 3C and S7, and as previously reported[40]). In myocytes pretreated with epinephrine, PTX was able to rescue and further enhance the depressed β_2AR-mediated positive responses (Figure 3A and S7). cAMP responses were decreased modestly in pretreated myocytes (Figure 3B), though less affected than contraction. However, PTX was able to rescue contractile responses with no significant effect on cAMP (Figure 3B), implying the existence of a separate negatively inotropic Gi-

dependent pathway. Inhibition of p38 MAPK produced similar and non-additive effects to PTX, consistent with the suggested role for this pathway as a Gi-dependent negatively inotropic modulator.

The epinephrine-dependent β_2AR-Gi mediated negative inotropism requires a preceding high β_1AR-Gs activation to initiate cAMP-dependent PKA- and GRK-phosphorylation of the β_2AR.[18, 41] This implies that, while norepinephrine does not directly couple receptors to Gi, the rise in cAMP it produces will predispose the β_2AR to traffic to Gi upon subsequent epinephrine binding. Here we demonstrate that PKA-mediated β_2AR phosphorylation is critical for Gi coupling as deleting the phosphorylation sites prevented both negative inotropism and cardioprotection attributable to β_2AR-Gi coupling (Figures 4B and S10). This also explains the reversibility of the Takotsubo cardiomyopathy syndrome. As the serum epinephrine levels fall, β_2AR dephosphorylation, or internalisation and replacement with de novo unphosphorylated β_2ARs, would reduced the β_2AR-Gi stimulus trafficking and restore normal contractile function in the surviving cardiomyocytes. Studies in model cell systems overexpressing fluorescently labelled β_2AR demonstrate the dependence of both PKA- and GRK-mediated β_2AR phosphorylation for β_2AR internalisation from the surface membrane and recycling to different surface microdomains.[19] Interestingly they also demonstrate the epinephrine-specific dependence of this trafficking.[41] This is relevant to the Takotsubo cardiomyopathy patients as to date there has been failure to identify any associated polymorphisms in the α_1ARs, β_1AR or β_2ARs,[42] but one study, albeit with small patient numbers, found an increased prevalence of the GRK polymorphism L41Q in the Takotsubo cardiomyopathy patient cohort compared to healthy matched controls.[20] This is a 'gain-of-

function' mutation, previously referred to as genetic betablockade,[43] confers reduced responsiveness to βAR agonists, and improved prognosis in the population carrying this polymorphism,[43] both conceivably consistent with enhanced myocardial β_2AR-Gi coupling.

Although the final outcome for the Takotsubo patient is generally good, they have been through an acute cardiac event requiring hospitalisation, and there is a significant incidence of cardiogenic shock (~4%), malignant ventricular arrhythmias (1-2%) and death (1-1.5%). It therefore seemed reasonable to try to block the depression of contraction with either a specific β_2AR antagonist or a p38 MAPK inhibitor. However the marked increase in mortality produced by this manoeuvre gave a clear indication that this was a counterproductive strategy (Figure 4A). The rapidity of the death, usually within 5-10 and always within 45 min, made it unlikely that apoptosis was the underlying mechanism. The β_2AR and/or Gi have been implicated in suppression of arrhythmias,[44] and β_2AR variants with sudden cardiac death.[45] β_2AR knockout mice went into cardiogenic shock following doxorubicin, through a β_1AR-related mechanism[46] and β_2AR/Gi mechanisms have also been implicated in post-ischemic stunning.[47] All these are potential mechanisms which could underlie the acute mortality. We suggest that the enhanced β_2AR-Gi coupling initiated by high epinephrine levels is protective to dampen the effects of toxic βAR-Gs coupling, which is left unchecked would be fatal.

Few β-blockers are pure neutral antagonists, with most having some other effect such as partial agonism (intrinsic sympathomimetic activity), inverse agonism (reduction in activity

of constitutively active receptors) or biased agonism (ligand directed trafficking to other pathways). It has been amply demonstrated that blockers of the β_2AR can activate other signalling pathways, both G-protein and non-G-protein-dependent.[48] We were first alerted to the possibility that the cardiodepressant effects in Takotsubo Cardiomyopathy were β_2AR-Gi dependent by their similarity to the β_2AR-Gi-mediated effect of β-blockers on myocytes from failing human heart.[29] We therefore hypothesised that β-blockers with strong β_2AR-Gi agonism would synergise with the negative inotropic effect of epinephrine. Propranolol, a particularly cardiodepressant agent, markedly enhanced and prolonged the negative phase when given after the epinephrine bolus in our *in vivo* model (Figure 5B). In support of the hypothesis of additive negative inotropic effects of propranolol and epinephrine, we note a recent report that an acute dilated cardiomyopathy was precipitated in a patient with phaeochromocytoma upon taking propranolol for migraine.[49] Carvedilol had a more modest effect, reversing the basal hypercontractility whilst having a neutral effect upon the apical hypokinesia (Figure 5C). Carvedilol (and propranolol) are also able to produce biased agonism through Gβγ mechanisms,[50] which would be PTX sensitive. Although possibly exacerbating the syndrome, carvedilol could be useful in treatment in the minority of Takotsubo cardiomyopathy patients with severe left ventricular outflow tract obstruction secondary to the basal hypercontractility. It should be noted that neither blocker had a deleterious effect on mortality in the Takotsubo Cardiomyopathy model. Bisoprolol, which is predominantly β_1AR selective, did not reproduce these effects to synergise with epinephrine.

We considered the implications for treating Takotsubo cardiomyopathy, and for heart failure therapy more generally. For the Takotsubo patient, strategies to raise cAMP (catecholaminergic inotropes or phosphodiesterase inhibitors) would clearly be contraindicated. Indeed dobutamine administered for stress echocardiography testing has precipitated Takotsubo Cardiomyopathy.[7] A cAMP-independent inotrope, levosimendan, was effective in reversing the negative inotropic effect of epinephrine and rescue occurred without increased (and a trend to decreased) mortality (Figures 4A and 6). We suggest that this is likely to be a safe supporting and bridging strategy for the sickest patients with cardiogenic shock until spontaneous recovery occurs, and preliminary clinical reports support this view.[51, 52] It should be noted that at the higher doses levosimendan can inhibit phosphodiesterases,[38] and increase cAMP, and thus we only would recommend the lower (non-vasodilatory) doses. The value of non-selective β-blockers, which may also act as agonists at β_2AR-Gi, is more difficult to predict, since they may amplify both the negative inotropic and the protective effects of epinephrine. It could further be suggested that the beneficial effects of β-blockers in heart failure has taken serendipitous advantage of cardioprotective β_2AR-Gi biased agonism. If those two effects could be modulated separately, this might point the way for an improved design of future β-blockers by selecting for cardioprotection through β_2AR-Gi biased agonism.

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Disclosures

None

Reference List

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Figure 1. Takotsubo cardiomyopathy is epinephrine-specific. Effects of $4.28x10^{-8}$ moles.$100g^{-1}$ epinephrine (dark red bars) and $1.43x10^{-7}$moles.$100g^{-1}$ norepinephrine (dark blue bars) on apical (**A**), mid left-ventricular (**B**) and basal myocardium contractility (**C**). Values are expressed as the mean percentage change in LV fractional shortening (FS) from baseline (untreated) levels ± SEM at each 5 minute time point following injection. N=6 (epinephrine), n=6 (norepinephrine) (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs baseline FS = 0). Abbreviation: B (baseline). RM| ANOVA (epinephrine vs norepinephrine): p<0.0001 (apex), p<0.01 (MLV), p<0.01 (base).

Figure 2. *In vivo* Takotsubo Cardiomyopathy model and prevention by Pertussis toxin pretreatment. Contractile responses after an intravenous bolus injection of epinephrine ($4.28x10^{-8}$ moles.$100g^{-1}$ - dark red bars) on left ventricular apical (**A**), mid left-ventricular (**B**) and basal myocardium (**C**). Values are expressed as the mean percentage change in LV fractional shortening (%ΔFS) from baseline (untreated) levels ± SEM at each 5 minute time point following injection. Light blue bars show time-matched inotropic responses of the apical, mid-left ventricular and basal myocardium in PTX ($25\mu g.Kg^{-1}$) pre-treated animals after equivalent i.v. epinephrine bolus, with loss of apical and MLV hypokinesis. N=6 (control

epinephrine), n=5 (epinephrine+PTX) (*p<0.05, **p<0.01, ***p<0.001 vs baseline FS = 0). Abbreviations: B (baseline). RM ANOVA (epinephrine vs epinephrine+PTX): p<0.001 (apex), p<0.01 (MLV), p<0.05 (base).

Figure 3. *In vitro* Takotsubo cardiomyopathy model induced by high dose epinephrine exposure. Effect of 20 min pretreatment with epinephrine (Epi-pre, 1μM), followed by 10 min wash, on subsequent β_2AR contractile (**A**) and cAMP (**B**) responses with Gi (PTX-sensitive) component. **A**, Contraction amplitude in isolated rat ventricular myocytes: peak fold increase over basal: Control (n=15); Epi-pretreated alone (n=15); Epi-pretreated +PTX (n=7). **B**, Whole cell cyclic AMP levels, measured using an EPAC2-FRET sensor. Control (n=40); Epi-pretreated alone (n=10); Epi-pretreated +PTX (n=9). *P<0.05, **P<0.01, ***P<0.001. **C**, β_2AR contractility response to 100nM isoproterenol in the presence of the β_1AR blocker CGP20712A (300nM), peak fold increase over basal in control (n=13) or PTX-treated rat ventricular myocytes (n=13), in the presence and absence of SB20380 2.5μM. **D** and **E**, Apically-derived cardiomyocytes demonstrate increased β_2AR levels and responses. **D**, Proportion of β_2ARs with respect to total βAR radioligand binding in ventricular myocytes from the apex and base of normal rat heart. N=4 preparations, **P<0.01 vs base. **E**, Apical cardiomyocytes (purple bars) show a larger increase in percentage cell shortening through the β_2AR compared to basal cardiomyocytes (green bars). Fold increase in shortening with 100nM isoproterenol + 300nM CGP20712A. (*p<0.05 apex vs base, paired t-test. Base: n=13 cells; Apex: n=13 cells, n=13 animals).

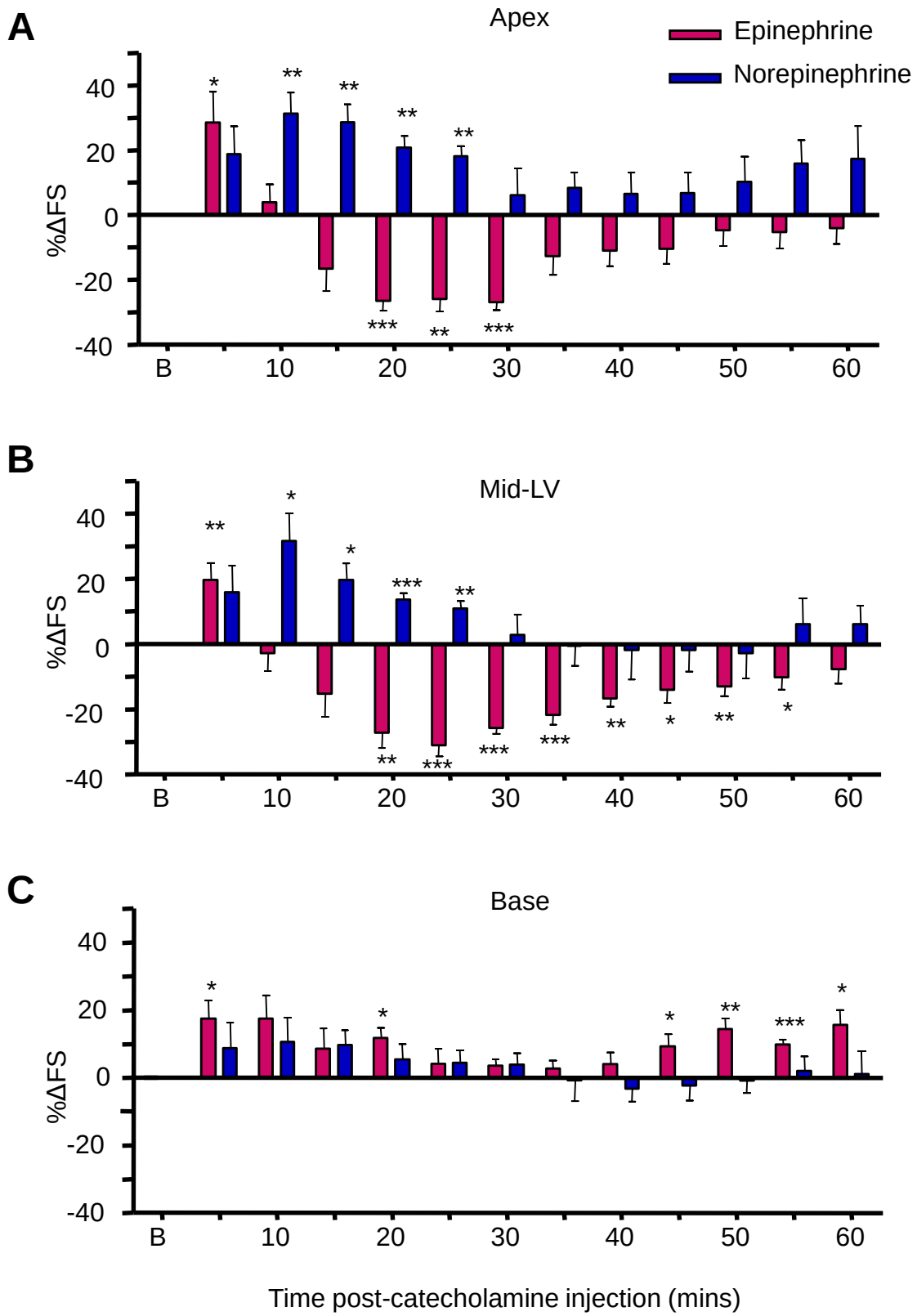
Figure 4. Epinephrine-mediated β_2AR-Gi signalling is cardioprotective. **A**, Mortality with *in vivo* bolus epinephrine ($4.28x10^{-8}$ moles.$100g^{-1}$) in the absence (n=14) or presence of 0.1-10mg.Kg^{-1} SB203580 (n=9), 1mg.Kg^{-1} ICI 118,551 (n=5), $1.43x10^{-11}$ moles.$100g^{-1}$ propranolol (n=9), $1.43x10^{-11}$ moles.$100g^{-1}$ carvedilol (n=12), 4.7 µg/kg/min levosimendan (n=5). *P<0.05 vs epinephrine alone. **B**, Survival of adult rat ventricular myocytes after exposure to 1µM isoproterenol (ISO) in the presence (light blue bars) and absence (red bars) of the β_1AR blocker CGP20712A (300nM), compared to untreated controls (white bars). Myocytes were transduced using adenoviral vectors with GFP (control), the wild-type β_2AR and β_2AR with mutations at the PKA phosphorylation sites 261, 262, 345, 346 S/A (β_2AR-PKA-KO) to prevent switching to Gi. N=6, # P<0.05 vs con/GFP, *p<0.05 vs GFP+ISO. **C**, Effect of Gi expression upon ISO-induced myocyte toxicity over 48hrs in culture. Myocytes were transduced using adenoviral vectors with GFP (control), or Gi-GFP (Gi) at Day 0. N=6 preparations, *P<0.05, **P<0.01 vs respective control, #P<0.05, ##P<0.01 vs ISO alone.

Figure 5. Agonist-independent negative inotropic effect of betablockers and potentiation of Takotsubo cardiomyopathy. **A**, Negative inotropic effect of βAR blockers on contraction of ventricular myocytes from failing human heart. Contraction amplitude relative to basal (open bar) for ICI 118,551 (3µM, n=21), propranolol (Prop, 5µM, n=9) and carvedilol (Carv, 3µM, n=24), *P<0.05, ***P<0.001 vs 100%. **B** and **C**, The β-blockers propranolol (**B**) and carvedilol (**C**) (both $1.43x10^{-11}$ moles.$100g^{-1}$ (i.v.)) either enhance or fail to prevent the negative inotropic effects of epinephrine ($4.28x10^{-8}$ moles.$100g^{-1}$ (i.v.)) at the apex and also reverse the positive effects of epinephrine at the base, in the *in vivo* rat model. Values are expressed as the mean percentage change in LV FS from baseline (untreated) levels ± SEM at each 5 minute time point following intravenous injection. N=6 (epinephrine), n=6

(epinephrine+propranolol), n=7 (epinephrine+carvedilol). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs baseline FS = 0).

Figure 6. Levosimendan rescues the Takotsubo cardiomyopathy model. Effects of 0.28mg/kg/h (4.7 μg/kg/min) levosimendan infusion (i.v.) (black bars) on the inotropic responses of the apical (**A**), mid left-ventricular (**B**) and basal myocardium contractility (**C**) after $4.26x10^{-8}$ moles.$100g^{-1}$ epinephrine (i.v.), compared to epinephrine alone (grey bars). Values are expressed as the mean percentage change in LV FS from baseline ± SEM at each 5 minute time point following injection. N=6 (epinephrine), n=5 (levosimendan + epinephrine) (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs baseline FS = 0). RM ANOVA Epinephrine vs epinephrine+levosimendan: p<0.01 (apex), p<0.01 (MLV), p=ns (base).

Figure 1


A
Apex
Epinephrine
Norepinephrine
%ΔFS
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20
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-20
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B
10
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60
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Mid-LV
%ΔFS
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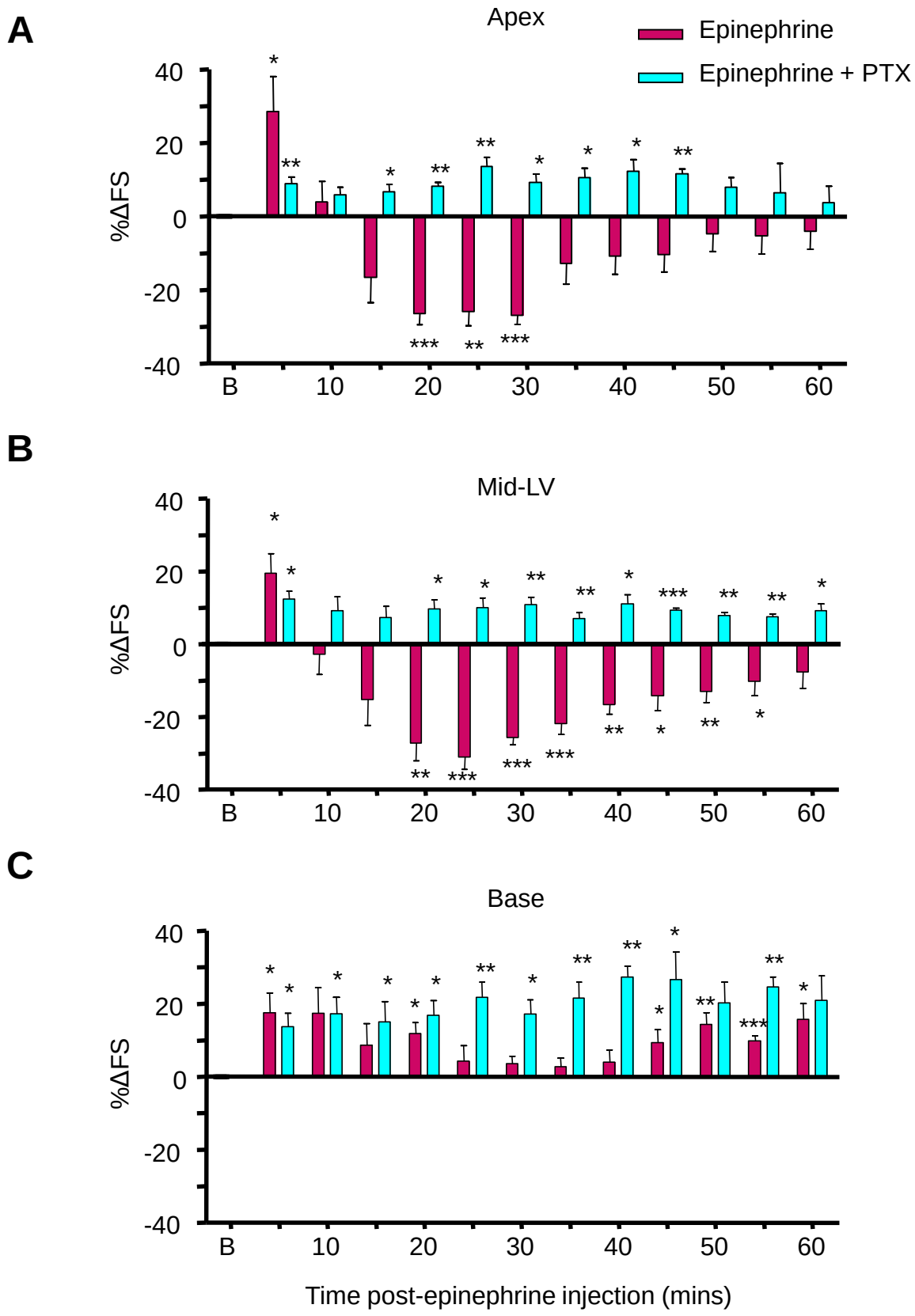
Base
%ΔFS
Time post-catecholamine injection (mins)

1

Figure 2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Epinephrine
Epinephrine + PTX
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40
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-20
-40
B
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30
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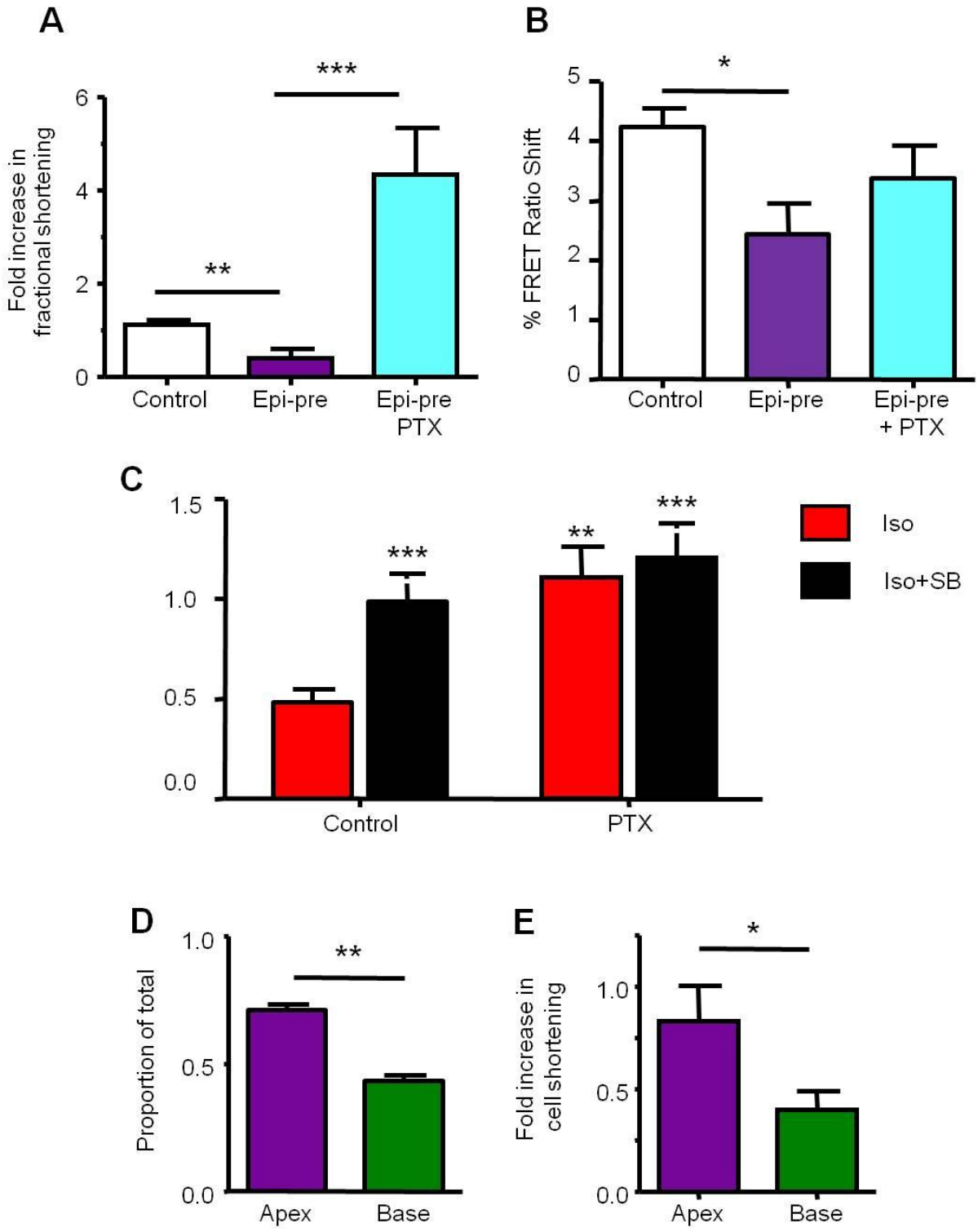
60
B
Mid-LV
%ΔFS
C
Base
%ΔFS
Time post-epinephrine injection (mins)

1

Figure 3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Epi-pre PTX
B
% FRET Ratio Shift
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3
2
1
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*
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1.0
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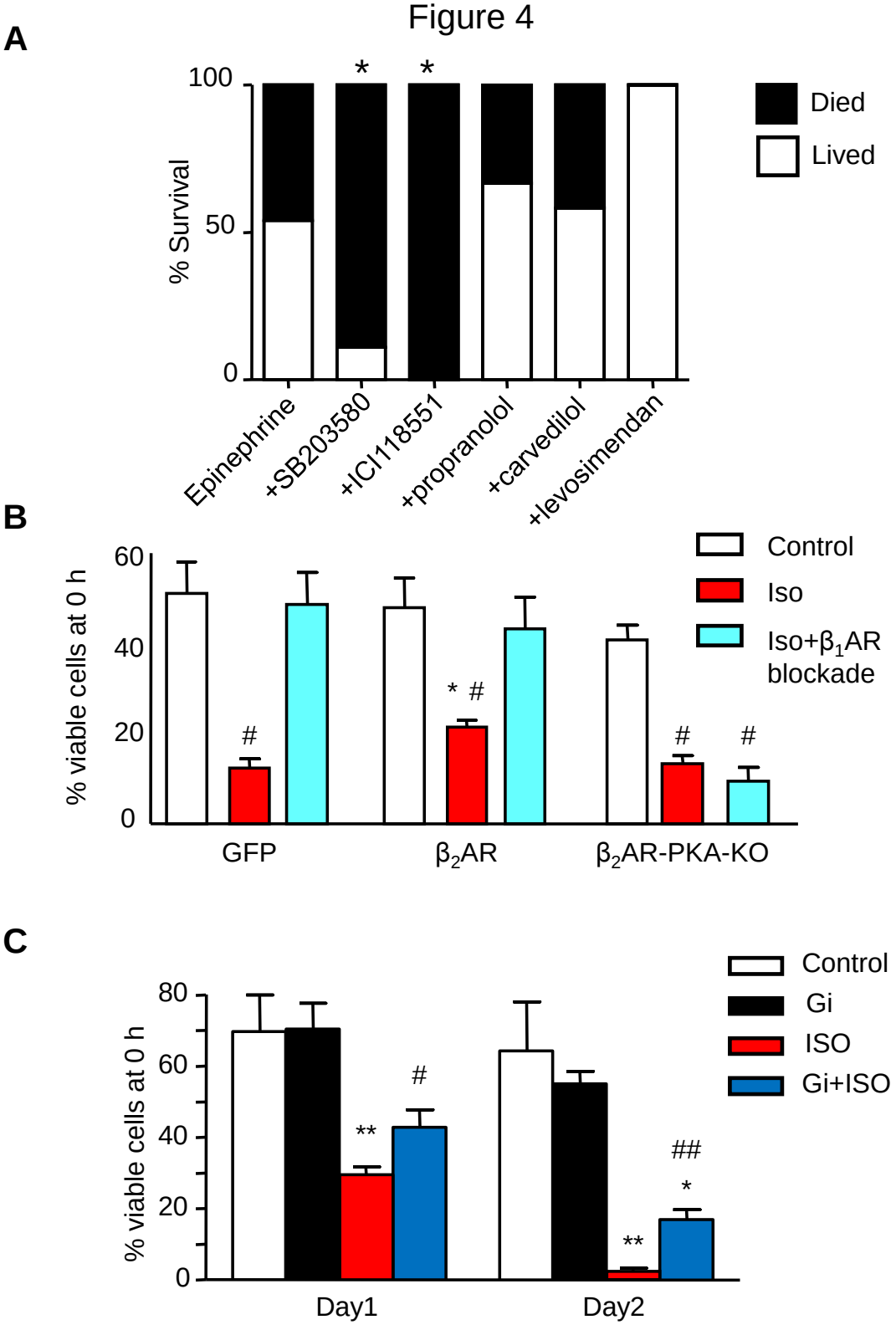
Figure 4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+SB203580
+ICI118551
+propranolol
+carvedilol
+levosimendan
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Iso+β1AR blockade
GFP
β2AR
β2AR-PKA-KO
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Gi+ISO
Day1
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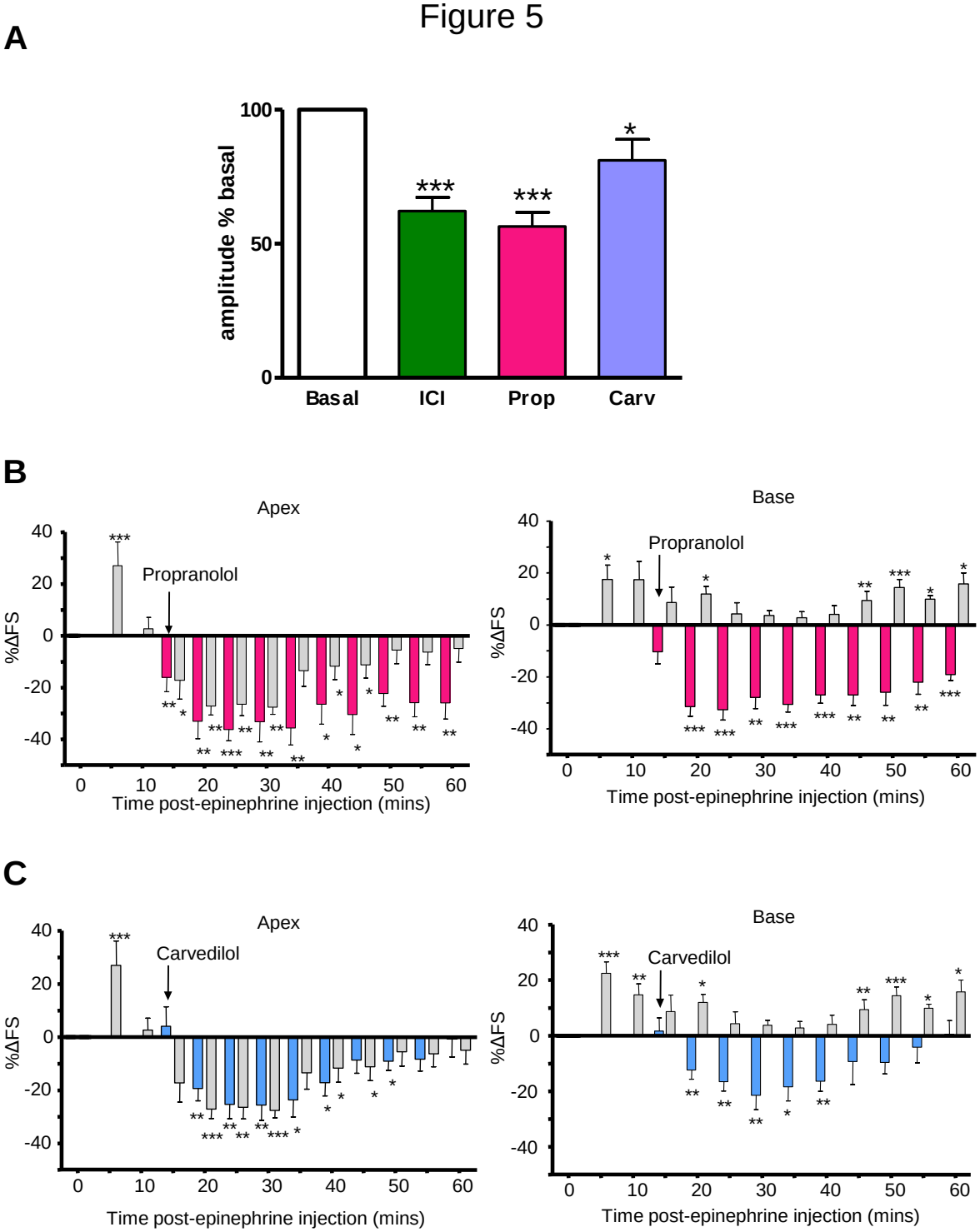
Figure 5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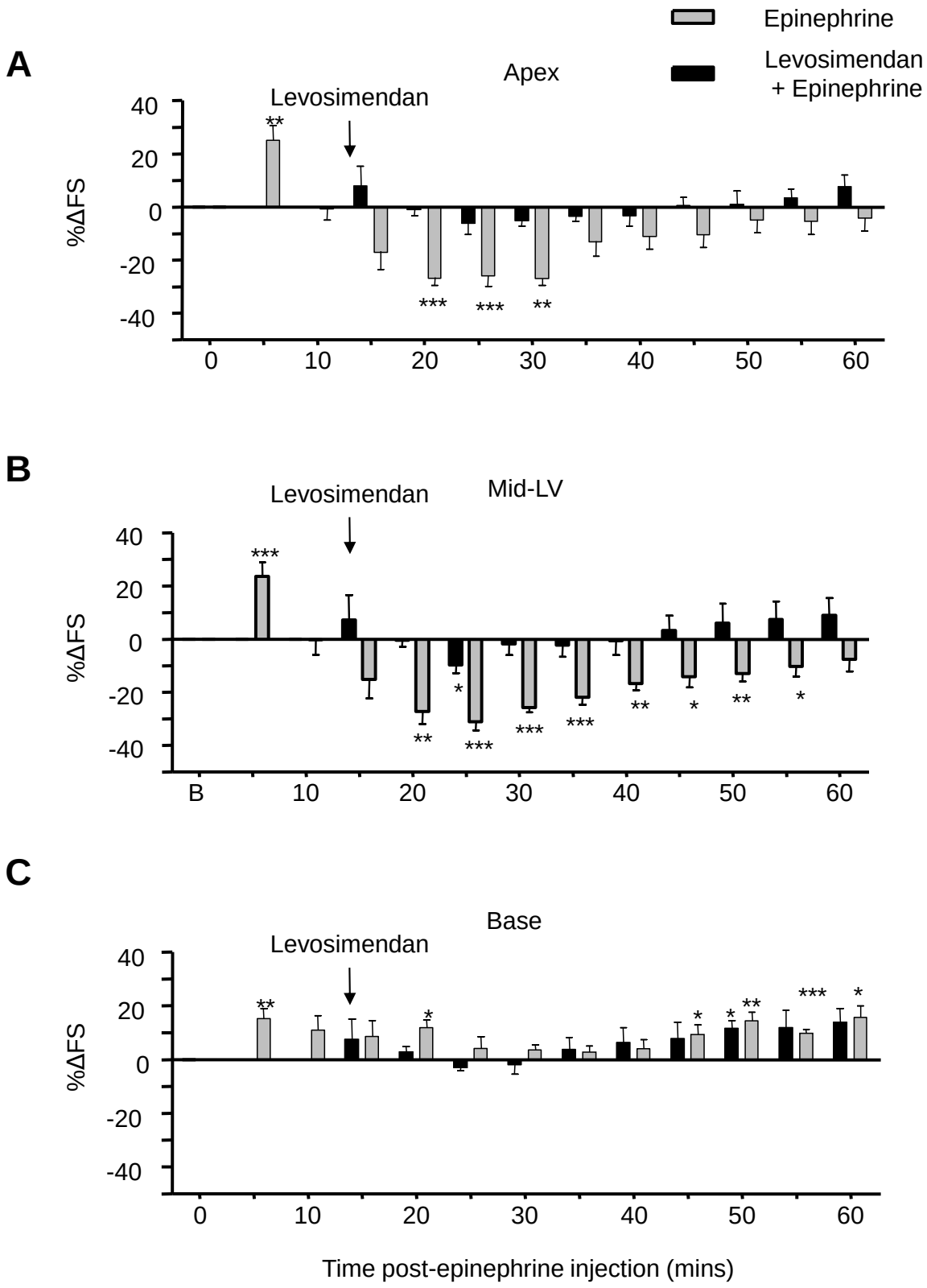
2

Figure 6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Levosimendan
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Levosimendan
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Base
Levosimendan
Time post-epinephrine injection (mins)

1