

RESEARCH PAPER

Reperfusion-induced myocardial dysfunction is prevented by endogenous annexin-A1 and its N-terminal-derived peptide Ac-ANX-A1₂₋₂₆

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BACKGROUND AND PURPOSE

Annexin-A1 (ANX-A1) is an endogenous, glucocorticoid-regulated anti-inflammatory protein. The N-terminal-derived peptide Ac-ANX-A1₂₋₂₆ preserves cardiomyocyte viability, but the impact of ANX-A1-peptides on cardiac contractility is unknown. We now test the hypothesis that ANX-A1 preserves post-ischaemic recovery of left ventricular (LV) function.

EXPERIMENTAL APPROACH

Ac-ANX-A1₂₋₂₆ was administered on reperfusion, to adult rat cardiomyocytes as well as hearts isolated from rats, wild-type mice and mice deficient in endogenous ANX-A1 (ANX-A1^{-/-}). Myocardial viability and recovery of LV function were determined.

KEY RESULTS

Ischaemia-reperfusion markedly impaired both cardiomyocyte viability and recovery of LV function by 60%. Treatment with exogenous Ac-ANX-A1₂₋₂₆ at the onset of reperfusion prevented cardiomyocyte injury and significantly improved recovery of LV function, in both intact rat and wild-type mouse hearts. Ac-ANX-A1₂₋₂₆ cardioprotection was abolished by either formyl peptide receptor (FPR)-nonselective or FPR1-selective antagonists, Boc2 and cyclosporin H, but was relatively insensitive to the FPR2-selective antagonist QuinC7. ANX-A1-induced cardioprotection was associated with increased phosphorylation of the cell survival kinase Akt. ANX-A1^{-/-} exaggerated impairment of post-ischaemic recovery of LV function, in addition to selective LV FPR1 down-regulation.

CONCLUSIONS AND IMPLICATIONS

These data represent the first evidence that ANX-A1 affects myocardial function. Our findings suggest ANX-A1 is an endogenous regulator of post-ischaemic recovery of LV function. Furthermore, the ANX-A1-derived peptide Ac-ANX-A1₂₋₂₆ on reperfusion rescues LV function, probably via activation of FPR1. ANX-A1-based therapies may thus represent a novel clinical approach for the prevention and treatment of myocardial reperfusion injury.

Abbreviations

ANX-A1, annexin-A1; CK, creatine kinase; CsH, cyclosporin H; FPR, formyl peptide receptors (including FPR1, FPR2 and FPR3 subtypes); HR, heart rate; H-R, hypoxia-reoxygenation; I-R, ischaemia-reperfusion; LV, left ventricular; LVDP, LV developed pressure; $LV \pm dP/dt$, first derivatives of LV pressure; LVEDP, LV end-diastolic pressure; MI, myocardial infarction; RM, repeated measures; RPP, rate-pressure product; SNK, Student–Newman–Keuls; SERCA, sarco(endo)plasmic reticulum Ca^{2+} -ATPase; SR, sarcoplasmic reticulum; Ac-ANX-A1₂₋₂₆, N-terminal-derived ANX-A1 peptide mimetic

Introduction

Over three decades ago, Flower and Blackwell discovered the 348-amino-acid glucocorticoid-regulated protein, annexin-A1 (ANX-A1), a second messenger in glucocorticoid actions that was originally termed lipocortin-1 (Flower and Blackwell, 1979). ANX-A1 is a 37 kDa protein expressed constitutively in respiratory, renal, brain, vascular and cardiac tissues, as well as circulating inflammatory cells such as neutrophils and monocytes (Dreier *et al.*, 1998; Flower and Blackwell, 1979; Perretti *et al.*, 1996). The unique biological action of annexins is attributed to their distinct N-terminal region (Flower and Blackwell, 1979). Since their original discovery, clarification of the considerable anti-inflammatory properties of both full-length ANX-A1 and its N-terminal-derived peptide mimetic Ac-ANX-A1₂₋₂₆ has emerged largely from the laboratories of Perretti and colleagues (Morand *et al.*, 2006; Perretti *et al.*, 1996; Perretti and Dalli, 2009; Yang *et al.*, 2004). These studies have demonstrated that these anti-inflammatory actions are mediated by the formyl peptide receptor (FPR) family and are largely attributed to FPR2 receptors (previously known as FPRL1 or aspirin-triggered lipoxin A₄ receptors) (Perretti *et al.*, 2002; Gavins *et al.*, 2003). Our own studies in contrast have focussed on the protective actions of ANX-A1 peptides specifically at the level of the myocardium *in vitro*, in settings devoid of inflammatory cells. We demonstrated that Ac-ANX-A1₂₋₂₆ directly protects myocardial preparations against endotoxic shock and metabolic injury (Ritchie *et al.*, 2003; 2005), and the FPR family was again implicated in these actions. Ac-ANX-A1₂₋₂₆ cardioprotection *in vitro* is comparable to that activated by adenosine or ischaemic preconditioning (Gordon *et al.*, 2003).

Despite clinical advances to accelerate reperfusion post myocardial infarction (MI), via thrombolysis or percutaneous revascularisation interventions, reperfusion injury is still evident post myocardial ischaemia (Moens *et al.*, 2005). An inflammatory response is a contributing component to reperfusion injury, which is independent at least in part, of circulating neutrophils (Goh *et al.*, 2007; Li *et al.*, 2009; Murphy and Steenbergen, 2008; Vinten-Johansen *et al.*, 2005). The myocardium can be further damaged by ischaemic contracture, resulting in incomplete recovery of left ventricular (LV) function as reperfusion ensues (Moens *et al.*, 2005; Goh *et al.*, 2007; Li *et al.*, 2009; Venardos *et al.*, 2009; Tawa *et al.*, 2010). Although the previous studies of Perretti, Gavins and colleagues have shown that ANX-A1 peptides powerfully limit neutrophil-dependent myocardial necrosis (D'Amico *et al.*, 2000; La *et al.*, 2001; Gavins *et al.*, 2005; 2006), their effect specifically on post-ischaemic recovery of LV contractile function is unknown. Interventions that preserve LV contractile

function following an ischaemic insult may be particularly relevant clinically, and affect prognosis. The aim of the present study was thus to test the hypothesis that endogenous ANX-A1 and its N-terminal derived peptide Ac-ANX-A1₂₋₂₆ preserve myocardial function post-ischaemic injury. Our *ex vivo* findings represent the first evidence that both endogenous and exogenous ANX-A1 peptides directly improve recovery of myocardial function during reperfusion, in the intact heart. ANX-A1-based therapies may represent a novel clinical approach for the treatment of myocardial reperfusion injury.

Methods

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publications no. 85-23, revised 1996) and the National Health and Medical Research Council of Australia guidelines, and was approved by the Animal Ethics Committee of the Baker IDI Heart & Diabetes Institute. All reagents were purchased from Sigma Aldrich (St. Louis, MO) except where indicated.

Simulated ischaemia-reperfusion (I-R) in adult rat cardiomyocytes

Cultured ventricular cardiomyocytes isolated from adult male Sprague–Dawley rats (~280 g, weight best suited for myocyte yield) (Ritchie *et al.*, 2005; Garreffa *et al.*, 2006; Goh *et al.*, 2007) were subjected to 90 min hypoxia (95% N₂-5% CO₂) and 2.5 h subsequent reoxygenation at 37°C as previously described (Goh *et al.*, 2007). The ANX-A1 N-terminal-derived peptide Ac-ANX-A1₂₋₂₆ (Ac-Ala-Met-Val-Ser-Glu-Phe-Leu-Lys-Gln-Ala-Trp-Phe-Ile-Glu-Asn-Glu-Glu-Gln-Glu-Tyr-Val-Gln-Thr-Val-Lys-OH, synthesized by Mimotopes, Clayton, Australia) was dissolved in Krebs buffer, at 0.3 μM, and added to cardiomyocytes at one of two different time points, either for the full duration of hypoxia-reoxygenation (H-R), or at the time of reoxygenation. Cardiomyocyte injury was determined at the end of reoxygenation, on LDH (IU·L⁻¹) activity in the culture medium (Goh *et al.*, 2007), expressed as % of paired control normoxic cardiomyocytes, as well as on cell viability using trypan blue exclusion (Ritchie *et al.*, 2005).

I-R in intact adult rat hearts ex vivo

Hearts isolated from adult male Sprague–Dawley rats (11 weeks old, 374 ± 8 g) were buffer-perfused at constant flow

(10 mL·min⁻¹) at 37°C as previously described (Goh *et al.*, 2007). Following equilibration (~20 min), hearts were subjected to 30 min global, no-flow ischaemia with 30 min reperfusion, conditions previously shown to limit recovery of LV function to approximately 60% of pre-ischaemic levels. Both isovolumic LV function (via intraventricular fluid-filled balloon catheter) and markers of myocardial injury were continuously assessed, including LDH (IU·L⁻¹, expressed fold of pre-ischaemic baseline) and creatine kinase (CK, IU·L⁻¹) (Ritchie *et al.*, 2005; Goh *et al.*, 2007). Ac-ANX-A1₂₋₂₆ (0.3 μM, dissolved in Krebs buffer), in the absence or presence of either the non-selective FPR1/FPR2 antagonist Boc2 (10 μM, synthesized by GL Biochem Shanghai Limited, China), the FPR1-selective antagonist cyclosporin-H (CsH, 1 μM, synthesized by Chemieliva Pharmaceutical Co Ltd, Hong Kong, China) or the FPR2-selective antagonist QuinC7 (10 μM, synthesized by Anthem Biosciences Private Limited, Bangalore, India). Antagonist concentrations were chosen based on previous studies (Dufton and Perretti, 2010; Ritchie *et al.*, 2005; Stenfeldt *et al.*, 2007; Zhou *et al.*, 2007), and were added to the perfusion buffer at the onset of reperfusion. Additional experimental groups included time-control normoxic (sham) hearts, studied in the absence or presence of Ac-ANX-A1₂₋₂₆ (added after 1 h drug-free perfusion, analogous to the time point in which reperfusion was commenced in the hearts subjected to ischaemia). Hearts were then snap-frozen and stored at -80°C until biochemical analysis. Post-ischaemic recovery of LV function was assessed on LV developed pressure (LVDP) and its derivatives LV ± dP/dt and rate-pressure product (RPP), all expressed as % of the within-heart pre-ischaemic baseline. The area under the curve (AUC) for each of these parameters during reperfusion was calculated using GraphPad Prism v5 (GraphPad Software Inc, La Jolla, CA, USA).

LV phosphorylation of the cell survival kinase Akt, as well as phosphorylation of phospholamban (which inhibits sarco(endo)plasmic Ca²⁺-ATPase, SERCA) were determined following I-R, using phospho-specific antibodies (Cell Signaling Technology, Beverly, MA), expressed as the ratio of phosphorylated to total protein (Goh *et al.*, 2007; Venardos *et al.*, 2009). Results were compared to drug-free hearts subjected to I-R and to sham (time control) normoxic hearts.

I-R in adult mouse hearts ex vivo

Male and female ANX-A1 wild-type (ANX-A1^{+/+}) and ANX-A1^{-/-} mice were generated on a mixed 129/SvJ × C57BL/6 background (kindly provided by Prof RJ Flower, William Harvey Research Institute) as described previously (Hannon *et al.*, 2003; Yang *et al.*, 2009) and studied at ~21 weeks of age. Hearts were removed from anaesthetized mice following thoracotomy and perfused with Krebs's buffer on a Langendorff perfusion apparatus under constant perfusion pressure (80 mmHg) and temperature (37°C). This setting of constant perfusion pressure is more physiologically relevant than the setting of constant flow used above. Isovolumic function was again determined using a fluid-filled latex balloon positioned in the LV, from which LVDP, LV ± dP/dt, RPP and LVEDP were derived. Following 20 min equilibration under normoxic perfusion, hearts were subjected to global no-flow ischaemia for 22.5 min, followed by restoration of flow for a further 40 min, conditions previously shown to limit recovery of LV function to approximately 60% of pre-ischaemic levels (Venardos *et al.*,

2009). ANX-A1₂₋₂₆ (0.3 μM, dissolved in Krebs buffer) was added to the perfusion buffer of subsets of either wild-type or ANX-A1^{-/-} mouse hearts, at the onset of reperfusion. At the end of the experiment, hearts were then snap-frozen and stored at -80°C until biochemical analysis. Expression of FPR1 and FPR2 in mouse myocardium was determined by real-time PCR. Results were expressed relative to within-heart pre-ischaemic baseline and were compared with drug-free wild-type hearts, subjected either to I-R or to shams.

Real-time PCR

Total RNA isolated from cells was prepared using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Total RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase and Oligo dT₂₀ (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using power SYBR Green PCR master mix (Applied Biosystems, Scoresby, VIC, Australia) according to the manufacturer's protocol. Mouse primers for the determination of FPR1, FPR2 and β-actin mRNA levels were used (Yang *et al.*, 2004; Rondepierre *et al.*, 2009). Analysis of relative change in gene expression was calculated according to the 2^{-ΔΔCt} method using the housekeeping gene (β-actin) as the control.

Statistical analysis

Results were expressed as mean ± SEM, with *n* representing the number of different cardiomyocyte preparations studied, or the number of animals per treatment group in the heart respectively. Statistical evaluation of the cardiomyocyte studies used one-way repeated-measures ANOVA with the Student–Newman–Keuls (SNK) method for multiple comparisons (cardiomyocyte studies). For the whole heart studies, two-way ANOVA was used to compare baseline characteristics across genotype and sex in mice, experimental groups over multiple time points, or two-way repeated-measures ANOVA with SNK *post hoc* analysis for multiple comparisons was used to compare experimental groups over multiple time points, or Mann–Whitney rank sum test for single, non-parametric time point comparisons. *P* < 0.05 was accepted as significant.

Results

Ac-ANX-A1₂₋₂₆ protects adult rat cardiomyocytes from H-R injury

H-R significantly increased LDH release from cardiomyocytes (*P* < 0.001) and significantly reduced cardiomyocyte viability (*P* < 0.05, Figure 1). Ac-ANX-A1₂₋₂₆ completely prevented cardiomyocyte LDH release, whether present for the duration of H-R, or when only added at the start of reoxygenation (Figure 1A, both *P* < 0.001). Similar Ac-ANX-A1₂₋₂₆ cardioprotective effects were evident on cardiomyocyte viability; the beneficial actions of Ac-ANX-A1₂₋₂₆ were comparable whether the peptide was administered from the start of H-R, or when administered only on reoxygenation (Figure 1B, *P* < 0.05). Ac-ANX-A1₂₋₂₆ had no effect when present for the analogous time points under normoxia. These results confirm that Ac-ANX-A1₂₋₂₆ elicits direct protective actions on cardiomyocytes subjected to metabolic injury comparable to those in

H-R injury, and which are comparable whether treatment is commenced concomitant with the insult or at the onset of post-insult recovery.

Ac-ANX-A1₂₋₂₆ protects adult rat hearts from I-R injury via FPRs

Sixty-five adult male rats, aged 10.8 ± 0.3 weeks old with bodyweight 374 ± 8 g, were used to study I-R *ex vivo*. At baseline, hearts exhibited LVDP of 68 ± 3 mmHg and LVEDP of 3.5 ± 0.5 mmHg. As shown in Figure 2, release of LDH from the myocardium was significantly elevated over the time course of reperfusion ($P < 0.005$). Similarly, the recovery of both LVDP and LV + dP/dt (both $P < 0.001$) were significantly impaired. At the onset of reperfusion, these markers of LV function were significantly reduced compared with their pre-ischæmic baseline, and remained depressed for the duration of reperfusion. Ac-ANX-A1₂₋₂₆ ($0.3 \mu\text{M}$), added to the reperfusion buffer, attenuated myocardial release of LDH during reperfusion (Figure 2A). Ac-ANX-A1₂₋₂₆ did not affect recovery of LVDP in the first 2 min of reperfusion. However, a significant improvement in LVDP was evident from 5 min of reperfusion, and LVDP was completely recovered by

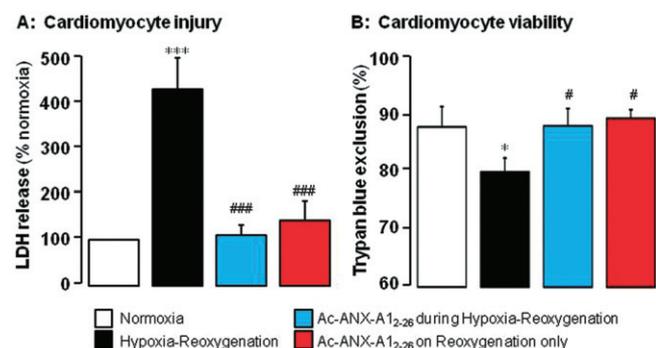


Figure 1

(A) Ac-ANX-A1₂₋₂₆ ($0.3 \mu\text{M}$) prevents adult rat cardiomyocyte hypoxia-reoxygenation (H-R) injury *in vitro* (assessed by measuring LDH activity) whether present for the full duration of H-R ($n = 11$) or only post-H-R ($n = 15$ cardiomyocyte preparations). (B) H-R-induced loss of cardiomyocyte viability (assessed by measuring trypan blue exclusion) was also prevented by Ac-ANX-A1₂₋₂₆ at both time points ($n = 8$ cardiomyocyte preparations). * $P < 0.05$ and *** $P < 0.001$ versus paired untreated control, and # $P < 0.05$ and ### $P < 0.001$ Ac-ANX-A1₂₋₂₆-treated versus untreated H-R cardiomyocytes from the same cardiomyocyte preparation on SNK.

Table 1

Effect of I-R, in the absence and presence of Ac-ANX-A1₂₋₂₆ (added at the onset of reperfusion), on release of the cardiac enzyme CK in the adult rat heart *ex vivo*

Parameter	Treatment	Min post reperfusion						n
		0	1	2	5	10	20	
CK (IU·L ⁻¹ , $P < 0.05$)	I-R	32 ± 8	32 ± 16	21 ± 9	17 ± 5	21 ± 6	17 ± 7	6
	I-R + Ac-ANX-A1 ₂₋₂₆	9 ± 5	7 ± 4	9 ± 6	8 ± 6	6 ± 4	3 ± 2	5

10 min reperfusion ($P < 0.001$, Figure 2B). The beneficial effects of Ac-ANX-A1₂₋₂₆ on both LDH release and the recovery of LVDP were also evident on area under curve (AUC) analysis. Similar Ac-ANX-A1₂₋₂₆ cardioprotection was evident on LV + dP/dt (Figure 2C), RPP ($P < 0.0001$, Figure 3A), LV-dP/dt ($P < 0.0001$, Figure 3B) and cardiac CK release (Table 1). Ac-ANX-A1₂₋₂₆ cardioprotection was accompanied by increased phosphorylation of the cell survival kinase Akt relative to total Akt content ($P < 0.05$, Figure 3C). Preliminary evidence of enhanced phosphorylation of the SERCA regulator, phospholamban was also shown ($P < 0.05$, Figure 3C). As shown in Table 2, Ac-ANX-A1₂₋₂₆ did not significantly affect LVDP or RPP in normoxic rat hearts, when added to the perfusion buffer after one hour of drug-free perfusion (the analogous time point in which ischaemic hearts underwent reperfusion), although LV \pm dP/dt were modestly elevated. Furthermore compared with shams, Ac-ANX-A1₂₋₂₆ did not enhance Akt phosphorylation in normoxic hearts ($n = 3$; result not shown).

To explore the contribution of FPRs to the cardioprotective actions of Ac-ANX-A1₂₋₂₆, we used the non-selective FPR-antagonist Boc2, the FPR1-selective antagonist CsH and the FPR2-selective antagonist QuinC7, added to the perfusion buffer at the onset of reperfusion concomitantly with Ac-ANX-A1₂₋₂₆. Our results clearly demonstrate that Ac-ANX-A1₂₋₂₆-mediated preservation of both LV viability (LDH release, Figure 4A) and LV function (LVDP, LV + dP/dt, RPP and LV-dP/dt; Figures 4B, C and 5A and B, respectively) were prevented by Boc2. A similar blunting of the cardioprotective actions of Ac-ANX-A1₂₋₂₆ was evident with CsH. In contrast, QuinC7 failed to attenuate the Ac-ANX-A1₂₋₂₆-induced cardioprotection on all of these variables, with the exception of LV-dP/dt (Figures 4 and 5), although both the FPR1- and FPR2-selective antagonists tended to blunt Ac-ANX-A1₂₋₂₆-induced Akt phosphorylation (Figure 5E). Moreover, FPR1 antagonism tended to blunt both the early and later stages of Ac-ANX-A1₂₋₂₆-induced cardioprotection. In contrast, a modest inhibition of Ac-ANX-A1₂₋₂₆ cardioprotection was evident with FPR2 antagonism, but this effect was relatively short-lived (evident at 2 min of reperfusion).

Ac-ANX-A1₂₋₂₆ protects adult wild-type mouse hearts from I-R injury

Male and female wild-type mice were well-matched on baseline characteristics, although a trend for female mice to be slightly younger and smaller was observed (Table 3). In the wild-type mouse heart perfused under constant pressure, baseline LVDP was 126 ± 6 mmHg and heart rate (HR) was

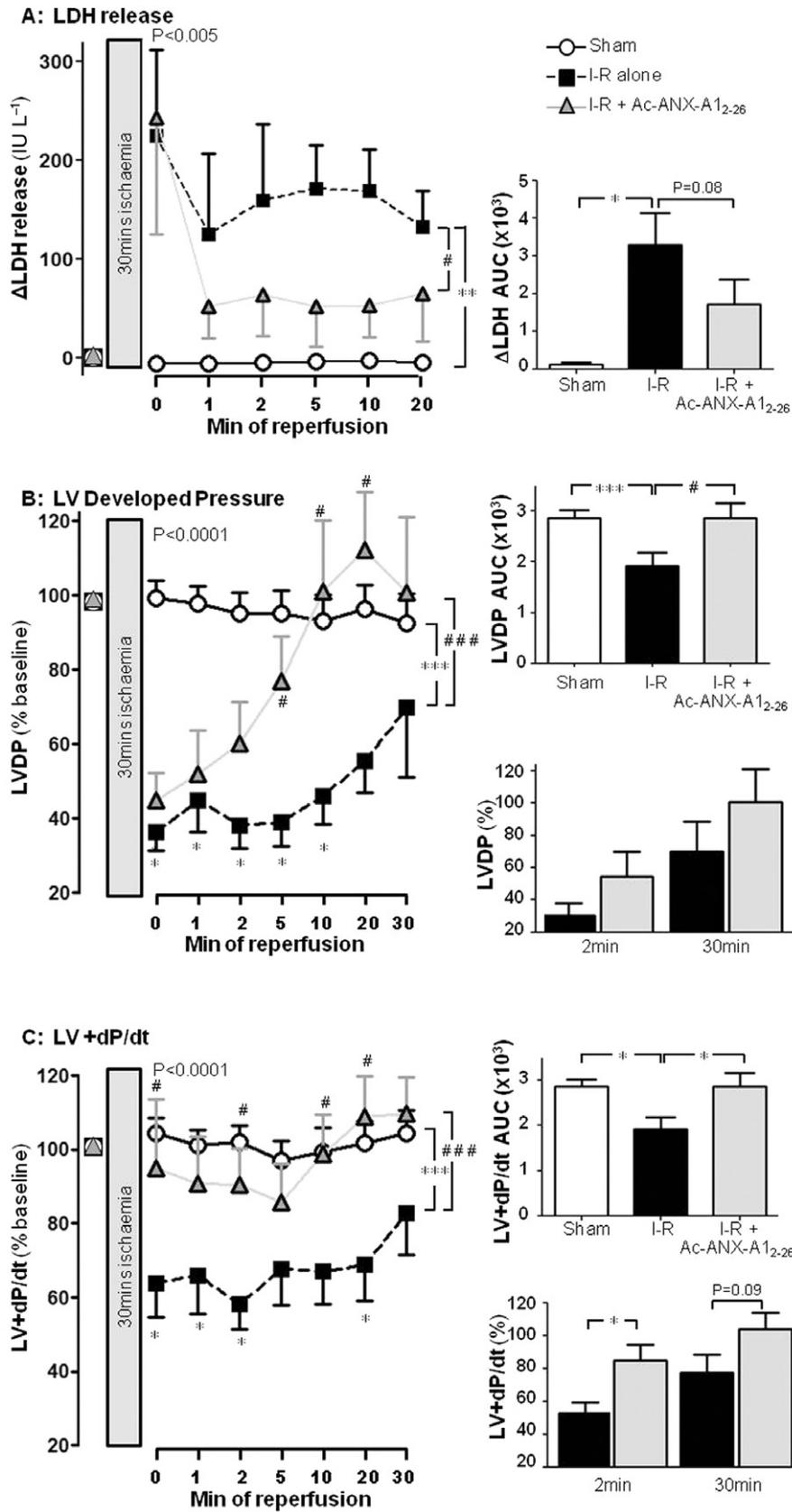


Figure 2

Ac-ANX-A1₂₋₂₆ (0.3 μM) addition at the onset of reperfusion attenuates I-R-induced impairment of myocardial viability and LV systolic function in rat isolated heart *ex vivo*. (A) Ac-ANX-A1₂₋₂₆ decreases the I-R-stimulated release of LDH from the myocardium ($P < 0.005$, untreated I-R, $n = 16$ vs. sham $n = 4$, and $P < 0.05$ untreated I-R vs. Ac-ANX-A1₂₋₂₆-treated I-R, $n = 15$, on two-way ANOVA). The untreated I-R group also had a significantly increased myocardial release of LDH (as assessed by AUC analysis; $P < 0.05$), a trend that was absent in the presence of Ac-ANX-A1₂₋₂₆ ($P = 0.08$, right panel). (B) Ac-ANX-A1₂₋₂₆ accelerates recovery of LVDP up to 30 min post reperfusion ($P < 0.001$ untreated I-R, $n = 15$ vs. sham $n = 9$, and $P < 0.001$ vs. Ac-ANX-A1₂₋₂₆-treated I-R, $n = 17$, on two-way ANOVA). The untreated I-R group also had a significantly impaired recovery of LVDP on AUC analysis ($P < 0.001$), which was prevented by Ac-ANX-A1₂₋₂₆ ($P < 0.05$, upper inset). Ac-ANX-A1₂₋₂₆-induced recovery of LVDP after 2 or 30 min reperfusion is shown in the lower inset (both $P = \text{NS}$). (C) Ac-ANX-A1₂₋₂₆ accelerates recovery of LV + dP/dt up to 30 min post reperfusion ($P < 0.001$ untreated I-R vs. sham, and $P < 0.001$ vs. Ac-ANX-A1₂₋₂₆-treated I-R, by two-way ANOVA). The untreated I-R group had a significantly impaired recovery of LV + dP/dt on AUC analysis ($P < 0.05$), which was prevented by Ac-ANX-A1₂₋₂₆ (upper inset). Ac-ANX-A1₂₋₂₆ significantly improved recovery of LV + dP/dt after 2 min reperfusion ($P < 0.05$, lower inset). * $P < 0.05$ and *** $P < 0.001$ untreated I-R versus sham; # $P < 0.05$ and ## $P < 0.001$ Ac-ANX-A1₂₋₂₆-treated I-R versus untreated I-R rat hearts at each time point on SNK respectively.

368 ± 17 beats min^{-1} , before ischaemia or normoxic time control. There were no differences in baseline cardiac parameters between hearts from male and female mice (Table 3), or in the time course of recovery of LV function on reperfusion (Table 4); hence, mice of either sex were studied.

In the wild-type mouse heart, I-R again significantly impaired the recovery of LVDP, LV + dP/dt and LV-dP/dt (Figure 6; all $P < 0.001$ vs. sham). Ac-ANX-A1₂₋₂₆ (0.3 μM) added to the reperfusion buffer significantly improved recovery of LV function, particularly in the latter stages of reperfusion (20 min onwards), in each of LVDP (Figure 6A), LV + dP/dt (Figure 6B) and LV-dP/dt (Figure 6C). RPP was similarly preserved by Ac-ANX-A1₂₋₂₆ ($P = 0.06$ vs. untreated I-R). Neither the time-to-ischaemic contracture, nor the extent of contracture, were affected by Ac-ANX-A1₂₋₂₆ (results not shown). These results confirm that Ac-ANX-A1₂₋₂₆ preserves LV function in a second species, and under the more physiologically relevant setting of constant pressure perfusion.

Deficiency of ANX-A1 exaggerates myocardial I-R injury

As outlined in Table 3, no systemic or cardiac phenotype was evident at baseline in ANX-A1^{-/-} mice, regardless of sex. The recovery of LV function in adult ANX-A1^{-/-} mouse hearts from I-R was significantly and markedly impaired compared with ANX-A1^{+/+} mice. Deficiency of endogenous ANX-A1 resulted in reduced recovery of LVDP, evident even in the earliest stages of reperfusion (Figure 7A). Recovery of LV + dP/dt (Figure 7B), LV-dP/dt (Figure 7C) and RPP ($P < 0.001$) were all significantly reduced in ANX-A1^{-/-} versus ANX-A1^{+/+} mouse hearts following ischaemia. There were no differences in the recovery of LV function in male versus female ANX-A1^{-/-} mouse hearts after I-R (Table 4). Furthermore, supplementing the reperfusion buffer with Ac-ANX-A1₂₋₂₆ (0.3 μM) in ANX-A1^{-/-} mouse hearts failed to restore the recovery of LV function following ischaemia (Table 5).

Effect of ANX-A1 deficiency on myocardial Akt and FPR

As shown in Figure 8A, the exaggerated impairment in LV function in the setting of deficiency of endogenous ANX-A1 was accompanied by reduced phosphorylation of Akt relative to total Akt content ($P < 0.05$, Figure 8A). Given that our results obtained in Figures 4–6 strongly implicate a role for at least FPR1 in the cardioprotective actions of Ac-ANX-A1₂₋₂₆, we determined the gene expression of both FPR1 and FPR2.

Our results indicate that both receptor subtypes are present in myocardium. FPR1 is selectively down-regulated in ANX-A1^{-/-} hearts, but there was no significant effect on FPR2 expression (Figure 8B).

Discussion

The therapeutic potential of the anti-inflammatory mediator ANX-A1 has been recognized in a range of inflammatory disorders (Hannon *et al.*, 2003; Perretti and Dalli, 2009; Ritchie *et al.*, 2003). Moreover, studies in ANX-A1^{-/-} mice demonstrate that endogenous ANX-A1 serves as a protective brake against inflammation and injury (Hannon *et al.*, 2003; Yang *et al.*, 2004; Ritchie *et al.*, 2005). We now present evidence that ANX-A1 has potent direct protective actions against cardiac dysfunction when administered after an ischaemic insult in the intact heart. Specifically, exogenous Ac-ANX-A1₂₋₂₆ treatment commencing concomitantly with reperfusion significantly potentiated recovery of LV function in the post-ischaemic heart *ex vivo*. The timing of this cardioprotection is analogous to a 'pharmacological post-conditioning'. These functional improvements were largely attributed to FPR1 activation by this ligand, based on the greater sensitivity to both the non-selective FPR1/FPR2 antagonist Boc2 and the FPR1-selective antagonist CsH, than observed with the FPR2-selective antagonist QuinC7. Ac-ANX-A1₂₋₂₆ also reduced myocardial necrosis and increased activation of the cell survival kinase, Akt. Given that deficiency of endogenous ANX-A1 exaggerated myocardial I-R injury, we propose that endogenous ANX-A1 contributes to maintaining normal cardiac physiology.

We have demonstrated that Ac-ANX-A1₂₋₂₆ can directly preserve myocardial inotropic responsiveness and cardiomyocyte viability, rather than secondary to any neutrophil-dependent mechanisms, *in vitro* (Ritchie *et al.*, 2003; 2005). The present study sheds the light, for the first time, on the beneficial impact of ANX-A1 mimetics on the recovery of LV function in the intact heart. Ac-ANX-A1₂₋₂₆ prevented cardiomyocyte injury and preserved LV systolic function (LVDP, LV + dP/dt) and LV diastolic function (LV-dP/dt) in both rat and mouse hearts. The preservation of LV function was significant within 10–20 min of reperfusion, and persisted for the duration of the experiment. Our results suggest that enhanced activation of cardioprotective signalling (e.g.

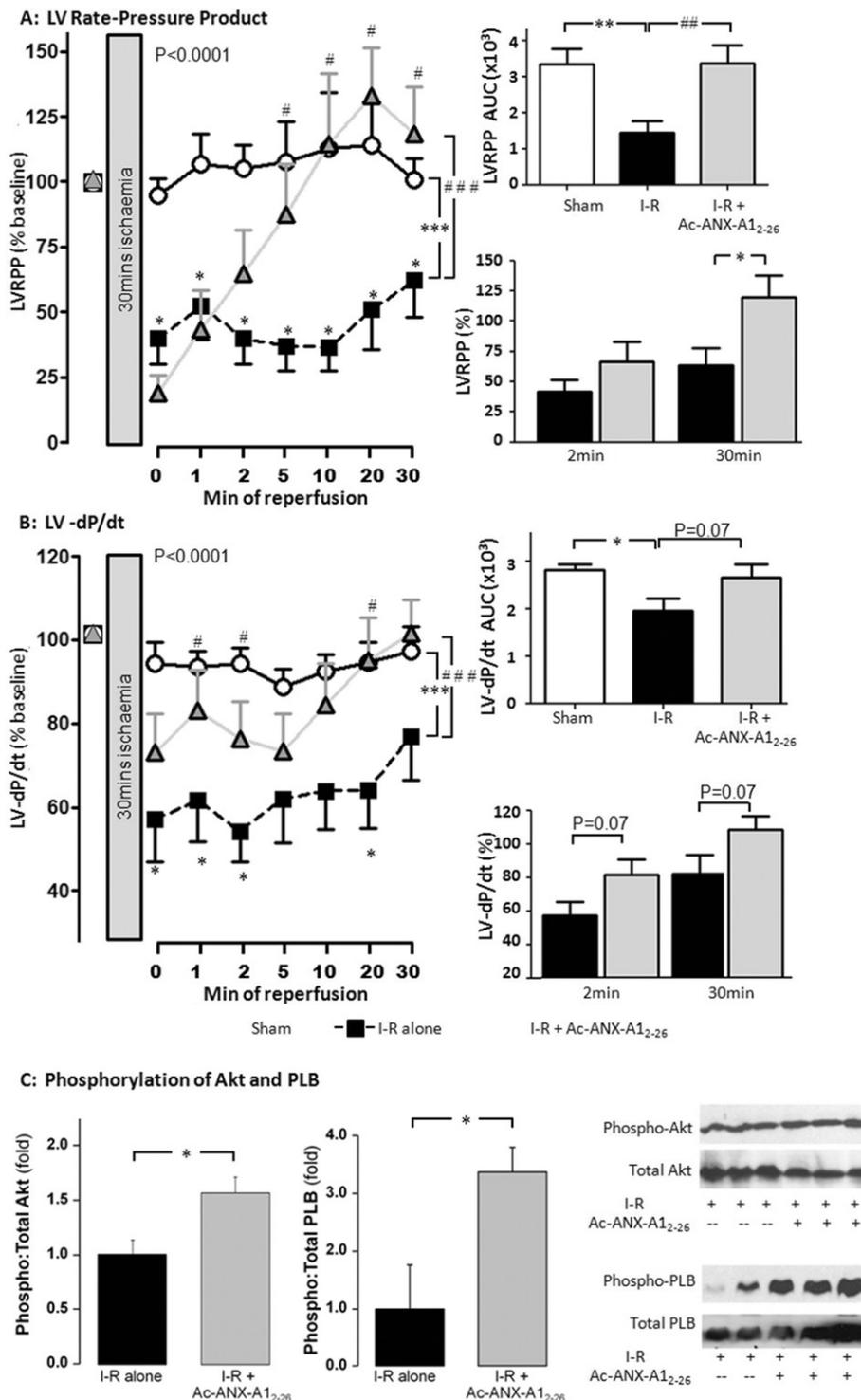


Figure 3

Ac-ANX-A1₂₋₂₆ (0.3 μM) attenuates I-R-induced impairment of (A) LVRPP and (B) LV-dP/dt up to 30 min post reperfusion in the rat isolated heart *ex vivo*. Recovery of RPP and LV-dP/dt on AUC analysis is shown on the upper inset of each panel, and relative recovery of both parameters after 2 and 30 min reperfusion on the lower insets. (C) ANX-A1₂₋₂₆ also increases phosphorylation of the cell survival kinase, Akt ($P < 0.05$ ANX-A1₂₋₂₆ I-R, $n = 4$ vs. untreated I-R rat hearts, $n = 10$, left-hand panel) and of the SERCA regulator, phospholamban ($P < 0.05$ ANX-A1₂₋₂₆ I-R, $n = 4$ vs. untreated I-R rat hearts, $n = 3$, right-hand panel), in rat hearts subjected to I-R. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$ untreated I-R versus sham; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ Ac-ANX-A1₂₋₂₆-treated I-R versus untreated I-R rat hearts at each time point on SNK respectively.

Table 2

Effect of Ac-ANX-A1₂₋₂₆ (added to the perfusion buffer after one hour of drug-free perfusion) on LV function in the normoxic adult rat heart *ex vivo*

Parameter	Treatment	Min post addition of Ac-ANX-A1 ₂₋₂₆										n
		0	1	2	5	10	20	30	AUC			
LVDP (% baseline, <i>P</i> = NS)	sham	99 ± 5	98 ± 5	95 ± 6	95 ± 6	93 ± 7	96 ± 7	92 ± 8	2850 ± 190	9		
	Ac-ANX-A1 ₂₋₂₆	121 ± 8	111 ± 14	111 ± 14	121 ± 15	124 ± 15	128 ± 12	127 ± 14	3720 ± 404	4		
LVRPP (% baseline, <i>P</i> = NS)	sham	93 ± 7	110 ± 15	107 ± 12	113 ± 20	117 ± 29	118 ± 22	102 ± 9	3340 ± 443	6		
	Ac-ANX-A1 ₂₋₂₆	127 ± 9	116 ± 13	117 ± 15	130 ± 15	134 ± 16	136 ± 12	131 ± 9	3950 ± 441	4		
LV + dIP/dt (% baseline, <i>P</i> < 0.05)	sham	99 ± 4	96 ± 4	96 ± 4	91 ± 4	94 ± 5	96 ± 6	99 ± 6	2860 ± 167	8		
	Ac-ANX-A1 ₂₋₂₆	117 ± 7	113 ± 11	111 ± 11	119 ± 12	123 ± 12	125 ± 10	121 ± 11	3650 ± 312	4		
LV-dP/dt (% baseline, <i>P</i> < 0.005)	sham	94 ± 5	93 ± 4	94 ± 4	89 ± 4	93 ± 4	95 ± 5	97 ± 6	2810 ± 119	8		
	Ac-ANX-A1 ₂₋₂₆	117 ± 3	115 ± 10	113 ± 9	119 ± 9	122 ± 9	123 ± 4	117 ± 7	3600 ± 188	4		
LDH (IU·L ⁻¹ , <i>P</i> < 0.05)	sham	-6 ± 4	-6 ± 6	-5 ± 4	-4 ± 4	-3 ± 4	-5 ± 3	ND	130 ± 45	4		
	Ac-ANX-A1 ₂₋₂₆	-19 ± 7	-33 ± 8	-32 ± 7	-39 ± 5	-44 ± 6	-13 ± 13	ND	670 ± 130	4		

Akt) and/or improved intracellular Ca²⁺ re-uptake by the sarcoplasmic reticulum (SR, as suggested by the reduced phospholamban brake on the activity of SERCA) may contribute to Ac-ANX-A1₂₋₂₆-induced cardioprotection, which thus targets contributing mechanisms specifically to death and dysfunction at the level of the myocardium (Moens *et al.*, 2005; Murphy and Steenbergen, 2008). Our findings suggest that ANX-A1 analogues represent novel alternative means to salvage recovery of post-ischaemic LV systolic and diastolic function post MI.

Ac-ANX-A1₂₋₂₆ is regarded as a non-subtype selective FPR agonist (Hayhoe *et al.*, 2006; Gavins, 2010; Dufton *et al.*, 2010). Previous studies have focussed predominantly on the anti-inflammatory actions of Ac-ANX-A1₂₋₂₆, which are largely FPR2-dependent (Perretti *et al.*, 2001; Gavins *et al.*, 2003; Maderna *et al.*, 2010; Dufton *et al.*, 2010). These FPR2 actions of Ac-ANX-A1₂₋₂₆ limit early myocardial I-R injury *in vivo*, as evidenced by reduced infarct size secondary to anti-neutrophil actions (La *et al.*, 2001). Given that the direct cardioprotective actions of exogenous Ac-ANX-A1₂₋₂₆ on the recovery of LV function in the adult male rat heart are inhibited by both the non-selective FPR1/FPR2 antagonist Boc2, as well as by the FPR1-selective antagonist CsH, but are considerably less sensitive to the FPR2-selective antagonist QuinC7, we now largely attribute these actions on the preservation of contractile function to FPR1, rather than FPR2, activation. The protective effects of the FPR2 receptor subtype probably become markedly more important *in vivo*, via their involvement in inflammatory cell-mediated events.

Systemic deficiency of the parent ANX-A1 protein exaggerates inflammatory responses *in vivo* (Hannon *et al.*, 2003; Yang *et al.*, 2009). Our findings in the present study reveal a previously unknown additional consequence of ANX-A1 deficiency, an augmented myocardial injury response specifically at the level of cardiac contractile function. Marked aggravation of post-ischaemic impairment of both LV systolic (LVDP, LV + dP/dt) and diastolic function (LVEDP, LV-dP/dt) were observed in ANX-A1^{-/-} mice. This suggests that endogenous ANX-A1 contributes significantly to the maintenance of normal cardiac physiology. Receptor utilization by the full length ANX-A1 protein remains contentious. Although ANX-A1 appears to show FPR2 selectivity relative to Ac-ANX-A1₂₋₂₆ (Hayhoe *et al.*, 2006), roughly 50% of the ANX-A1 effects are lost in FPR1-deficient mice. Furthermore, FPR1-transfected HEK293 cells bind ANX-A1 (Perretti *et al.*, 2001). Some residual protective ANX-A1 actions may thus remain in FPR2-deficient mice (Dufton *et al.*, 2010). It is hence possible that although most evidence suggests that ANX-A1 predominantly binds to FPR2 (Hayhoe *et al.*, 2006), under some settings, FPR1 may also bind ANX-A1 (Dufton and Perretti, 2010). We now confirm that both FPR1 and FPR2 subtypes are present in the heart, as previously suggested (Migeotte *et al.*, 2006). Interestingly, our results suggest that a deficiency of endogenous annexin-A1 down-regulates myocardial FPR1 (but not FPR2) expression, which probably accounts for the marked impairment in recovery of LV contractile function following the ischaemic insult. Indeed this marked down-regulation (by >80%) of LV FPR1 expression in ANX-A1^{-/-} probably accounts for the inability of exogenous Ac-ANX-A1₂₋₂₆ to protect LV contractile function (Table 5). The subtype of FPR responsible for the cardio-

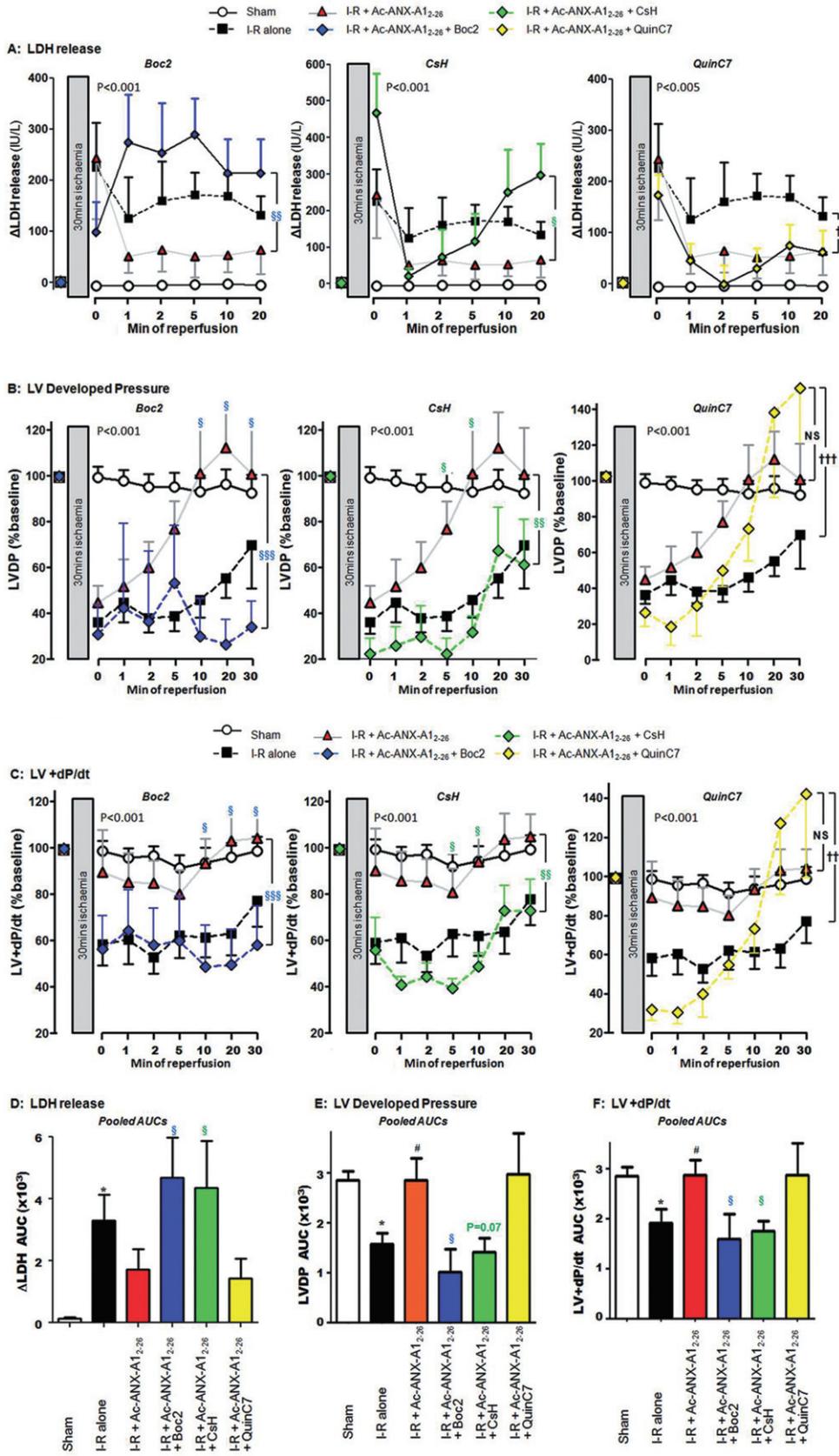


Figure 4

Role of FPR subtypes in Ac-ANX-A1₂₋₂₆ cardioprotection in the rat isolated heart *ex vivo*, on the time course of protection in the presence of Ac-ANX-A1₂₋₂₆ (0.3 μM), alone or in the presence of the non-selective FPR-antagonist Boc2 (10 μM), the FPR1-selective antagonist CsH (1 μM), or the FPR2-selective antagonist QuinC7 (10 μM) during reperfusion. (A) Both Boc2 and CsH (both $P < 0.05$, $n = 6$) significantly inhibited Ac-ANX-A1₂₋₂₆-induced protection of myocardial LDH release, but QuinC7 had no effect ($P = \text{NS}$, $n = 6$). Similar attenuation of the Ac-ANX-A1₂₋₂₆ effect by Boc2 and CsH, but not QuinC7, was also evident on recovery of both (B) LVDP and (C) LV + dP/dt. The AUC results for Ac-ANX-A1₂₋₂₆ cardioprotection ± FPR antagonists are also shown, on (D) LDH release, (E) LVDP and (F) LV + dP/dt. Sham $n = 9$, untreated I-R, $n = 15$ and Ac-ANX-A1₂₋₂₆-treated I-R, $n = 17$. * $P < 0.05$ untreated I-R versus sham; [#] $P < 0.05$ Ac-ANX-A1₂₋₂₆-treated I-R versus untreated I-R rat hearts; [§] $P < 0.05$, ^{§§} $P < 0.01$ and ^{§§§} $P < 0.001$ antagonist + Ac-ANX-A1₂₋₂₆-treated I-R versus Ac-ANX-A1₂₋₂₆-treated I-R and [†] $P < 0.05$, ^{††} $P < 0.01$ and ^{†††} $P < 0.001$ antagonist + Ac-ANX-A1₂₋₂₆-treated I-R versus untreated I-R rat hearts respectively.

Table 3

Baseline characteristics of annexin-A1 wild-type and ANX-A1^{-/-} mice prior to I-R *ex vivo*

Genotype and sex	Age (weeks)	BW (g)	LVDP (mmHg)	LV + dP/dt (mmHg·s ⁻¹)	LV-dP/dt (mmHg·s ⁻¹)
Wild-type male ($n = 14$)	21.9 ± 0.7	25.2 ± 0.8	121 ± 8	3757 ± 290	-2982 ± 171
Wild-type female ($n = 13$)	20.1 ± 0.6 ^a	23.9 ± 0.3 ^b	132 ± 7	3925 ± 273	-3236 ± 176
ANX-A1 ^{-/-} male ($n = 16$)	20.7 ± 0.3	24.9 ± 0.4	129 ± 8	3966 ± 227	-3358 ± 211
ANX-A1 ^{-/-} female ($n = 16$)	21.4 ± 0.4	24.0 ± 0.3	124 ± 5	3818 ± 267	-3159 ± 178
P (genotype)	NS	NS	NS	NS	NS
P (sex)	NS	$P < 0.05^b$	NS	NS	NS
P (genotype x sex)	$P < 0.05$	NS	NS	NS	NS

^aOn two-way ANOVA with SNK *post hoc* analysis, male wild-type mice tended to be older than female wild-type mice. ^bOn two-way ANOVA, male mice tended to be heavier than females. SNK *post hoc* analysis indicated this was largely evident in wild-type mice. Note the absence of sex and genotype effects on cardiac phenotype at baseline

Table 4

The extent of LV dysfunction following I-R injury was not affected by sex, in either hearts isolated from annexin-A1 wild-type or ANX-A1^{-/-} mice *ex vivo*

Experimental group	LVDP (% baseline, min post reperfusion)								AUC × 10 ³	n
	0	1	2	5	10	20	30	40		
Wild-type male I-R	51 ± 19	94 ± 27	57 ± 9	53 ± 9	50 ± 9	56 ± 11	63 ± 12	65 ± 13	2.33 ± 0.43	7
Wild-type female I-R	34 ± 9	75 ± 13	60 ± 7	45 ± 9	44 ± 13	60 ± 9	65 ± 8	63 ± 5	2.27 ± 0.31	4
ANX-A1 ^{-/-} male I-R	27 ± 4	56 ± 8 ^a	37 ± 6	10 ± 3 ^a	13 ± 3 ^a	28 ± 6	29 ± 7	29 ± 7 ^a	1.00 ± 0.20 ^a	8
ANX-A1 ^{-/-} female I-R	17 ± 5	57 ± 9	33 ± 9	16 ± 5	21 ± 7	30 ± 6	33 ± 5	34 ± 5	1.13 ± 0.20	6

Analysis of the time course of recovery of LVDP over 40 min of reperfusion on two-way ANOVA indicates that $P_{(\text{min post reperfusion})} < 0.0001$; $P_{(\text{experimental group})} < 0.0001$; $P_{(\text{interaction})} = \text{NS}$. Analysis of AUC for LVDP on two-way ANOVA indicates that $P_{(\text{gender})} = \text{NS}$; $P_{(\text{experimental group})} < 0.001$; $P_{(\text{interaction})} = \text{NS}$.

^aOn two-way ANOVA with SNK *post hoc* analysis, hearts from male ANX-A1^{-/-} have poorer recovery from I-R injury than male wild-type mice ($P < 0.01$). No significant differences were observed between male and female mice of either genotype.

protective actions of endogenous ANX-A1 on myocardial function demonstrated in the present study remains to be resolved *in vivo*. However, our results support the hypothesis that targeting ANX-A1 is a potential strategy to maintain myocardial function in settings of I-R. ANX-A1-based therapeutic strategies may facilitate both direct effects on myocardial function, as well as those downstream of anti-inflammatory actions.

Until relatively recently, the anti-inflammatory and other protective actions of full-length ANX-A1 and Ac-ANX-A1₂₋₂₆ were regarded as virtually interchangeable (Flower and Rothwell, 1994). However, evidence is now emerging that different mechanisms may mediate some components of their actions (Hayhoe *et al.*, 2006; Migeotte *et al.*, 2006; Yang *et al.*, 2009). For example, ANX-A1-mediated up-regulation of glucocorticoid-induced leucine zipper protein is FPR-

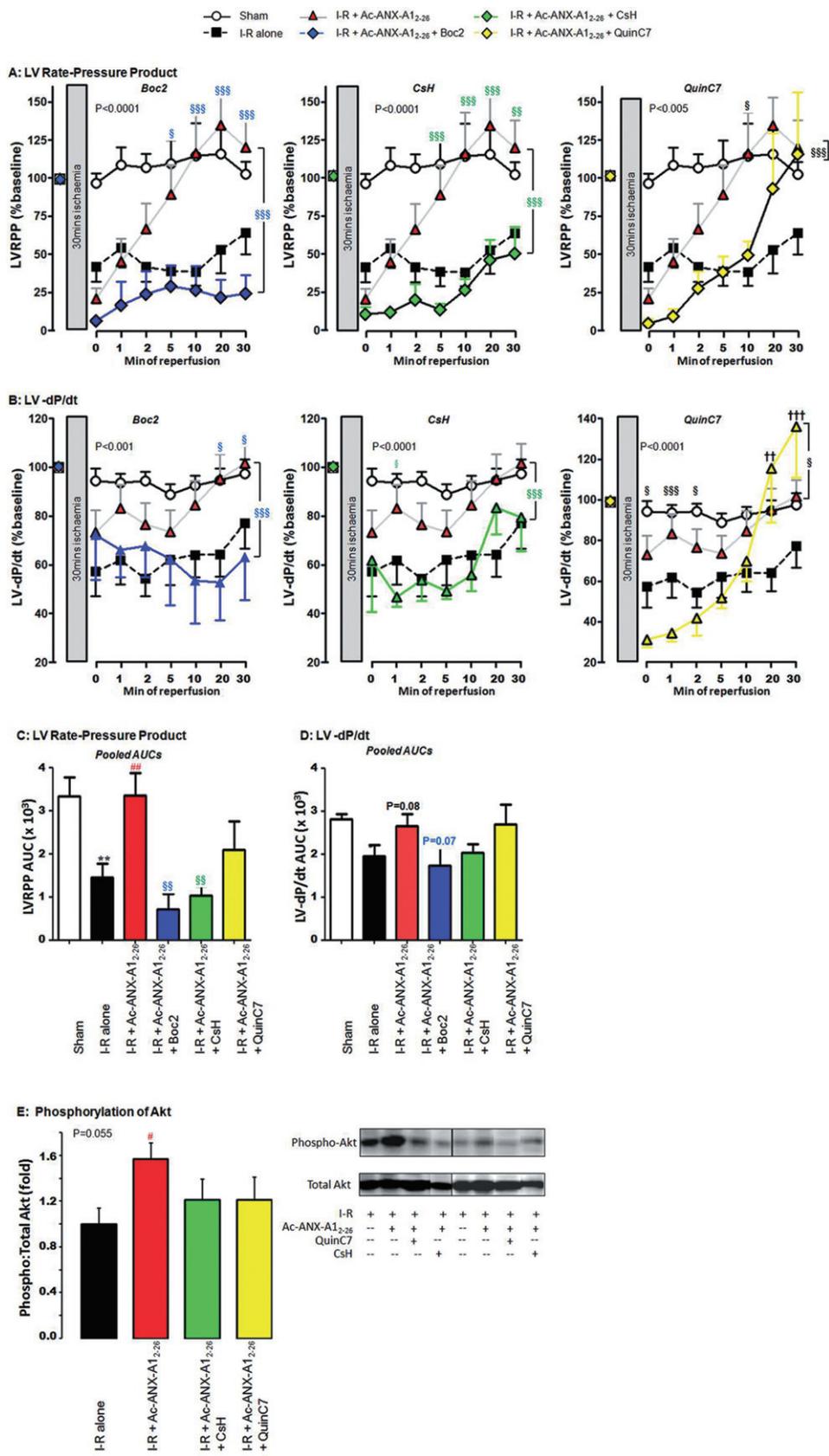


Figure 5

FP antagonism attenuates the cardioprotective effects of Ac-ANX-A1₂₋₂₆ on recovery of both (A) RPP and (B) LV-dP/dt (sham $n = 9$, untreated I-R $n = 15$, Ac-ANX-A1₂₋₂₆-treated I-R $n = 17$ and all antagonist groups $n = 6$). Recovery of (C) RPP and (D) LV-dP/dt on AUC analysis is also shown, as well as (E) Ac-ANX-A1₂₋₂₆-induced phosphorylation of Akt (all $n = 5$). * $P < 0.05$ and ** $P < 0.005$ untreated I-R versus sham; ## $P < 0.01$ Ac-ANX-A1₂₋₂₆-treated I-R versus untreated I-R rat hearts, § $P < 0.05$, §§ $P < 0.005$ and §§§ $P < 0.001$ antagonist + Ac-ANX-A1₂₋₂₆-treated I-R versus Ac-ANX-A1₂₋₂₆-treated I-R and †† $P < 0.01$ and ††† $P < 0.001$ antagonist + Ac-ANX-A1₂₋₂₆-treated I-R versus untreated I-R rat hearts respectively.

Table 5

Ac-ANX-A1₂₋₂₆ fails to restore recovery of LV function following I-R injury in hearts isolated from both male and female ANX-A1^{-/-} mice *ex vivo*

Experimental group	LVDP (% baseline, min post reperfusion)									AUC × 10 ³	n
	0	1	2	5	10	20	30	40			
Untreated wild-type I-R	45 ± 12	87 ± 17	58 ± 6	50 ± 7	48 ± 7	57 ± 7	63 ± 8	64 ± 8	2.31 ± 0.29	11	
Ac-ANX-A1 ₂₋₂₆ -treated wild-type I-R	25 ± 4	87 ± 11	67 ± 4	52 ± 8	53 ± 10	77 ± 8	81 ± 6	82 ± 6	2.89 ± 0.27	6	
Untreated ANX-A1 ^{-/-} I-R	23 ± 3	57 ± 6 ^a	35 ± 5 ^a	13 ± 3 ^a	16 ± 3 ^a	29 ± 4 ^a	31 ± 4 ^a	31 ± 4 ^a	1.05 ± 0.14 ^a	14	
Ac-ANX-A1 ₂₋₂₆ -treated ANX-A1 ^{-/-} I-R	51 ± 32	59 ± 7	62 ± 16	20 ± 5	23 ± 5	35 ± 8 ^b	36 ± 7 ^b	37 ± 7 ^b	1.36 ± 0.22 ^b	8	

Analysis of the time course of recovery of LVDP over 40 min of reperfusion on two-way ANOVA indicates that: $P_{(\text{min post reperfusion})} < 0.001$; $P_{(\text{experimental group})} < 0.001$; $P_{(\text{interaction})} = \text{NS}$. Analysis of AUC for LVDP on two-way ANOVA indicates that $P_{(\text{genotype})} < 0.0001$; $P_{(\text{experimental group})} = 0.08$; $P_{(\text{interaction})} = \text{NS}$. ^a $P < 0.05$ versus untreated wild-type. ^b $P < 0.05$ versus Ac-ANX-A1₂₋₂₆-treated wild-type mice.

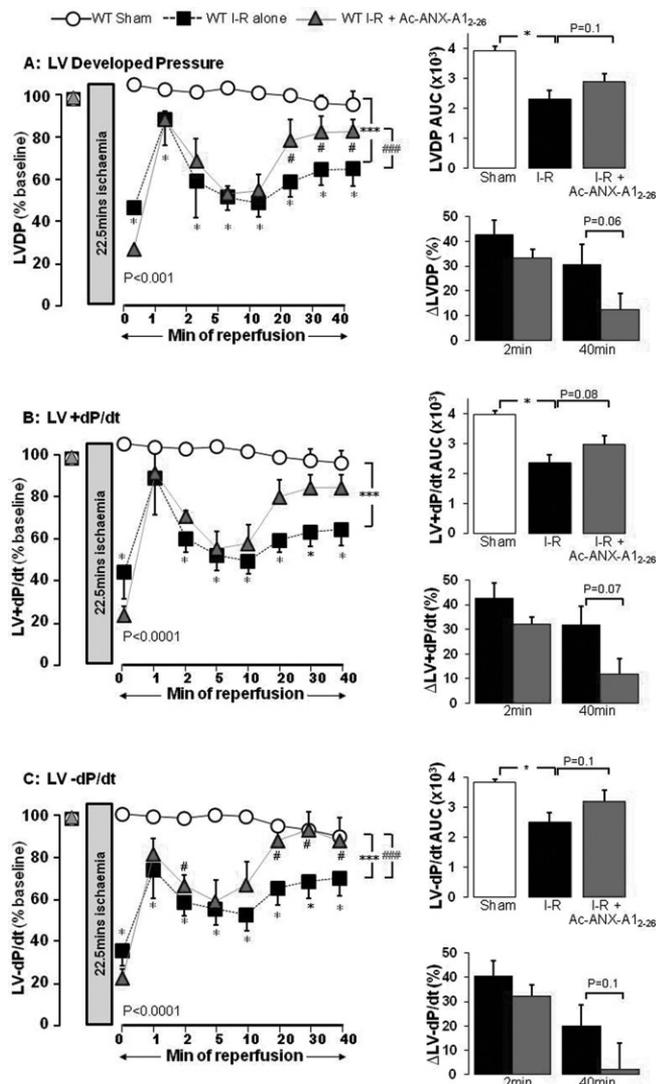


Figure 6

ANX-A1₂₋₂₆ (0.3 μM) addition at the onset of reperfusion attenuates I-R-induced impairment of LV systolic function in the isolated wild-type mouse heart *ex vivo*. Recovery of LVDP, LV + dP/dt and LV-dP/dt on AUC analysis is shown on the upper inset of each panel, and the relative impairment (i.e. the relative reduction in LV function as % of baseline) of all parameters after 2 and 30 min reperfusion are shown on the lower insets. (A) Ac-ANX-A1₂₋₂₆ accelerates recovery of LVDP up to 40 min post reperfusion ($P < 0.001$ untreated I-R, $n = 11$ versus sham $n = 10$ and $P < 0.001$ versus ANX-A1₂₋₂₆-treated I-R, $n = 6$, both on two-way ANOVA). Untreated I-R also significantly impaired recovery of LVDP on AUC analysis ($P < 0.05$), which tended to be prevented by Ac-ANX-A1₂₋₂₆ ($P = 0.1$, upper inset). The impairment in recovery of LVDP in untreated versus Ac-ANX-A1₂₋₂₆-treated I-R was not different after 2 min reperfusion ($P = \text{NS}$), but a greater recovery of LVDP in Ac-ANX-A1₂₋₂₆-treated hearts was evident after 40 min reperfusion ($P = 0.06$, lower inset). (B) Ac-ANX-A1₂₋₂₆ accelerates recovery of LV + dP/dt up to 40 min post reperfusion (untreated I-R, $n = 11$ $P < 0.001$ versus sham, $n = 10$ and $P = 0.089$ versus ANX-A1₂₋₂₆-treated I-R, $n = 6$, on two-way ANOVA). Untreated I-R significantly impaired recovery of LV + dP/dt on AUC analysis ($P < 0.05$), which tended to be prevented by Ac-ANX-A1₂₋₂₆ ($P = 0.08$, upper inset). Ac-ANX-A1₂₋₂₆ tended to improve recovery of LV + dP/dt after 40 min reperfusion ($P = 0.08$) but not after 2 min reperfusion ($P = \text{NS}$, lower inset). (C) Ac-ANX-A1₂₋₂₆ accelerates recovery of LV-dP/dt up to 40 min post reperfusion (untreated I-R $n = 11$ $P < 0.001$ vs. sham $n = 10$ and $P < 0.001$ vs. ANX-A1₂₋₂₆-treated I-R $n = 6$, both by two-way ANOVA). Untreated I-R significantly impaired recovery of LV-dP/dt on AUC analysis ($P < 0.05$), which Ac-ANX-A1₂₋₂₆ tended to prevent ($P = 0.1$, upper inset). Ac-ANX-A1₂₋₂₆ tended to improve recovery of LV-dP/dt after 40 min reperfusion ($P = 0.1$) but not after 2 min reperfusion ($P = \text{NS}$, lower inset). * $P < 0.05$ and *** $P < 0.001$ untreated I-R versus sham and # $P < 0.05$ and ### $P < 0.001$ ANX-A1₂₋₂₆-treated I-R versus untreated I-R mouse hearts respectively.

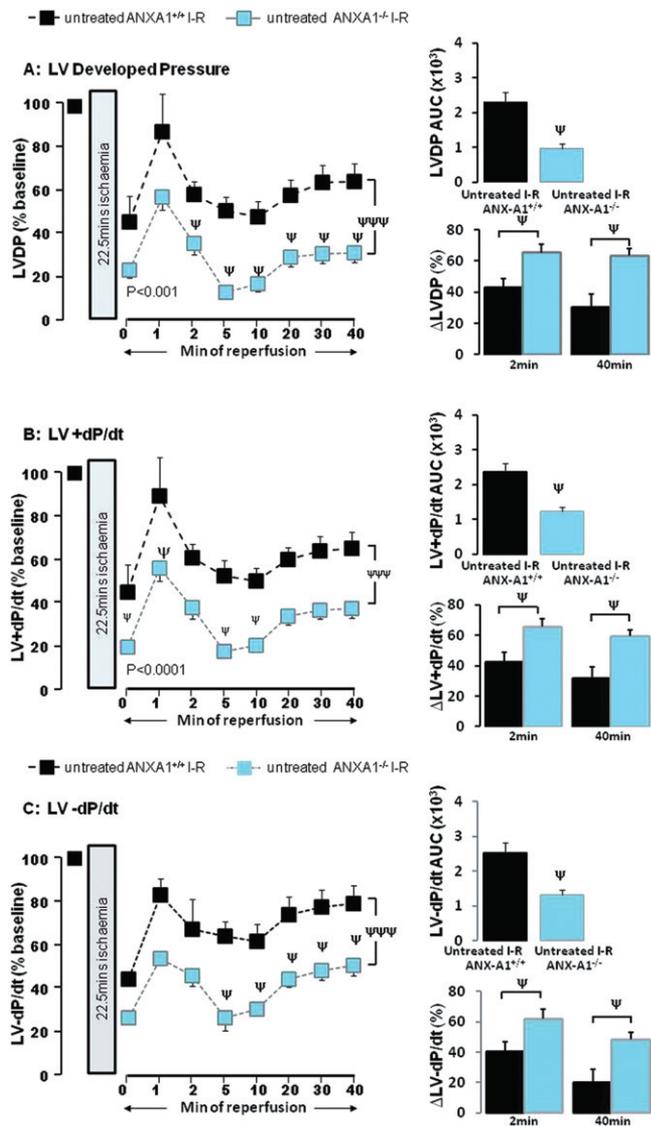
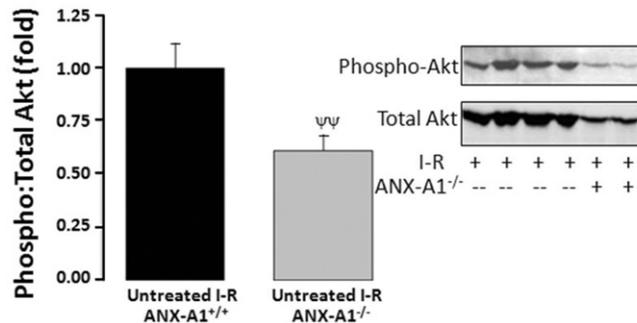


Figure 7

Deficiency of endogenous ANX-A1 exaggerates I-R-induced impairment of LV systolic function in the intact mouse heart *ex vivo*. Recovery of LVDP, LV + dP/dt and LV-dP/dt on AUC analysis is shown on the upper inset of each panel, and the relative impairment (i.e. the relative reduction in LV function as % of baseline) of all parameters after 2 and 40 min reperfusion are shown on the lower insets. (A) The time course of recovery of LVDP is significantly impaired in ANX-A1^{-/-} ($n = 14$) versus ANX-A1^{+/+} mouse hearts ($n = 11$, $P < 0.001$ on two-way ANOVA). ANX-A1^{-/-} also significantly exaggerated the impaired recovery of LVDP on AUC analysis ($P < 0.05$, upper inset), which was evident at both 2 and 40 min reperfusion (both $P < 0.05$, lower inset). (B) ANX-A1^{-/-} also significantly delayed recovery of LV + dP/dt compared with ANX-A1^{+/+} mouse hearts ($P < 0.0001$ on two-way ANOVA), which was also evident on AUC analysis ($P < 0.05$, upper inset), and at both 2 and 40 min reperfusion (both $P < 0.05$, lower inset). (C) ANX-A1^{-/-} similarly delayed recovery of LV-dP/dt compared with ANX-A1^{+/+} mouse hearts ($P < 0.001$ on two-way ANOVA), which was also evident on AUC analysis ($P < 0.05$, upper inset), and at both 2 and 40 min reperfusion (both $P < 0.05$, lower inset). $\Psi P < 0.001$ and $\Psi\Psi P < 0.001$ untreated I-R ANX-A1^{+/+} versus untreated I-R ANX-A1^{-/-} mouse hearts.

A: Phosphorylation of Akt



B: LV FPR expression in ANX-A1^{-/-}

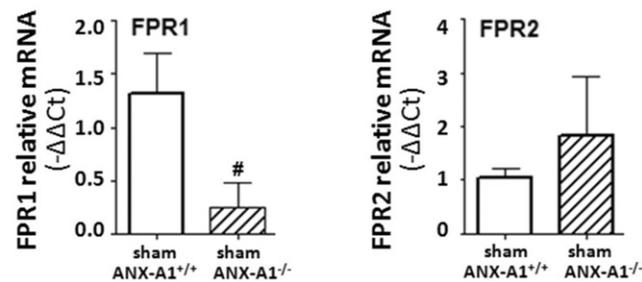


Figure 8

Deficiency of ANX-A1 (A) suppresses phosphorylation of the cell survival kinase Akt following I-R in mouse hearts (both $n = 6$, $\Psi\Psi P < 0.01$) and (B) down-regulates gene expression of FPR1; FPR2 expression is unaffected ($n = 4-5$ per group).

independent and cannot be rescued by Ac-ANX-A1₂₋₂₆ treatment *in vitro* (Yang *et al.*, 2009). Perretti and Gavins previously postulated that a component of the cardioprotective actions of Ac-ANX-A1₂₋₂₆ might be mediated by competing with full-length ANX-A1 protein for binding to the (as yet unidentified) enzyme responsible for ANX-A1 catabolism, preserving ANX-A1 bioavailability (La *et al.*, 2001). Such a mechanism would also be absent in ANX-A1^{-/-} mice and could not be restored by Ac-ANX-A1₂₋₂₆. We now demonstrate that the exaggerated impairment of LV function seen in ANX-A1^{-/-} mouse hearts subjected to I-R injury *ex vivo* also persists in the face of acute Ac-ANX-A1₂₋₂₆ treatment. Our studies thus reveal detrimental consequences of life-long systemic deficiency of ANX-A1-mediated basal myocardial FPR1 and/or FPR2 activation, or suggest FPR-independent myocardial effects of ANX-A1, analogous to those in macrophages (Yang *et al.*, 2009). Elucidating which of these possible phenomena contributed to the exaggerated impairment of LV function post I-R in ANX-A1^{-/-} mouse hearts was beyond the scope of the present study. However, our results clearly demonstrate the protective role of endogenous ANX-A1 in the myocardial response to I-R injury in the intact heart.

Cardiac necrosis, inflammation and dysfunction all increase the risk of ischaemic cardiomyopathy, heart failure and death (Moens *et al.*, 2005); each of these is targeted by ANX-A1 analogues. We thus propose that ANX-A1 analogue-based therapies represent potential cardioprotective agents

for treating I-R injury. In addition to their established inhibition of the systemic inflammatory response (D'Amico *et al.*, 2000; La *et al.*, 2001; Gavins *et al.*, 2005; 2006; Hecht *et al.*, 2009), ANX-A1 analogues address damage by acting directly on the myocardium, limiting its ability to contract and relax, as evidenced by our own observations, as well as preventing cardiomyocyte death, an important determinant of outcome from MI (Murphy and Steenbergen, 2008; Peart and Headrick, 2009). Furthermore, this protection is effective when treatment is initiated subsequent to the injury. In conclusion, our results suggest that ANX-A1-based strategies may offer novel potential therapeutic approaches for the treatment of myocardial I-R injury and dysfunction. This could be applied to both planned open heart surgery and angioplasty, unplanned injury during the development of infarction, as well as to injury such as in other cardiomyopathies associated with inflammation. Development of treatment strategies that are effective when initiated subsequent to injury, and target inflammatory cell-dependent and -independent causes of myocardial injury may ultimately reduce progression to heart failure and death in patients suffering MI. The findings presented here warrant future studies exploring the cardioprotective actions of ANX-A1 analogues on LV function *in vivo*, as well as on late myocardial necrosis (days to weeks post onset of reperfusion). With the development of synthetic, orally-active non-peptide ANX-A1 analogues (Burli *et al.*, 2006), such studies are now possible.

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Conflict of interest

The authors have no conflicts of interest with respect to this work.

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