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Annexin-1 peptide Anx-$1_{2–26}$ protects adult rat cardiac myocytes from cellular injury induced by simulated ischaemia

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1 The anti-inflammatory properties of annexin-1 peptides have been largely ascribed to their powerful antineutrophil actions *in vivo*. We have recently reported that the N-terminal fragment of annexin-1, Anx-$1_{2–26}$, preserves contractile function of cardiac muscle *in vitro*. The aim of the present study was to determine if Anx-$1_{2–26}$ elicits protective actions specifically on the cardiac myocyte (in the absence of neutrophils), using a model of metabolic inhibition to simulate ischaemia.

2 Metabolic inhibition of cardiac myocytes (4 h incubation at 37°C in HEPES-containing buffer supplemented with 2-deoxy-D-glucose, D,L-lactic acid and pH adjusted to 6.5) followed by 2.5 h recovery in normal medium markedly increased creatine kinase (CK) and lactate dehydrogenase (LDH) levels by 179 ± 39 and 26 ± 7 IU L^{-1} (both $n = 40$, $P < 0.001$), respectively. However, cellular injury was significantly decreased when Anx-$1_{2–26}$ (0.3 μM) was present during metabolic inhibition, CK by $74 \pm 10\%$ and LDH by $71 \pm 6\%$ (both $n = 31$, $P < 0.001$), respectively.

3 Boc 2 (10 μM), a nonselective formyl peptide receptor antagonist, present during metabolic inhibition, abolished the cardioprotective effect of Anx-$1_{2–26}$.

4 Addition of chelerythrine (10 μM), 5-hydroxydecanoate (500 μM) or SB202190 (1 μM) during metabolic inhibition also abolished Anx-$1_{2–26}$-induced cardioprotection.

5 Cellular injury induced by metabolic inhibition was also largely prevented when myocytes were incubated with Anx-$1_{2–26}$ for 5 min with 10 min recovery prior to the insult, or when Anx-$1_{2–26}$ was present only during the recovery period following drug-free metabolic inhibition.

6 In conclusion, the annexin-1 peptide Anx-$1_{2–26}$ potently prevents cardiac myocyte injury induced by metabolic inhibition, an action that was dependent at least in part on the activation of the formyl peptide receptor family of G-protein-coupled receptors, protein kinase C, p38 mitogen-activated protein kinase and ATP-sensitive potassium channels.
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Abbreviations: ACCT culture medium, medium 199 supplemented with albumin; L-carnitine, creatine and taurine; Akt, a protein kinase downstream of PI3 kinase; ALXR, aspirin-triggered lipoxin A_4 receptors; ANOVA, analysis of variance, Anx-$1^{-/-}$, annexin-1-null mice; Anx-$1_{2–26}$, N-terminal fragment of annexin-1; Boc 2, nonselective formyl peptide receptor antagonist BOC-2 *N*-t-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe; Chel, chelerythrine; CK, creatine kinase; CRF, corticotrophin-releasing factor; Δ, change; Erk 1/2, extracellular signal-regulated kinases (also known as p42/p44 mitogen-activated protein kinase); fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FPR, formyl peptide receptors; FPRL-1 and FPRL-2, FPR-like receptors subtypes 1 and 2; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulphonic acid); 5-HD, 5-hydroxydecanoate; IFNγ, interferon-γ; K_{ATP} channels, ATP-sensitive potassium channels; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; LXA_4, lipoxin A_4; MI, metabolic inhibition; PI3 kinase, phosphatidylinositol-3-OH kinase; p38MAP kinase, p38 mitogen-activated protein kinase; SB, water-soluble p38MAP kinase inhibitor, SB202190

Introduction

The annexin protein superfamily is comprised of over 20 distinct Ca^{2+} and phospholipid-binding proteins, at least 12 of which occur in humans. The unique spectrum of biological actions of each annexin is attributed to its specific N-terminal sequence (Flower & Rothwell, 1994). Annexin-1 (also known as lipocortin-1) is expressed constitutively in many tissues; this expression is markedly upregulated by ischaemia/reperfusion, oxidative stress and glucocorticoids (Fava *et al*., 1989; Yang *et al*., 1997; Dreier *et al*., 1998). Both annexin-1 and the peptide derived from its N-terminal, Anx-$1_{2–26}$, exert potent anti-inflammatory actions in a range of experimental models such as rheumatoid arthritis (Yang *et al*., 1997; 2004). Much of this efficacy has been attributed to actions on inflammatory phagocytes, and this includes prevention of injury induced by

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ischaemia/reperfusion (Relton *et al*., 1991; Cuzzocrea *et al*., 1997; D'Amico *et al*., 2000; La *et al*., 2001).

Myocardial ischaemia/reperfusion injury remains a major cause of morbidity and mortality despite development of treatment strategies that permit more rapid reperfusion (thrombolysis, percutaneous coronary interventions). Although the inflammatory response that results from this injury is caused in part by pathways activated by recruitment of blood-borne neutrophils into the injured myocardium (Jordan *et al*., 1999; Wang *et al*., 2002), marked damage still occurs even in the absence of neutrophils (Litt *et al*., 1989; Sheridan *et al*., 1991; Pannangpetch & Woodman, 1996). Thus, neutrophil-independent mechanisms of myocardial ischaemia/reperfusion injury are also clearly present. To date, the only investigations of annexin-1 peptides in myocardial ischaemia/reperfusion injury have focussed on reduced infiltration of neutrophils into the heart (D'Amico *et al*., 2000; La *et al*., 2001). In contrast, we have previously shown that in the absence of neutrophils, the N-terminal annexin-1 peptide Anx-$1_{2–26}$ is capable of preventing the loss of contractile function response of the myocardium induced by the inflammatory mediators endotoxin (lipopolysaccharide, LPS) and interferon-γ (IFNγ) *in vitro* (Ritchie *et al*., 1999; 2003).

The aim of the present study was to test the hypothesis that the annexin-1-derived peptide Anx-$1_{2–26}$ elicits cardioprotective actions directly on the cardiac myocyte (i.e. in the absence of circulating inflammatory cells), using our myocyte model of metabolic inhibition to simulate ischaemia (Gordon *et al*., 2003). This model incorporates a number of the physiological and metabolic changes that rapidly appear in the ischaemic myocardium, including anaerobic glycolysis and accumulation of both lactate and H^+ ions (Piper *et al*., 2003). We now demonstrate that Anx-$1_{2–26}$ powerfully prevents cardiac myocyte injury, whether administered during the ischaemic insult, as a preconditioning pulse, or during recovery from the insult. This protective action was dependent at least in part on the activation of the formyl peptide receptor family of G-protein-coupled receptors, protein kinase C, p38 mitogen-activated protein (p38MAP) kinase and ATP-sensitive potassium (K_{ATP}) channels.

Methods

Metabolic inhibition in cardiac myocytes

Male Sprague–Dawley rats (230–280 g) were anaesthetised intraperitoneally with ketamine hydrochloride (100 mg kg^{-1}) and xylazine (12 mg kg^{-1}) and hearts rapidly removed. Cardiac myocytes were freshly isolated as described previously (Gordon *et al*., 2003) and resuspended in ACCT culture medium, a serum-free bicarbonate-buffered medium 199 (Trace Scientific, Australia) supplemented with 0.2% albumin (bovine fraction V), 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 25 μg ml^{-1} gentamicin (Life Technologies, NY, U.S.A.), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (CSL Biosciences, Australia). Cells were placed in multiwell tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), with $\sim 7 \times 10^4$ rod-shaped cells ml^{-1}, and were allowed to equilibrate at 37°C with 95% air and 5% CO_2 for 48 h prior to study. This technique yields <7% nonmyocyte contamination, as described previously (Gordon *et al*., 2003). Each cardiac myocyte preparation allowed comparison of six to 12 treatment conditions. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and was approved by the Animal Ethics Committees of the Howard Florey and Baker Heart Research Institutes.

The cardiac myocyte model of metabolic inhibition-induced injury used was a modification of a previous technique (Nayeem *et al*., 1997; Gordon *et al*., 2003). On the day of study, paired cardiomyocytes were exposed to either control, HEPES (*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulphonic acid)-containing buffer comprising (in mM): NaCl (137), KCl (3.5), $CaCl_2 \cdot 2H_2O$ (0.88), $MgS0_4 \cdot 7H_2O$ (0.51), D-glucose (5.55) and HEPES (4), with 2% foetal calf serum (pH 7.4), or a buffer designed to metabolically inhibit the myocytes, for 4 h at 37°C. The metabolic inhibition buffer consisted of HEPES-containing buffer supplemented with 2-deoxy-D-glucose (10 mM) and D,L-lactic acid (20 mM), with pH adjusted to 6.5. At the end of this 4 h incubation, both control and metabolically inhibited myocytes were then allowed to recover for 2.5 h in ACCT medium. At the conclusion of the recovery period, aliquots of culture medium were collected on ice for immediate determination of both lactate dehydrogenase (LDH) and creatine kinase (CK) activity, as measures of cardiomyocyte injury. The ability of cells to exclude trypan blue was determined as a measure of cell viability.

Cardioprotection with annexin-1 peptide Anx-$1_{2–26}$: mechanism of action

Potential cardioprotective actions of the annexin-1 peptide Anx-$1_{2–26}$ were determined by supplementing the metabolic inhibition buffer with Anx-$1_{2–26}$. Myocytes were then allowed to recover in normal, drug-free culture medium. Pilot studies investigating the actions of Anx-$1_{2–26}$ over the concentrations of 0.1–30 μM demonstrated that 0.3 μM Anx-$1_{2–26}$ was the lowest concentration that effectively limited cellular injury, and this concentration was used for all subsequent experiments.

To evaluate the mechanism of the Anx-$1_{2–26}$-induced cardioprotection, both at the receptor level and on intracellular signal transduction processes, we used a range of pharmacological tools, including the nonselective formyl peptide receptor family antagonist Boc 2 (BOC-2 *N*-t-butoxy carbonyl Phe-Leu-Phe-Leu-Phe) (10 μM), and selective inhibitors of protein kinase C (chelerythrine (Chel), 10 μM) (Armstrong *et al*., 1995), K_{ATP} channels (5-hydroxydecanoate (5-HD), 500 μM) (Ishida *et al*., 2001) and p38MAP kinase (SB202190, 1 μM) (Lenoir *et al*., 2002). All inhibitors were added to the metabolic inhibition buffer in the presence and absence of Anx-$1_{2–26}$, at the lowest concentration that prevented the protective actions of the annexin-1 peptide in preliminary experiments (results not shown).

Cardioprotective actions of Anx-$1_{2–26}$ at other time points

We determined whether treating cardiac myocytes with Anx-$1_{2–26}$ (0.3 μM) at time points other than during metabolic inhibition itself, specifically as a pretreatment prior to the insult, or administration during the recovery period following the insult, would also protect cardiac myocytes against cellular injury. Cardiac myocytes were pretreated with

HEPES-containing buffer supplemented with Anx-1_{2-26} (0.3 μM) for 5 min with 10 min recovery in normal medium, immediately prior to 4 h drug-free metabolic inhibition. We have previously demonstrated that this time point mimics ischaemic preconditioning (Gordon *et al*., 2003). As this pretreatment process involved complete replacement of culture medium at the start and end of the Anx-1_{2-26} pretreatment period, an additional mechanical (sham pretreatment) control was included in these studies, whereby culture medium was replaced with drug-free HEPES-containing buffer for 5 min with 10 min recovery in normal medium, immediately prior to 4 h drug-free metabolic inhibition. Finally, in a separate series of experiments, we also determined the effect of Anx-1_{2-26} (0.3 μM) added to the serum-free defined medium during the 2.5 h recovery phase, following drug-free metabolic inhibition.

Measurement of cellular injury

At the conclusion of each experiment, LDH and CK levels were determined by spectrophotometric detection using commercially available assay kits (Sigma) as described previously (Gordon *et al*., 2003). In brief, an aliquot (250 μl) of cell suspension was assessed for each of LDH (absorbance at 340 nm, indicating oxidation of lactate to pyruvate) and CK (absorbance at 340 nm, indicating reduction of nicotinamide adenine dinucleotide) enzyme levels. For both LDH and CK determination, the paired control sample baseline level (IU l^{-1}) was subtracted from each sample to obtain absolute changes in enzyme level (i.e. ΔIU l^{-1}). The level of LDH or CK for each treatment was expressed relative to the number of cells studied and as a percent of that induced by metabolic inhibition alone (% of metabolic inhibition; where cellular injury caused by metabolic inhibition was defined as 100%) (Nayeem *et al*., 1997).

At the conclusion of the postmetabolic inhibition recovery period, cell viability was determined using trypan blue (ICN Biosciences, Aurora, OH, U.S.A.). An aliquot (250 μl) of the cardiac myocyte suspension was resuspended in 0.5 ml of phosphate-buffered saline (PBS, CSL Biosciences) and 0.5 ml of 0.4% trypan blue were added to each well. After 5 min, PBS and trypan blue were removed, and the number of stained *versus* nonstained cardiomyocytes was counted under a light microscope (Labovert FS, Leitz Wetzlar, Germany). A total of 100 cells were counted in each well. The percentage of viable cells was calculated as nonstained (live) cells per 100 counted for each treatment.

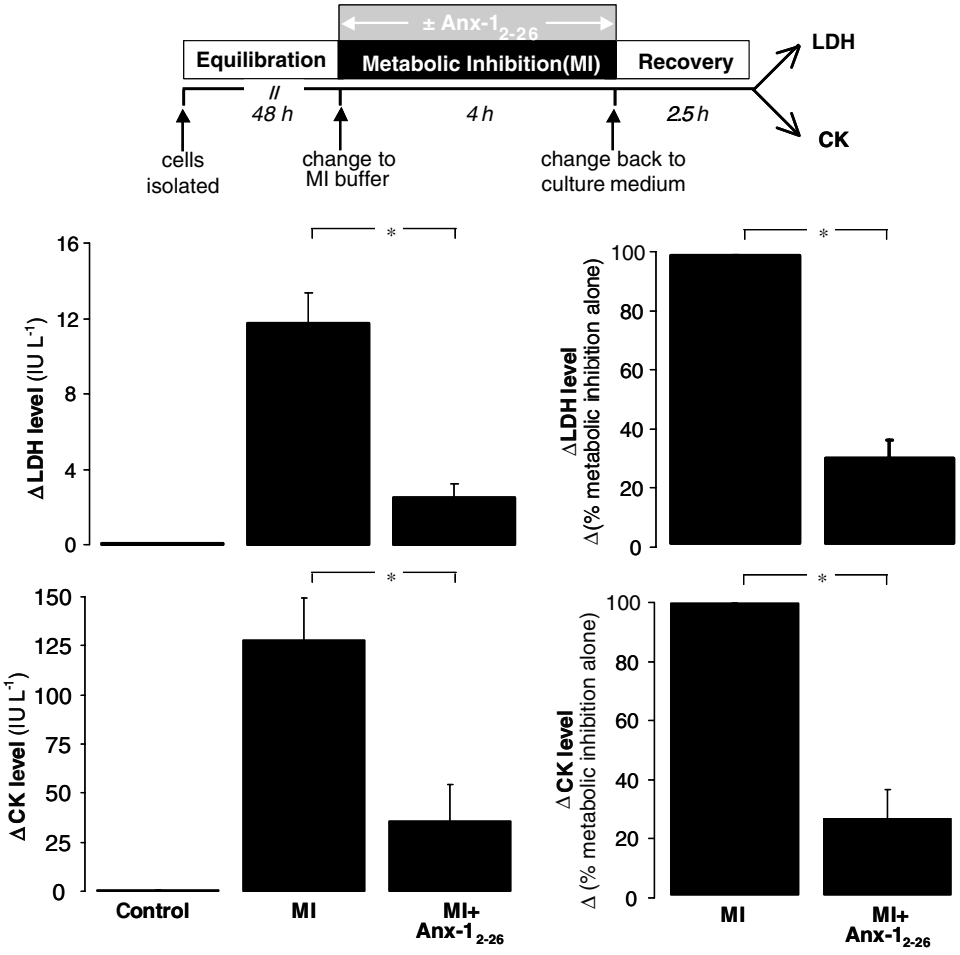

Figure 1 Exposure of adult rat ventricular cardiomyocytes to 4 h metabolic inhibition (MI) followed by 2.5 h recovery reproducibly increased levels of LDH (upper panels) and CK (lower panels). Treatment with the annexin-1 peptide Anx-1_{2-26} (0.3 μM) during MI markedly reduced this LDH and CK release. Left-hand panels show absolute increases in enzyme activity above paired control, and right-hand panels show change in enzyme activity as a percent of the change in response to MI alone (all $n=31$, $P<0.001$ on one-way ANOVA).

Data analysis

Results were expressed as mean ± standard error of the mean (s.e.m.). Evaluation of the data comparing treatment effects with metabolic inhibition, in paired cardiomyocytes from the same preparation, was performed using Kruskal–Wallis one-way analysis of variance (ANOVA), with Dunn's *post hoc* analysis for multiple comparisons where appropriate. $P<0.05$ was considered to be statistically significant.

Materials

Foetal calf serum, penicillin, streptomycin, gentamicin (CSL Biosciences, Parkville, Australia), medium 199 (JRH Biosciences, Lenexa, KS, U.S.A.), collagenase type II (Worthington, Lakewood, NJ, U.S.A.), hyaluronidase, trypsin (Sigma Aldrich, St Louis, MO, U.S.A.), bovine albumin (fraction V), L-carnitine, creatine and taurine (Sigma Aldrich, St Louis, MO, U.S.A.) were of tissue culture grade. Boc 2, Chel, 5-HD, and enzyme activity kits (CK and LDH) were of analytical grade and were obtained from Sigma Aldrich. Anx-1_{2-26} (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) was synthesised by Ian Moss, Imperial College, London, U.K.

Results

Annexin-1 peptide Anx-1_{2-26} prevents cardiac myocyte injury

Metabolic inhibition (4 h) followed by 2.5 h recovery markedly increased both CK and LDH levels above those in paired control cardiomyocytes, from 187 ± 39 to $332\pm55\,IU\,l^{-1}$, and from 5.5 ± 1.9 to $19.3\pm3.3\,IU\,l^{-1}$ (both $n=40$, $P<0.001$), respectively. However, as shown in Figure 1, cellular injury was markedly decreased when the annexin-1 peptide Anx-1_{2-26} (0.3 μM) was present during metabolic inhibition. LDH levels were reduced by $71\pm6\%$ and CK levels by $74\pm10\%$ compared with metabolic inhibition alone (both $n=31$, $P<0.001$ on repeated measures one-way ANOVA). Although some evidence of protection was observed at 0.1 μM Anx-1_{2-26} on LDH analysis (LDH was reduced to $65\pm11\%$ of metabolic inhibition alone, $n=3$), this was not evident on CK analysis (which was $110\pm34\%$ of metabolic inhibition alone, $n=3$). All further studies utilised 0.3 μM Anx-1_{2-26}, the optimal concentration for cardioprotection in this model.

Mechanism of Anx-1_{2-26} cardioprotective actions

Coincubation of cardiac myocytes with the combination of Anx-1_{2-26} (0.3 μM) and Boc 2 (10 μM), a nonselective formyl peptide receptor family antagonist, during metabolic inhibition, abolished the cardioprotective effect. LDH levels and CK levels were 118 ± 15 and $89\pm8\%$ of that induced by metabolic inhibition, respectively (Figure 2, both $n=11$, $P<0.001$ on one-way ANOVA). Supplementation of metabolic inhibition buffer with Boc 2 in the absence of Anx-1_{2-26} did not significantly modulate cellular injury on LDH or CK analysis (Figure 2, $P=$NS) and failed to decrease cell viability on trypan blue exclusion (results not shown).

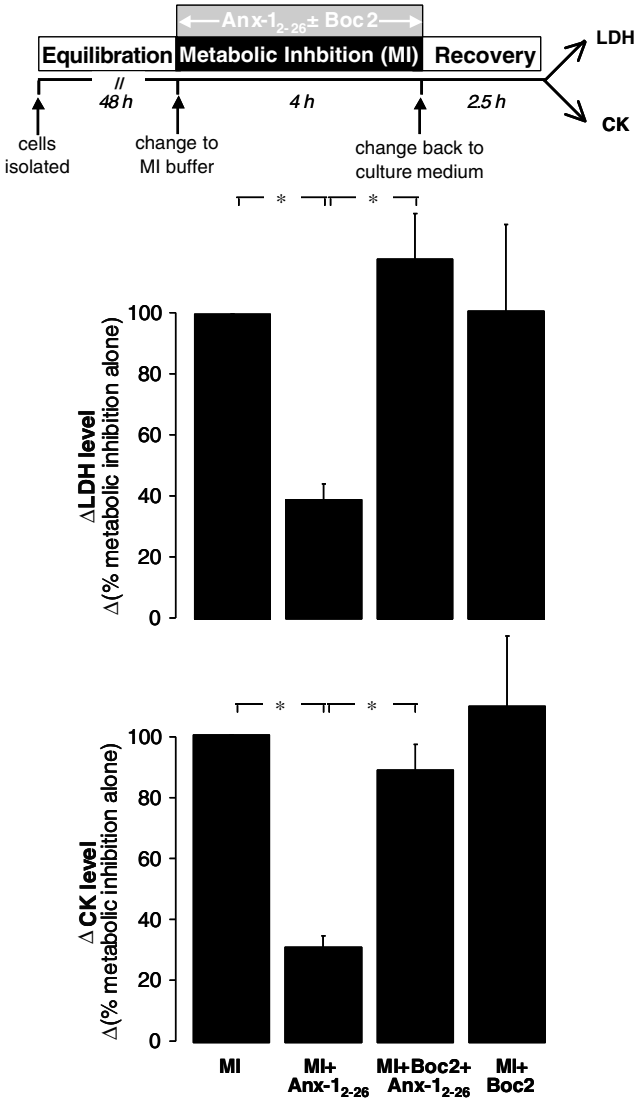

Figure 2 The nonselective formyl peptide receptor family antagonist Boc 2 (10 μM, $n=11$), present during metabolic inhibition (MI), prevents the protective actions of Anx-1_{2-26} (0.3 μM, $n=11$) on change in both LDH (upper panel) and CK (lower panel) enzyme activity, expressed as a percent of the change in response to MI alone (both $P<0.001$ on repeated measures one-way ANOVA). Boc 2 alone during MI had no effect on either LDH ($n=6$) or CK ($n=4$) activity.

Coincubation of cardiac myocytes with the combination of Anx-1_{2-26} (0.3 μM) and the protein kinase C inhibitor Chel (10 μM) during metabolic inhibition abolished the cardioprotective effect. LDH levels and CK levels were 91 ± 14 and $70\pm9\%$ of metabolic inhibition alone, respectively (Figure 3a, both $n=9$, $P<0.001$ on one-way ANOVA). Similar results were obtained with the selective mitochondrial K_{ATP} channel blocker 5-HD (500 μM) and the water-soluble p38MAP kinase inhibitor, SB202190 (SB, 1 μM). In the presence of Anx-1_{2-26} and 5-HD, LDH and CK levels were 99 ± 11 and $76\pm8\%$ of those induced by metabolic inhibition alone (Figure 3b, both $n=15$, $P<0.001$ on repeated measures one-way ANOVA). Likewise, in the presence of Anx-1_{2-26} and SB, LDH and CK levels were 87 ± 14 and $96\pm22\%$ of those induced by metabolic inhibition alone (Figure 3c, both $n=10$, $P<0.001$ on repeated measures one-way ANOVA). Supplementation of

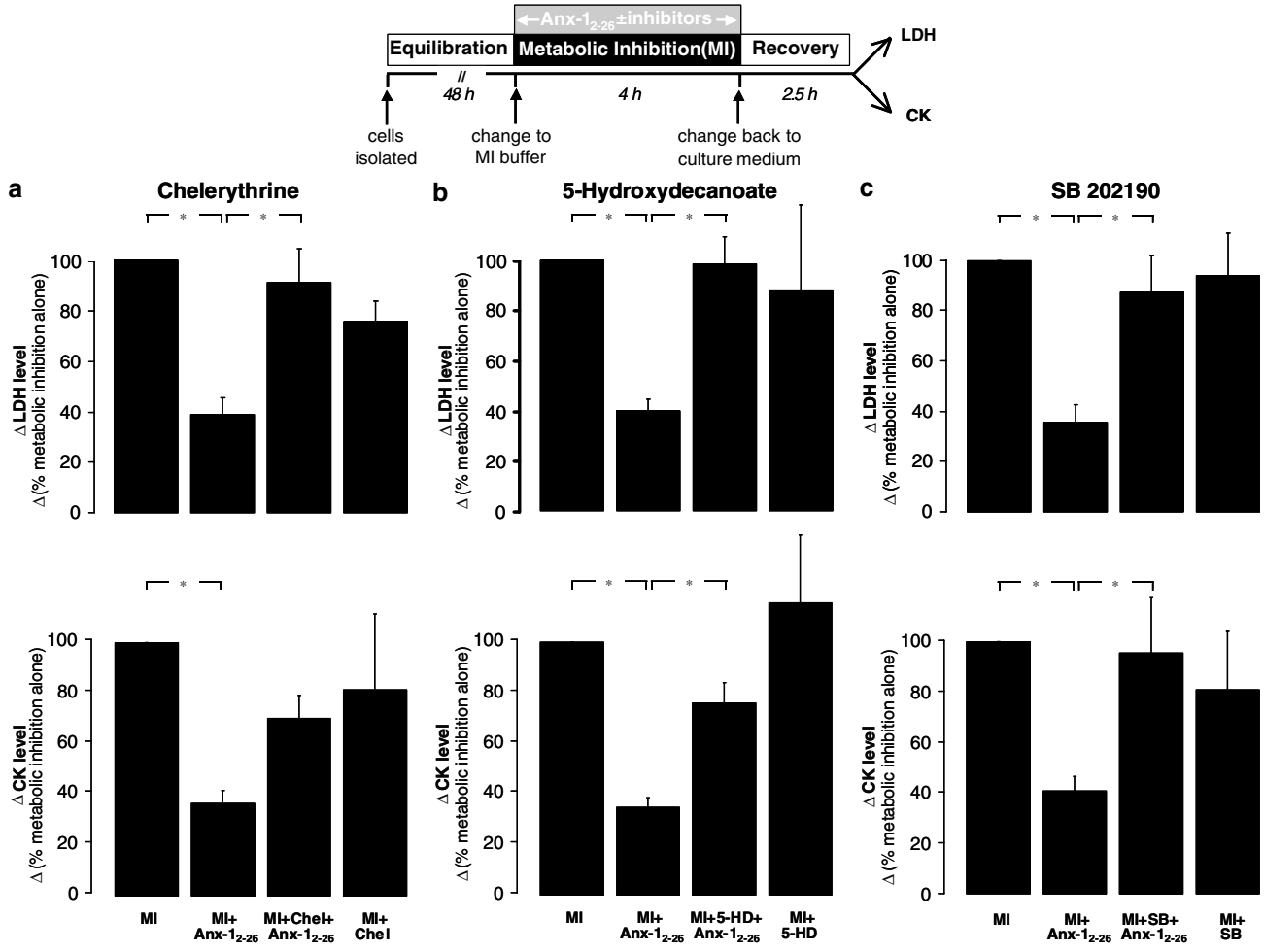

Figure 3 Selective inhibitors of protein kinase C, mitochondrial K_{ATP} channels or of p38MAPK, present during metabolic inhibition (MI), prevent the protective actions of Anx-1_{2-26} (0.3 μM) on both LDH (upper panels) and CK (lower panels). (a) Chelerythrine (chel, 10 μM) inhibited Anx-1_{2-26} actions during MI ($n=9$, $P<0.001$ on one-way ANOVA), but had no effect on its own on either LDH ($n=6$) or CK ($n=4$) activity. (b) 5-HD (500 μM) inhibited Anx-1_{2-26} actions during MI ($n=15$, $P<0.001$ on one-way ANOVA), but had no effect on its own on either LDH ($n=6$) or CK ($n=3$) activity. (c) SB202190 (SB, 1 μM) inhibited Anx-1_{2-26} actions during MI ($n=10$, $P<0.01$ on one-way ANOVA), but had no effect on its own on either LDH ($n=5$) or CK ($n=3$) activity.

metabolic inhibition buffer with any of these inhibitors (Chel, 5-HD or SB) in the absence of Anx-1_{2-26} did not significantly modulate cellular injury on LDH or CK analysis (Figure 3, $P=$NS) and failed to decrease cell viability on trypan blue exclusion (results not shown).

Cardioprotective actions of Anx-1_{2-26} at other timepoints

Pretreatment of cardiac myocytes for 5 min with annexin-1 peptide Anx-1_{2-26} (0.3 μM in normal HEPES-containing buffer) followed by 10 min washout in normal culture medium prior to drug-free metabolic inhibition buffer caused a marked decrease in cellular injury. LDH and CK levels were reduced to levels comparable to levels observed in control myocytes, to $20\pm18\%$ above control and $3\pm20\%$ below control, respectively (Figure 4a). However, sham-pretreated cardiac myocytes (exposed to drug-free HEPES-containing buffer for 5 with 10 min washout in normal culture medium prior to metabolic inhibition) were not protected: LDH levels and CK levels were $83\pm6\%$ and $75\pm6\%$ of metabolic inhibition alone, respectively. Exposure of cardiac myocytes to Anx-1_{2-26} (0.3 μM) only during the 2.5 h recovery period following drug-free metabolic inhibition significantly reduced cellular injury. LDH levels were reduced by $85\pm27\%$ and CK levels by $67\pm21\%$ (Figure 4b). On repeated measures one-way ANOVA, the protection elicited by Anx-1_{2-26} either prior to the start or following the completion of the metabolic inhibition period was significant for both LDH ($P<0.005$, $n=6$) and CK ($P<0.05$, $n=8$) analyses.

Discussion

In the current study, we demonstrated that Anx-1_{2-26} prevents injury to cardiac myocytes induced by mimicking the anaerobic glycolysis of ischaemia. This is the first report that the N-terminal annexin-1 peptide fragment Anx-1_{2-26} has a direct protective action at the level of the cardiac myocyte. Prevention of injury induced by simulated ischaemia was dependent at least in part on the activation of the formyl

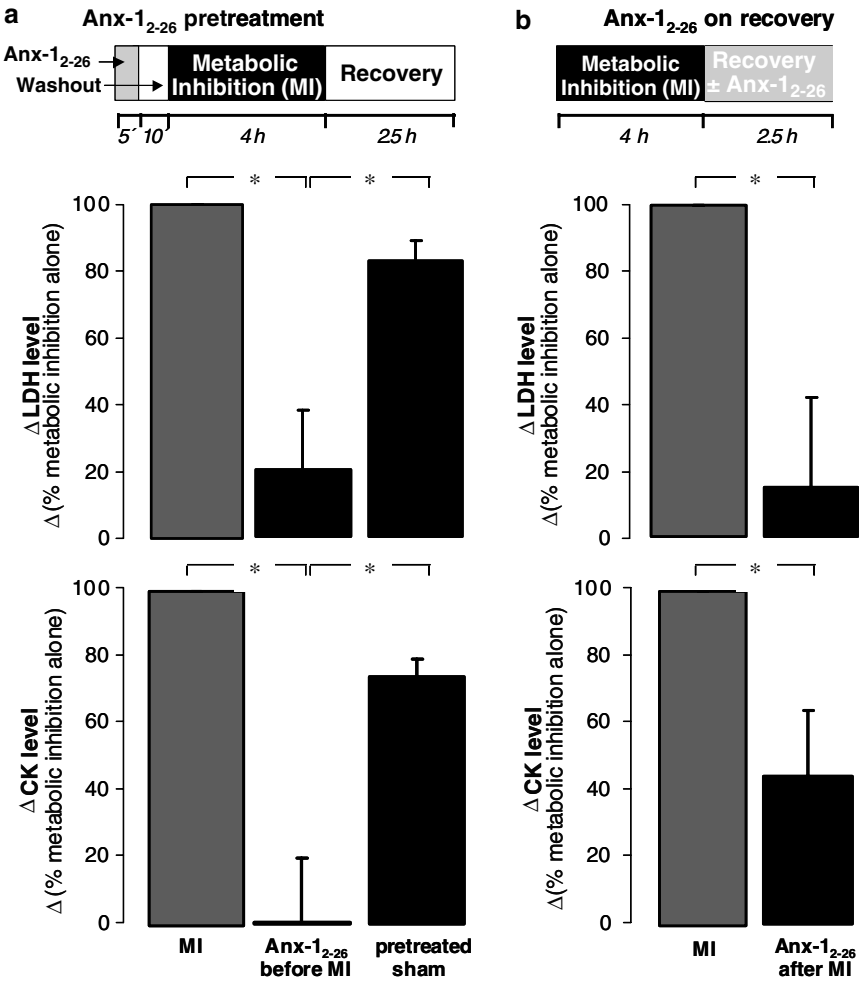

Figure 4 Treatment with the annexin-1 peptide Anx-1_{2-26} (0.3 μM) at other time points also markedly reduced LDH and CK release. (a) Anx-1_{2-26} or sham (mechanical control in which culture medium is replaced) added for 5 mins, with 10 mins washout (culture medium again replaced) immediately prior to metabolic inhibition (MI). (b) Anx-1_{2-26} added to culture medium during recovery from MI. Upper panels show change in LDH (both $n=6$, $P<0.005$ on one-way ANOVA) and lower panels show change in CK (both $n=8$, $P<0.05$ on one-way ANOVA), both as a percent of the change in response to MI alone.

peptide receptor family of G-protein-coupled receptors, as well as protein kinase C, mitochondrial K_{ATP} channels and p38MAP kinase. Furthermore, Anx-1_{2-26} protected cardiac myocytes whether given before, during or after metabolic inhibition, and was comparable to the benefit we have previously observed with adenosine or by pretreating cardiac myocytes with a transient episode of metabolic inhibition (which could be regarded as a preconditioning stimulus: Gordon *et al*., 2003).

The mechanisms by which annexin-1 and related peptides exert their anti-inflammatory actions are still not fully understood. Recruitment of neutrophils by injured tissues, their subsequent attachment to activated endothelium and migration from the vascular lumen into underlying tissues are all inhibited by annexin-1 peptides. Phospholipase A_2 activity, and release of arachidonic acid and prostanoid release are also suppressed (Cirino & Flower, 1987; Goulding *et al*., 1990; Flower & Rothwell, 1994; Yang *et al*., 1997), which can limit the infiltration of neutrophils from the vasculature into the myocardium (D'Amico *et al*., 2000; La *et al*., 2001). Thus, annexin-1 peptides are clearly effective in limiting the neutrophil-mediated component of ischaemia/reperfusion injury, which certainly contributes to cardioprotection. From our studies both in cardiac myocytes subjected to metabolic inhibition in the present investigation, and in myocardial preparations suppressed by endotoxin and IFN-γ (Ritchie *et al*., 1999; 2003), it is clear, however, that annexin-1 peptides also exert direct, neutrophil-independent, cardioprotective actions. Development of new treatment strategies that protect against both neutrophil-dependent and neutrophil-independent mechanisms of ischaemia/reperfusion injury could have major clinical impact, and targeting annexin-1-related mechanisms represents such a strategy. Consistent with this proposal, recent studies in annexin-1-null (Anx-$1^{-/-}$) mice indicate that disruption of annexin-1 upregulates expression of proinflammatory genes including cyclooxygenase-2 and phospholipase A_2, with no overt effect on development (Hannon *et al*., 2003). These mice also have an exaggerated response to both acute (zymosan, carrageenin) and chronic (arthritis) inflammatory stimuli, characterized by increased neutrophil migration and cytokine production (Hannon *et al*., 2003; Yang *et al*., 2004). Furthermore, cultured lung fibroblasts from Anx-$1^{-/-}$ mice exhibit hyper-responsiveness to endotoxin (Alldridge *et al*., 1999). Whether *in vivo* responses to myocardial ischaemia/

reperfusion injury, both neutrophil-dependent and those exerted at the level of the cardiac myocyte, are exaggerated in Anx-$1^{-/-}$ mice, remains to be addressed.

The signal transduction pathways utilised by annexin-1 peptides, including its putative receptors, have not been fully elucidated. Evidence is now emerging from both receptor antagonists (Boc 1, Boc 2) and transgenic knockout approaches that the annexin-1 peptides bind and activate the formyl peptide receptor family of receptors to exert their anti-inflammatory, antineutrophil and other actions (Perretti *et al*., 2001; 2002). This family comprises formyl peptide receptors (FPR) and the FPR-like receptors, aspirin-triggered lipoxin A_4 receptors (known as FPR-like receptor subtype 1, FPRL-1 or ALXR) and FPR-like receptor subtype 2 (FPRL-2), all of which have seven transmembrane-spanning domains functionally coupled to guanine nucleotide-binding protein G_i (Perretti, 2003). Homology between these receptors is relatively high at both the nucleotide and amino-acid levels (Gavins *et al*., 2003; Perretti & Gavins, 2003). The bacterial surrogate peptide, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), binds to FPR to mediate its proinflammatory chemotactic actions but is not a ligand at ALXR (Perretti *et al*., 2001; Perretti, 2003). Neutrophil-derived eicosanoids including lipoxin A_4 and 15-epi-LXA_4 are selective agonists at ALXR, and elicit potent antineutrophil and other anti-inflammatory actions subsequent to receptor binding (Chiang *et al*., 2000; Levy & Serhan, 2002). Annexin-1 and Anx-$1_{2–26}$ are thought to bind to both FPR and ALXR (Gavins *et al*., 2003). Moreover, both exhibit specific and saturable binding to recombinant human ALXR ($K_d \sim 0.9\,\mu$M, in line with concentrations used in our myocyte studies). Recent receptor knockout studies suggest, however, that the anti-inflammatory actions of these annexin-1 peptides are mediated solely by activation of ALXR (Gavins *et al*., 2005). Boc2 is a nonselective FPR family antagonist used in the present study to oppose the actions of Anx-$1_{2–26}$. It was derived from fMLP, and the bulky butoxyl-carbonyl moiety is thought to block receptor function (Perretti, 2003). At present, no antagonists are available to distinguish between FPR family subtypes, thus we were unable to identify the precise FPR receptor subtype mediating Anx-$1_{2–26}$ actions in cardiac myocytes. Our studies have confirmed, however, that this receptor family are involved in annexin-1 actions on the cardiac myocyte.

In neutrophils, FPR signalling downstream of G-protein coupling has been postulated to include stimulation of inositol phosphate turnover, mobilization of calcium from intracellular stores and modulation of other intracellular signals such as protein kinase C and p38 MAP kinase (Le *et al*., 2004). Results from the present study, using a metabolic inhibition model of cardiac myocyte injury and selective pharmacological inhibitors indicate that stimulation of both protein kinase C and p38MAP kinase are implicated in the actions of Anx-$1_{2–26}$, at least when present during a myocardial insult such as metabolic inhibition. Opening of mitochondrial K_{ATP} channels, together with protein kinase C activation, is an important upstream trigger of p38MAP kinase stimulation in cardioprotection mediated by many stimuli, including ischaemic preconditioning, adenosine and the corticotrophin-releasing factor (CRF) ligand urocortin (Liu *et al*., 1997; Gordon *et al*., 2003; Yellon & Downey, 2003). Using 5-HD, our data also indicate opening of mitochondrial K_{ATP} channels in the actions of Anx-$1_{2–26}$. One limitation of the study is that our experiments were not designed to elucidate precisely whether the signals mediating the protective actions of Anx-$1_{2–26}$ treatment during metabolic inhibition occurred in parallel or in series. Furthermore, the mechanisms of Anx-$1_{2–26}$ cardioprotection, when administered as a pretreatment or during recovery from injury, were not investigated. For example, activation of phosphatidylinositol-3-OH kinase (PI3 kinase), Akt (downstream of PI3 kinase) and/or p42/p44 extracellular signal-regulated kinases (Erk 1/2) at the onset of reperfusion have also been shown to reduce markedly ischaemia/reperfusion injury (Hausenloy & Yellon, 2004). Whether the same mechanisms that mediate cardioprotection with Anx-$1_{2–26}$ treatment during a myocardial insult (protein kinase C, p38MAP kinase, mitochondrial K_{ATP} channels) are implicated in the protective actions of Anx-$1_{2–26}$ treatment at these other time points and/or the role of other signalling cascades such as PI3 kinase/Akt and Erk 1/2 remains to be elucidated.

In conclusion, our studies demonstrated that the annexin-1 peptide Anx-$1_{2–26}$ exerts direct (i.e. neutrophil-independent) protective actions in cardiac preparations *in vitro*. The peptide Anx-$1_{2–26}$ is effective whether present during the insult itself, administered as a preconditioning pulse beforehand or during recovery from the insult (analogous to postconditioning). To our knowledge, this is the first report in which treatment commenced at the onset of ischaemia is evaluated in the same study as preconditioning and postconditioning treatments. We propose that annexin-1 peptides reduce myocardial damage *in vivo via* two actions: a direct effect on the myocardium and an indirect action *via* inhibition of neutrophil activation. Further, the annexin-1 peptides likely represent a novel therapeutic approach for several reasons: myocardial ischaemia/reperfusion injury is an inflammatory disorder ameliorated by annexin-1, and there is potential to regulate its activity in the body. Given that the peptide Anx-$1_{2–26}$ was effective prior to, during or after the insult, our findings are of particular significance, and the potential for exploiting this mechanism of cardioprotection will be further extended when synthetic, nonpeptide annexin-1 mimetics or inhibitors of annexin-1 degradation become available. Such new strategies could be utilized for preserving postischaemic myocardium in humans, in terms of both evolving infarction and controlled ischaemic episodes such as bypass surgery and balloon angioplasty.

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