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Cardioprotective actions of an N-terminal fragment of annexin-1 in rat myocardium in vitro

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Abstract

We have previously shown that the glucocorticoid dexamethasone prevents the cardiodepressant actions of interferon-γ plus lipopolysaccharide in cardiac tissue in vitro. We now demonstrate that an N-terminal fragment of annexin-1 (Ac2-26, 1 μM), a putative mediator of glucocorticoid actions, completely protects against interferon-γ + lipopolysaccharide-induced depression of the inotropic response to isoprenaline in rat isolated papillary muscles. However, Ac2-26 does not preserve resting contractile function. Fifteen hours incubation with interferon-γ + lipopolysaccharide also markedly induced mRNA expression (by real time polymerase chain reaction, PCR) of both the nitric oxide synthase 2 (NOS2) isoform of nitric oxide synthase (by 6.7 ± 1.7-fold, $P < 0.01$) and cyclo-oxygenase-2 (by 3.4 ± 0.6-fold, $P < 0.05$) in cardiomyocytes. Pretreatment with Ac2-26 (1 μM) prevented the induction of cyclo-oxygenase-2 mRNA, but not NOS2 mRNA, whereas dexamethasone (1 μM) suppressed the expression of both NOS2 mRNA and cyclo-oxygenase-2 mRNA. Co-incubation of dexamethasone with an anti-annexin-1 antibody did not attenuate the suppression of NOS2 mRNA. Thus, Ac2-26 reproduces some, but not all, of the cardioprotective effects of glucocorticoids in vitro in the absence of neutrophils. These protective actions are independent of changes in NOS2 expression.

Keywords: Cardiomyocyte; Cyclo-oxygenase-2; Cytokine; Annexin-1; Glucocorticoid; Nitric oxide (NO) synthase 2

1. Introduction

Annexin-1, also known as lipocortin-1, is a 37-kDa member of the annexin family of Ca^{2+} and phospholipid-binding proteins, expressed constitutively in many cells, including granules within the neutrophil cytoplasm (Dreier et al., 1998; Flower and Rothwell, 1994; La et al., 2001b). The full-length annexin-1 protein consists of 348 amino acids, comprising 4 series of repeated amino acid sequences attached to an N-terminal sequence. The N-terminal sequence of annexin-1 is thought to contain important regulatory phosphorylation sites (Flower and Rothwell, 1994; La et al., 2001b). Annexin-1 is released upon neutrophil adhesion to endothelial cells, and is then thought to bind to extracellular neutrophil receptors to suppress inflammatory signalling cascades, such as phospholipase A_2 and arachidonic acid release (Cirino and Flower, 1987; Flower and Rothwell, 1994; La et al., 2001b). Exogenous annexin-1 administration has also been shown to elicit protective antiproliferative and anti-inflammatory actions via these anti-neutrophil mechanisms both in vitro and in vivo, but the intracellular mechanisms involved in these actions have not been further elucidated (Flower and Rothwell, 1994; Hirata et al., 1980; Perretti and Flower, 1994). Annexin-1 is also thought to function as a second messenger for many glucocorticoid actions, particularly in circulating inflammatory cells such as neutrophils and macrophages, following glucocorticoid induction of annexin-1 gene expression (Flower and Rothwell, 1994). Many N-terminal annexin-1-derived peptides, such as the 25 amino acid fragment Ac2-26, display similar activity to the full-length protein, although there are some anti-inflammatory effects dependent on another sequence known as antiflammin-2 (Moreno, 1996). N-terminal peptides reproduce potent inhibition of neutrophil function, including their expression of adhesion molecules, attachment to activated endothelium and migra-

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tion out of the vascular lumen (Flower and Rothwell, 1994; La et al., 2001a; Lim et al., 1998; Perretti et al., 1993). Recently, annexin-1 peptides have been shown to exert cardioprotective actions against myocardial ischaemia and reperfusion injury in the rat, and these are attributed at least in part to actions on neutrophils (D'Amico et al., 2000; La et al., 2001a). We have now looked for actions of an N-terminal peptide directly on cardiac tissue in vitro.

Myocardial depression in septic shock results from the action of endotoxins, particularly the lipopolysaccharide component of the bacterial cell wall, which induces the release of cytokines such as interleukin-1β, interferon-γ and tumour necrosis factor α into the bloodstream (Danner et al., 1991; Kumar et al., 1996). The induction of nitric oxide synthase (isoform NOS2) and subsequent increased release of nitric oxide is believed responsible for the hypotension and the cardiac contractile dysfunction (both resting and in response to β-adrenoceptor stimulation) observed in septic shock or direct exposure to endotoxin or cytokines (Balligand et al., 1994; Cannon et al., 1998; Thiemermann, 1994). We have previously demonstrated that the glucocorticoid dexamethasone protects inotropic responsiveness from endotoxin-induced myocardial depression in isolated hearts: this cardioprotection is mimicked by exposure of these hearts to NOS2 inhibitors (including both N^{ω}-nitro-L-arginine and mercaptoethylguanidine) in vitro (Sun et al., 1997). Other anti-inflammatory mechanisms of glucocorticoids include inhibition of phospholipase A_2 activation, inhibition of pro-inflammatory lipid mediator production (leukotrienes, prostaglandins and platelet-activating factor) and suppression of neutrophil migration, all of which are elevated in response to endotoxin or cytokines (Cannon et al., 1998; Newton et al., 1997; Perretti and Flower, 1994).

Some time ago, we demonstrated that pretreatment with dexamethasone prevents the cardiodepressant effects of interferon-γ + lipopolysaccharide on both resting contractile function and inotropic responsiveness to isoprenaline in vitro. Western blot analysis suggested the cardioprotective effect was mediated by suppression of NOS2 protein expression (Sun et al., 1998). We have now sought to determine whether an N-terminal fragment of annexin 1, Ac2-26, also elicited direct cardioprotective actions on rat myocardial tissue. In a short communication as part of the proceedings of a scientific meeting, we reported that Ac2-26 preserved the inotropic β-adrenergic peak tension response at least as well as dexamethasone (Ritchie et al., 1999). We now expand these findings to a full analysis of the cardioprotective actions of Ac2-26 on inflammatory mediator-induced myocardial dysfunction, and determine the role of NOS2 mRNA expression in this protection, all in the absence of neutrophils. We find that Ac2-26, like glucocorticoids, preserves inotropic responsiveness at the level of ventricular muscle, but this action is independent of NOS2 expression. However, Ac2-26 does not protect baseline contractile function.

2. Materials and methods

2.1. Materials

Lipopolysaccharide, recombinant murine interferon-γ, dexamethasone, L-arginine and isoprenaline were obtained from Sigma (St. Louis, MO). The N-terminal fragment of annexin-1, Ac2-26 (Ac-Ala-Met-Val-Ser-Glu-Phe-Leu-Lys-Gln-Ala-Trp-Phe-IIe-Glu-Asn-Glu-Glu-Gln-Glu-Tyr-Val-Gln-Thr-Val-Lys-OH) was obtained from Bachem (Bubendorf, Switzerland), and tetrahydrobiopterin from ICN Biomedicals (Costa Mesa, CA). Reagents for Western blot analysis and for real time polymerase chain reaction (PCR) analysis, including TaqMan® Reverse Transcription Reagents, TaqMan® Universal PCR master mix, primers and probes, were of molecular biology grade and were purchased from Applied Biosystems (Branchburg, NJ). Lyophilised sheep anti-human annexin-1 antisera raised against a peptide epitope derived from the annexin-1 N-terminus was kindly provided by Prof. R.J. Flower, William Harvey Research Institute, London, UK. All reagents for cell culture were of tissue culture grade, and for RNA extraction, reagents were of molecular biology grade. All other materials were purchased from Sigma except where indicated, and were of analytical grade.

2.2. Papillary muscle studies

The investigation conforms to the European Community guidelines for the use of experimental animals, and was approved by the Howard Florey Institute Animal Experimentation Ethics Committee. Papillary muscles were isolated from male Sprague–Dawley rat hearts (250–400 g) under ether/oxygen anaesthesia and attached by silk thread to a Grass FT03C force-displacement transducer (Grass Instruments, Quincy, MA). Tissue baths containing Krebs buffer (composition in mM: NaCl 118; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; glucose 11) were bubbled with 95% O_2/5% CO_2 throughout the experiment to maintain pH at 7.4. Tissue bath temperature was maintained at 30 °C, as previously described (Sun et al., 1998).

Muscles were electrically driven by S-44 stimulators (Grass Instruments) at 0.2 Hz. Rectangular pulses 5 ms in duration and 20% above threshold (< 20 V) were delivered through a pair of longitudinal platinum electrodes in close proximity to the muscles. After an equilibration period of about 30 min, baseline isometric contraction parameters (peak isometric developed tension and its first derivatives, $+dT/dt_{max}$ and $-dT/dt_{min}$) were recorded using Maclab (Analog Digital Instruments, Castle Hill, Australia). Muscle length was maintained at maximum active tension for the duration of the experiment.

Cumulative concentration–response curves to isoprenaline (0.001–10 μM) were generated for peak tension, $+dT/dt_{max}$ and $-dT/dt_{min}$. Five consecutive contractions were sampled and averaged for each measurement. Beginning 30

min after washout of isoprenaline, papillary muscles were incubated for 15 h in control conditions (no drugs present) or in the presence of interferon-γ (100 U/ml) + lipopolysaccharide (100 μg/ml, added 1 h after interferon-γ). During this long incubation, stimulation frequency was reduced to 0.1 Hz. The bathing buffer was supplemented with tetrahydrobiopterin (5 μM) and L-arginine (0.1 μM) for the 15 h incubation period. After the incubation, the frequency was readjusted to 0.2 Hz for 30 min prior to construction of a second isoprenaline concentration–response curve. The influence of Ac2-26 (1 μM, added 30 min before interferon-γ) on changes in isometric contraction parameters induced by interferon-γ + lipopolysaccharide, both resting and inotropic response to isoprenaline, were determined.

2.3. Isolation of adult rat cardiomyocytes

Cardiomyocytes from adult male Sprague–Dawley rats (200–300 g) were freshly dissociated with >93% cardiomyocyte content as previously described (Ritchie et al., 1998), for measurement if changes in mRNA and protein expression. Cardiomyocytes were suspended in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY). The medium was supplemented with 12.5 U/ml penicillin, 12.5 μg/ml streptomycin, 10% heat-inactivated foetal calf serum and 1% glutamine (TRACE Scientific, Noble Park, Australia). Cardiomyocytes were incubated at 37 °C in six-well plates (Falcon/Becton Dickinson, Lincoln Park, NJ) for 15 h allowing paired comparisons of the various treatments with control. Treatment conditions comprised (i) control (no drugs present), (ii) interferon-γ (100 U/ml) + lipopolysaccharide (100 μg/ml), (iii) interferon-γ + lip-lipopolysaccharide + Ac2-26 (1 μM), and (iv) interferon-γ + lipopolysaccharide + dexamethasone (1 μM). Ac2-26 and dexamethasone were added 30 min prior to interferon-γ + lipopolysaccharide. The mRNA studied was extracted from up to seven separate cardiomyocyte preparations, isolated from seven different rats. In three preparations, the effect on mRNA expression of the N-terminally directed antibody to annexin-1 (1:60 dilution) was determined, added 3.5 h prior to dexamethasone.

2.4. Real time PCR analysis of NOS2 and cyclo-oxygenase-2 mRNA

Cardiomyocytes were washed with Dulbecco's phosphate-buffered saline (CSL, Parkville, Australia) twice prior to total RNA extraction, using the single-step acid guanidine–thiocyanate method (Chomczynski and Sacchi, 1987). The cardiomyocyte pellet was denatured (4 M guanidine thiocyanate/0.5% *N*-lauroyl-sarcosine/25 mM sodium citrate/0.1 M 2-mercapto-ethanol) prior to addition of 50 μl 2 M sodium acetate (pH 4), 500 μl water-saturated phenol and 200 μl chloroform/isoamyl alcohol (49:1). After 15 min incubation on ice and centrifugation, the aqueous phase was diluted 1:1 in ice-cold 100% isopropanol and stored at −20 °C for 30 min. Following centrifugation, the pellet was resuspended in 0.6 ml denaturing solution (above). RNA was then precipitated with one volume of 100% isopropanol (30 min at −20 °C prior to centrifugation), washed with 100 μl 75% ethanol, and resuspended in diethyl-pyrocarbonate-treated water. Total RNA was then reverse-transcribed to a final cDNA concentration of 10 ng/μl using TaqMan® reverse transcription reagents (Applied Biosystems). Reverse transcription was performed at 48 °C for 30 min followed by RT inactivation at 95 °C for 5 min (Perkin-Elmer GeneAmp 9600).

Real time PCR detects the accumulation of PCR product at every cycle of amplification using fluorescently labelled hybridisation probes (Heid et al., 1996; Rosenkranz et al., 2003). Relative quantification of changes in NOS2 and cyclo-oxygenase-2 mRNA expression were determined using real time PCR analysis and the ABI Prism® 7700 Sequence Detection System, with 18S ribosomal RNA as the endogenous control. In brief, forward and reverse primers and a fluorogenic probe were constructed for each test gene (NOS2 and cyclo-oxygenase-2), according to Perkin Elmer TaqMan® PCR specifications, from rat-specific sequences published on GenBank. Control TaqMan® 18S ribosomal RNA primers and probe were purchased from Applied Biosystems. The sequences of primers and probes used for PCR analysis are shown in Table 1. TaqMan® probes were labelled at the 5′ end with a fluorescent reporter dye, either FAM for the two test genes, or VIC for the endogenous control 18S (Table 1). A second fluorescent label was positioned at the 3′ end of all the probes, the quencher dye TAMRA, which suppresses the fluorescence emission of the reporter dyes until the probe is cleaved during PCR. Validation experiments were performed to determine the optimal primer and probe concentration for each gene, to prevent a more plentiful target using up common reagents. The final concentrations of primers and

Table 1
Sequences of primers and probes used for PCR analysis

		Sequence
NOS2	Forward primer	5′-GGT GGG TGG CCT CGA GTT-3′
	Reverse primer	5′-CAG AAG TCT CGG ACT CCA ATC TC-3′
	Fluorogenic probe	5′-*FAM* CCT GCC CCT TCA ATG GTT GGT ACA TG *TAMRA*-3′
COX2	Forward primer	5′-CCA TCT GTT CTC CTC AAT ACT GGA A-3′
	Reverse primer	5′-TTG AGG CAG TGT TGA TGA TCC TA-3′
	Fluorogenic probe	5′-*FAM* ACC CAC TTC TCC TCC GAA GGT GCT AGG *TAMRA*-3′
18S	Forward primer	5′-CGG CTA CCA CAT CCA AGG AA-3′
	Reverse primer	5′-GCT GGA ATT ACC GCG GCT-3′
	Fluorogenic probe	5′-*VIC* TGC TGG CAC CAG ACT TGC CCT C *TAMRA*-3′

Probes are labelled with the fluroescent dyes FAM, VIC and TAMRA to enable detection of PCR product.

probes used for real-time PCR analysis were (i) 40 nM forward NOS2 primer, 120 nM reverse NOS2 primer and 100 nM NOS2 probe; (ii) 300 nM forward cyclo-oxygenase-2 primer, 300 nM reverse cyclo-oxygenase-2 primer and 100 nM cyclo-oxygenase-2 probe; and (iii) 120 nM forward 18S primer, 120 nM reverse 18S primer and 125 nM 18S probe. Both the test gene (either NOS2 or cyclo-oxygenase-2) and 18S were amplified in the same tube to determine the relative increases in their expression relative to 18S. The final PCR reaction mix (total volume 25 μl) contained cDNA template (2 ng and 0.5 ng for NOS2 and cyclo-oxygenase-2 expression, respectively), 1× TaqMan® Universal PCR master mix (Applied Biosystems), as well as optimal concentrations of probes and forward and reverse primers. Thermocycler conditions comprised 2 min at 50 °C and 10 min at 95 °C prior to 40 cycles of amplification, each comprising 30 s at 95 °C and 60 s at 60 °C (ABI Prism® 7700 sequence detector). Changes in NOS2 and cyclo-oxygenase-2 mRNA were then quantified by real time PCR and the ΔΔCt method with 18S ribosomal RNA as the internal standard (Heid et al., 1996; Rosenkranz et al., 2003).

2.5. Western blot analysis of NOS2 protein

Western blot analysis of NOS2 protein in cardiomyocytes was determined as previously described (Sun et al., 1998). Briefly, cardiomyocytes were lysed and cytosolic fractions stored at −80 °C until required. Total protein was determined using the Bradford assay. Samples were diluted 1 in 2 in sample buffer, boiled for 5 min to denature protein, and loaded onto an 8% SDS-polyacrylamide gel (25 μg protein per lane). Following electrophoresis, proteins were transferred to a nitrocellulose membrane and incubated for 1 h in blocking buffer (Tris-buffered saline containing 5% non-fat dried milk and 0.05% Tween 20), prior to overnight incubation at 4 °C with rabbit polyclonal antibody to mouse macrophage NOS2 (1:1000, Transduction Laboratories, Lexington, KY). The blot was washed and incubated for 2 h with the secondary antibody, horseradish peroxidase-labelled goat anti-rabbit immunoglobulin (1:1000, Sigma). Proteins were visualised by enhanced chemiluminescence (Kit for Amersham, Buckinghamshire, UK), anticipating a single band of the predicted size for NOS2 protein of 130 kDa.

2.6. Statistics

Baseline characteristics were presented as mean ± S.E.M. Two-way analysis of variance (ANOVA) was used to statistically compare resting contractile parameters prior to and following overnight incubation. Responses to both isoprenaline challenges were expressed as a percentage of the maximum response to the first isoprenaline challenge. Concentration–response curves for isoprenaline were compared using two-way repeated measures ANOVA. One-way ANOVA with post-hoc analysis for multiple comparisons (Tukey test) was used to statistically compare the treatment groups for both the maximum response, and the log EC_{50} values, from the isoprenaline concentration–response curves. The real time PCR results were compared using two-way repeated measures ANOVA. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Resting contractile function of papillary muscles after 15 h

The influence of interferon-γ + lipopolysaccharide, on contractile indices of papillary muscles at rest, prior to and following 15 h incubation with Ac2-26 (1 μM) is illustrated in Fig. 1. As previously observed (Ritchie et al., 1999; Sun et al., 1998), 15 h incubation of papillary

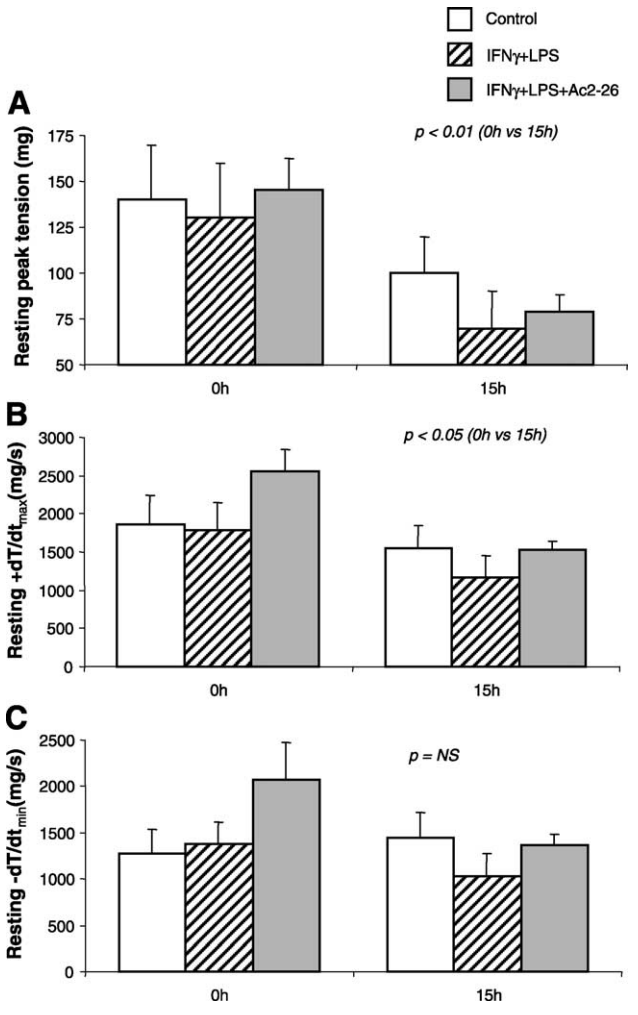

Fig. 1. Influence of interferon-γ + lipopolysaccharide in the presence and absence of Ac2-26 on contractile indices of papillary muscles at rest, prior to and following 15 h incubation. (A) Peak tension; (B) $+dT/dt_{max}$; (C) $-dT/dt_{min}$. Two-way analysis of variance (ANOVA) was used to compare statistically the resting contractile parameters prior to (0 h) and following (15 h) overnight incubation.

muscles under control conditions induced a 26 ± 4% decline in peak tension (Fig. 1A, $n=5$, $P<0.05$ two-way ANOVA) and an 18 ± 4% decline in $+dT/dt_{max}$ (Fig. 1B, $P<0.05$ two-way ANOVA). After incubation with interferon-γ + lipopolysaccharide for 15 h, a 46 ± 8% decline in peak tension, and a 36 ± 6% decline in $+dT/dt_{max}$ was observed. Although this decline in papillary muscle contractile function appeared slightly greater in the presence of interferon-γ + lipopolysaccharide compared with control, in this series of experiments it did not attain statistical significance. However, in contrast to dexamethasone (Sun et al., 1998), pretreatment with Ac2-26 did not prevent the decline in either peak tension or $+dT/dt_{max}$ under resting conditions: $+dT/dt_{max}$ decreased from 2.6 ± 0.3 to 1.5 ± 0.1 g/s (Fig. 1B, $n=5$). No significant changes in resting $-dT/dt_{min}$ were observed (Fig. 1C, $n=5$).

3.2. Inotropic responsiveness to isoprenaline

We have previously shown that 15 h incubation of interferon-γ + lipopolysaccharide impairs the inotropic responsiveness to isoprenaline in papillary muscles (Sun et al., 1998). As shown in Fig. 2, this concentration-dependent impairment of the inotropic response to isoprenaline (0.001–10 μM) is particularly evident at concentrations of isoprenaline greater than 0.1 μM. We now show that pretreatment with Ac2-26 completely prevented the interferon-γ + lipopolysaccharide-induced decline in the responses of both $+dT/dt_{max}$ and $-dT/dt_{min}$ to isoprenaline (Fig. 3B $P<0.005$, and C $P<0.001$, on two-way repeated measures ANOVA, respectively), comparable to its preservation of peak tension (Fig. 2A $P<0.005$, on two-way repeated measures ANOVA). As shown in Fig. 3, pretreatment with Ac2-26 completely prevented the reduction in both the maximum response (the upper limit of the concentration–response curve) and sensitivity (as shown by EC_{50}) of responsiveness to isoprenaline induced by interferon-γ + lipopolysaccharide. The maximum response of peak tension (Fig. 3A), $+dT/dt_{max}$ (Fig. 3B) and $-dT/dt_{min}$ (Fig. 3C) with Ac2-26 was significantly greater than interferon-γ + lipopolysaccharide alone (all $P<0.01$). The maximum response in the presence of Ac2-26 was not significantly different from the control tissues ($P>0.05$). In terms of sensitivity, log EC_{50} values with Ac2-26 for both peak tension and $+dT/dt_{max}$ were significantly lower than observed in papillary muscles treated with interferon-γ + lipopolysaccharide alone (Fig. 3D and E, both $P<0.01$). No significant changes in log EC_{50} values for $-dT/dt_{min}$ were observed (Fig. 3F). Thus, the results demonstrate that Ac2-26 dramatically protects β-adrenergic responsiveness from the action of interferon-γ + lipopolysaccharide.

3.3. Induction of NOS2 expression

As shown in Fig. 4A, RNA extracted from cardiomyocytes subjected to reverse transcription and real time PCR

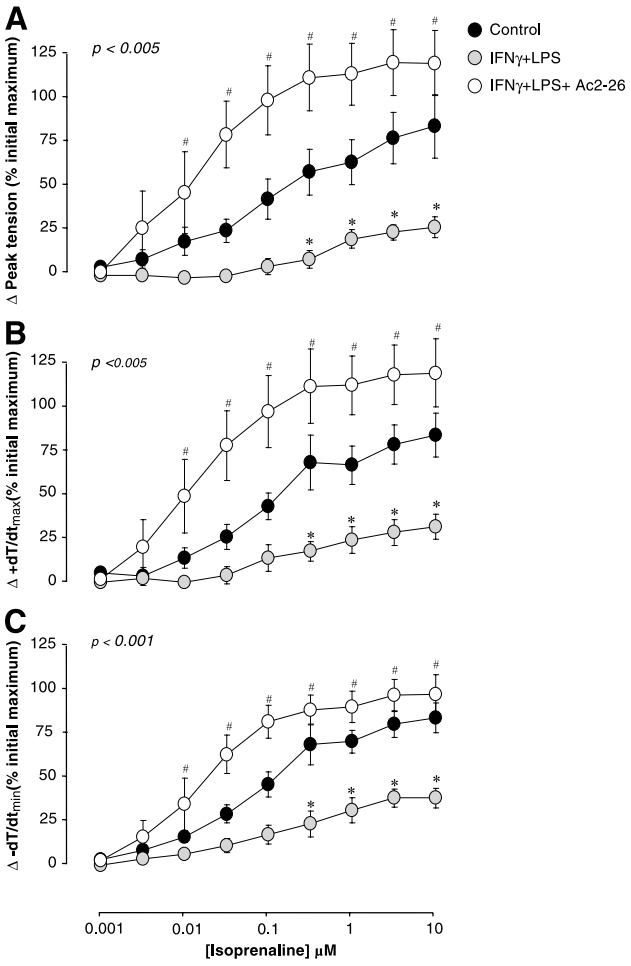

Fig. 2. Influence of pretreatment with Ac2-26 prior to incubation with interferon-γ + lipopolysaccharide on inotropic responsiveness to isoprenaline (0.001–10 μM) in papillary muscles. Results for each papillary muscle is expressed as a percent of the maximum response to isoprenaline prior to the overnight incubation. (A) Peak tension; (B) $+dT/dt_{max}$; (C) dT/dt_{min}. Concentration–response curves for isoprenaline were compared using two-way repeated measures ANOVA. Asterisks (*) indicate $P<0.05$ for interferon-γ + lipopolysaccharide versus control, and hashmarks (#) indicate $P<0.05$ for interferon-γ + lipopolysaccharide + Ac2-26 versus interferon-γ + lipopolysaccharide.

analysis indicated that interferon-γ + lipopolysaccharide markedly induced mRNA expression of NOS2 by approximately 7-fold ($n=7$, $P<0.01$ on two-way repeated measures ANOVA). Pretreatment with Ac2-26 did not prevent the induction of NOS2 mRNA: NOS2 mRNA expression was still increased by 6-fold ($n=7$, $P=$NS). Conversely, pretreatment with dexamethasone suppressed the expression of NOS2 mRNA, by 52 ± 4% ($n=6$). Furthermore, because annexin-1 is postulated to act like a second messenger for some of the anti-inflammatory actions of glucocorticoids (Hirata et al., 1980; Perretti and Flower, 1994; Philip et al., 1998), we sought to determine whether an N-terminally directed antibody to annexin-1 prevented the cardioprotective action of dexamethasone on rat cardiomyocytes (in terms of NOS2 mRNA expression). We used the same

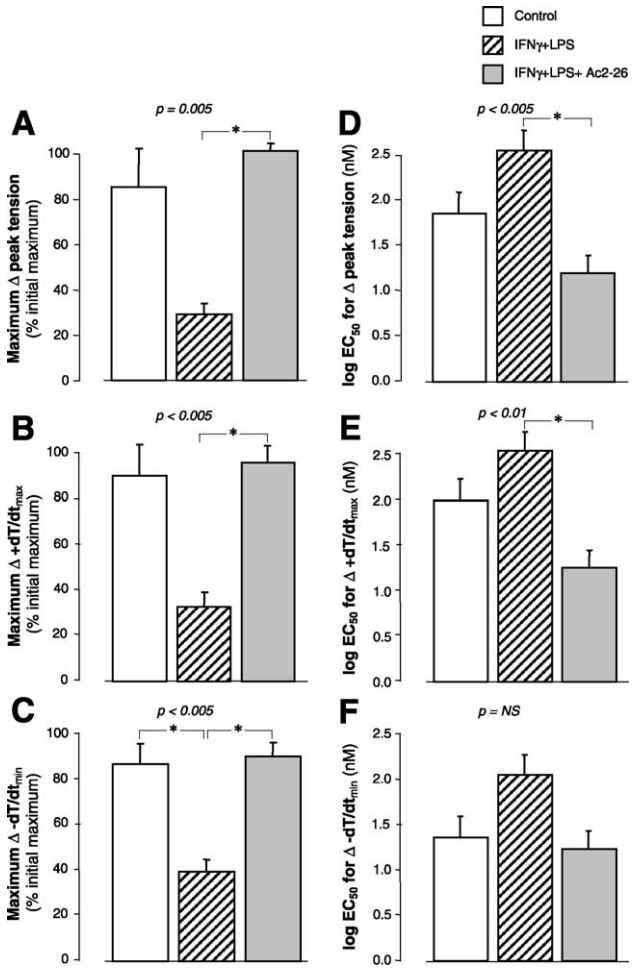

Fig. 3. Effect of the various treatments on the maximum responsiveness and sensitivity of contractile indices to isoprenaline. The parameters maximum response (reflecting magnitude of response) and EC_{50} (reflecting sensitivity of response) were determined from the full concentration/response curves illustrated in Fig. 2. Pretreatment with Ac2-26 prevents the impairment in maximum responsiveness and sensitivity of contractile indices to isoprenaline induced by interferon-γ + lipopolysaccharide in papillary muscles. (A) Maximum peak tension response at 15 h; (B) maximum $+dT/dt_{max}$ response at 15 h; (C) maximum $-dT/dt_{min}$ response at 15 h; (D) log EC_{50} for peak tension at 15 h; (E) log EC_{50} for $+dT/dt_{max}$ at 15 h; (F) log EC_{50} for $-dT/dt_{min}$ at 15 h. Asterisks (*) indicate $P<0.05$ for individual comparisons.

concentration of the same antibody previously shown to prevent suppression by dexamethasone of NO generation and NOS2 expression in J774 macrophages (Wu et al., 1995). The results ($n=3$, Fig. 4A) indicate that suppression of NOS2 mRNA by dexamethasone was preserved when co-incubated with the anti-annexin-1 antibody.

Corresponding results were obtained on Western blot analysis. A prominent band at 130 kDa was observed in interferon-γ + lipopolysaccharide-treated cardiomyocytes, indicating NOS2 protein expression in these samples. No NOS2 protein was evident in control cardiomyocytes. However, pretreatment with Ac2-26 did not affect interferon-γ + lipopolysaccharide-induced NOS2 protein expression, in contrast to dexamethasone, which reduced the NOS2 band. A representative immunoblot of NOS2 protein expression in cardiomyocytes is shown in Fig. 4B.

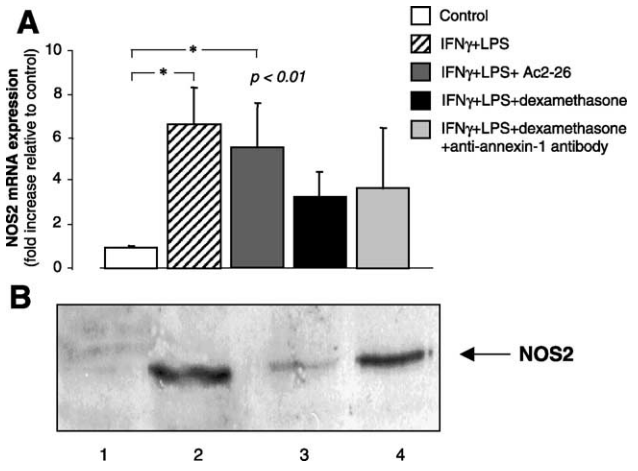

Fig. 4. Interferon-γ + lipopolysaccharide induces NOS2 mRNA (real time PCR) and protein (Western blot) expression in cardiomyocytes. (A) This NOS2 mRNA induction is blocked by dexamethasone but not by Ac2-26. The suppression of NOS2 mRNA expression by dexamethasone is not blocked by an N-terminally directed antibody to annexin-1. Asterisks (*) indicate $P<0.05$ for individual comparisons. (B) Induction of NOS2 protein expression is also blocked by dexamethasone, but not by Ac2-26. Lane 1, control; Lane 2, 15 h incubation with interferon-γ + lipopolysaccharide; Lane 3, interferon-γ + lipopolysaccharide + dexamethasone; Lane 4, interferon-γ + lipopolysaccharide + Ac2-26.

3.4. Induction of cyclo-oxygenase-2 mRNA expression

The influence of interferon-γ + lipopolysaccharide in the presence and absence of Ac2-26 on cardiomyocyte cyclo-oxygenase-2 mRNA expression is illustrated in Fig. 5. Interferon-γ + lipopolysaccharide induced mRNA expression of cyclo-oxygenase-2 by 3-fold on real time PCR analysis ($n=5$, $P<0.001$ on two-way repeated measures ANOVA, Fig. 5). However, in contrast to NOS2 mRNA

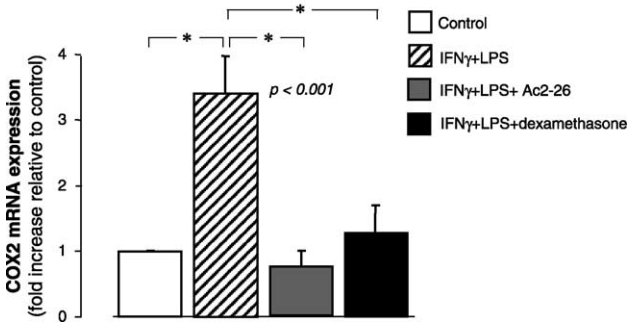

Fig. 5. Using real time PCR analysis on RNA extracted from cardiomyocytes, interferon-γ + lipopolysaccharide induces cyclo-oxygenase-2 mRNA expression relative to control. This is blocked by both dexamethasone and Ac2-26 ($P<0.001$). Asterisks (*) indicate $P<0.05$ for individual comparisons.

expression, the expression of cardiomyocyte cyclo-oxygenase-2 mRNA was strongly suppressed by pretreatment with Ac2-26, by 73 ± 6% ($n = 4$). Similar results were observed following pretreatment with dexamethasone (Fig. 5).

4. Discussion

In the current study, we have demonstrated that an N-terminal fragment of the annexin-1 peptide, Ac2-26, completely abrogates the detrimental effect of interferon-γ + lipopolysaccharide on myocardial inotropic responsiveness to β-adrenergic stimulation (Fig. 2), in terms of each of peak tension, $+dT/dt_{max}$ and $-dT/dt_{min}$. The cardioprotective effect of Ac2-26, observed at the level of both sensitivity (EC_{50}) and maximum response (Fig. 3) for all three contractile indices was at least equal to that which we have previously observed with the glucocorticoid dexamethasone (Sun et al., 1998). However, in contrast to dexamethasone, Ac2-26 did not prevent the impairment of resting contractile function (Fig. 1) nor did it affect induction of NOS2 expression induced by interferon-γ + lipopolysaccharide (Fig. 4). Furthermore, suppression of NOS2 mRNA still occurred after co-incubation with dexamethasone and an N-terminally directed antibody to annexin-1. These data suggest that although annexin-1 exhibits marked protective effects against endotoxin-induced cardiac depression, it does not account for the full spectrum of cardioprotective effects of glucocorticoids.

The intracellular mechanisms involved in the antiproliferative, anti-inflammatory, and cardioprotective actions of annexin-1 have not been fully elucidated, but have been best characterised in inflammatory cells. Annexin-1 acts by binding to extracellular receptors to block the neutrophil expression of adhesion molecules, neutrophil attachment to activated endothelium and neutrophil proliferation and migration out of the vascular lumen (Croxtall and Flower, 1992; La et al., 2001a; Lim et al., 1998; Perretti et al., 1993; Perretti and Flower, 1994). Identification of annexin-1 receptors in neutrophils has been hampered by loss of biological activity of annexin-1 once radiolabelled (Goulding et al., 1990), but it has recently been suggested that they may be related to neutrophil formyl peptide receptors normally activated by bacterial products like fMet-Leu-Phe (fMLP) (D'Amico et al., 2000; La et al., 2001a). Whether these receptors are present on other cell types, such as cardiomyocytes, has not been investigated. However, endogenous annexin-1 is certainly detected in the heart (albeit modestly), and this expression markedly increases in response to stress (Dreier et al., 1998; La et al., 2001a). Cardioprotective actions of annexin-1 peptides in myocardial ischaemia/reperfusion injury in vivo have previously been attributed solely to anti-neutrophil actions (D'Amico et al., 2000; La et al., 2001a), but these mechanisms clearly cannot be involved in the protective action now demonstrated in cardiac tissue in vitro. Recent evidence suggests that the intracellular mechanism of annexin-1 actions may be linked to alterations in Ca^{2+} handling, probably at the level of release from cytosolic stores rather than influx into the cell (Frey et al., 1999; Willmott et al., 1997), as are the myocardial actions of dexamethasone (Reilly et al., 1999). Furthermore, annexin-1 is also thought to behave like a stress protein (Rhee et al., 2000).

Our findings with an N-terminal fragment of annexin-1 clearly contrast with earlier reports that indicate that selective annexin-1 antibodies block the protective action of dexamethasone on lipopolysaccharide-induced vasodilatation in vivo and ex vivo, as well as on macrophage NOS2 induction in vitro (Cannon et al., 1998; Wu et al., 1995). Although another portion of the annexin-1 peptide (termed antiflammin-2) also has some anti-inflammatory effects (Miele et al., 1988; Moreno, 1996), the protective action of Ac2-26 in cardiomyocytes clearly indicates that the N-terminus, which contains the majority of phosphorylation sites (Flower and Rothwell, 1994; La et al., 2001b) exerts protection, and this is independent of NOS2. Our studies are therefore the first to indicate a direct protective action of annexin-1 on the heart muscle, which is independent of neutrophils or other inflammatory cells (Ritchie et al., 1999). Previous studies have suggested that dexamethasone's anti-inflammatory actions may be mediated by the release of intermediates such as annexin-1 (Cannon et al., 1998; Hirata et al., 1980; Perretti and Flower, 1994; Philip et al., 1998; Wu et al., 1995). Dexamethasone clearly has additional protective actions in cardiac tissue that are not reproduced by Ac2-26, such as the preservation of resting contractile function.

Evidence of sepsis-associated depression of cardiovascular function independent of NOS2 (Meng et al., 1997; Oyama et al., 1998) is consistent with our data, for Ac2-26 did not suppress expression of NOS2 mRNA (Fig. 4A) or protein (Fig. 4B). Endotoxin and cytokines alter the cardiac expression of many important biological mediators other than NOS2: the cyclo-oxygenase-2 isoform and intracellular adhesion molecule-1 (ICAM-1) are upregulated, whilst the constitutively expressed cyclo-oxygenase-1, NOS3 and NOS1 isoforms are downregulated (Komatsu et al., 1997; Liu et al., 1996a,b). Induction of cardiac haem-oxygenase1, endothelin-1 and apolipoprotein expression, and alterations in the intracellular handling of fatty acids have also been observed (Memon et al., 1998; Pellacani et al., 1998). Our own results indicate that the increased expression of cyclo-oxygenase-2 induced by interferon-γ + lipopolysaccharide is completely suppressed by both Ac2-26 and dexamethasone (Fig. 5). Although beyond the scope of the present study, the impact of Ac2-26 on expression of cyclo-oxygenase-1, ICAM-1, other NOS isoforms, haem-oxygenase1 and endothelin-1 is not known at present, and is also worthy of investigation.

Dexamethasone also protects cardiac function in the face of cytokine-induced depression, and attenuates NOS2 induction in cardiac myocytes, as in other cell types. The

increased production of nitric oxide resulting from cytokine action in the heart may impair responsiveness to β-adrenoceptor activation via inhibition of either L-type Ca^{2+} channel current, cyclic GMP or cyclic AMP (Mery et al., 1993; Sulakhe et al., 1996). Our results with dexamethasone suggest that changes in basal and β-adrenergic stimulated inotropy induced by interferon-γ + lipopolysaccharide may be modulated in parallel with NOS2 mRNA expression, confirming the potential protection of myocardial function when NOS2 induction is prevented, as shown with both dexamethasone and with inhibition of NOS (Sun et al., 1998). Thus, activation of NOS2 might well contribute to cardiac dysfunction in septic shock as well as in other forms of cardiac contractile failure. However, our finding that Ac2-26 can prevent the cytokine-induced impairment of inotropic responsiveness despite continued expression of NOS2 suggests that this annexin fragment can preserve myocardial function by means not dependent on NOS2.

It has previously been suggested that dexamethasone cardioprotection may be mediated by release of another intermediate, osteopontin (Singh et al., 1995). The role of osteopontin in the cardioprotective actions of dexamethasone remains unclear: however, a small osteopontin peptide analogue blocks NOS2 induction in cardiomyocytes (Singh et al., 1995). Other potentially cardioprotective actions of glucocorticoids distinct from their suppression of NOS2 include inhibition of cytokine expression (Knudsen et al., 1987), L-arginine influx and tetrahydrobiopterin biosynthesis (Simmons et al., 1996). Glucocorticoid-mediated increases in cardiac catecholamine synthesis (Kennedy and Ziegler, 1991) and upregulation of myocardial L-type calcium channel subunits (Takimoto et al., 1997) may also contribute to cardioprotective actions in endotoxin-shock. The contribution of annexin-1 to these effects has not been investigated.

In conclusion, an N-terminal fragment of the annexin-1 peptide Ac2-26 protects β-adrenergic inotropic responsiveness in the heart. This effect is not mediated by inhibition of NOS2 induction, and is independent of any action on circulating inflammatory cells such as neutrophils. The mechanism responsible for this cardioprotective effect, like the spectrum of effects of annexin-1 on cellular physiology, remains to be further explored. N-terminal peptides based on annexin-1 may prove useful in restoring responsiveness to β-adrenergic inotropic stimulation in the depressed heart, as observed in septic shock and perhaps in other forms of heart failure.

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