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Inotropic response to endothelin-1, isoprenaline and calcium in cardiomyocytes isolated from endotoxin treated rats: effects of ethyl-isothiourea and dexamethasone

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1 The contractile effects of endothelin-1, isoprenaline and extracellular calcium were assessed on ventricular cardiomyocytes isolated from lipopolysaccharide-treated rats. The involvement of nitric oxide was investigated using dexamethasone (*in vivo*) and ethyl isothiourea (*in vitro*).

2 Male Wistar rats ($n=70$) were injected with either saline (1 ml kg^{-1}) or lipopolysaccharide (LPS; 5 mg kg^{-1}) alone, or following pre-treatment with dexamethasone (DEX + LPS; 5 mg kg^{-1}). Ventricular cell shortening was recorded using a video edge detection system, and concentration-response relationships were established for endothelin-1, isoprenaline and calcium, in the absence or presence of ethyl isothiourea (ETU; 10 μM). iNOS expression was assessed using reverse transcription-polymerase chain reaction.

3 iNOS mRNA expression was greater ($P<0.001$) in the LPS (iNOS/GAPDH ratio: 0.90 ± 0.09) treated group compared to saline (iNOS/GAPDH ratio: 0.36 ± 0.02). Baseline contractile amplitude was reduced ($P<0.05$) in the LPS (7.3 ± 0.2 μm) and DEX + LPS groups (6.7 ± 0.3 μm) compared to saline (8.0 ± 0.2 μm).

4 The concentration-dependent contractile response to endothelin-1 was attenuated ($P<0.05$) in the LPS group compared to saline (maximum change: 0.45 ± 0.2 vs 1.8 ± 0.2 μm). Neither ETU nor dexamethasone improved contractile function in the LPS-treated animals.

5 The concentration-dependent increase in the contractile response to isoprenaline was attenuated in the LPS-treated group compared to saline ($P<0.05$; maximum change: 1.7 ± 0.4 vs 3.1 ± 0.4 μm). This effect was reversed by ETU (maximum change: 3.7 ± 0.6 μm). Pre-treatment with dexamethasone prevented a significant fall in contraction amplitude (maximum change: 2.4 ± 0.4 μm).

6 The contractile response to calcium was reduced ($P<0.05$) in the LPS group compared to saline (maximum change: 8.7 ± 0.6 vs 10.7 ± 0.8 μm). Neither ETU nor dexamethasone restored contractile function in the LPS-treated group.

7 In conclusion, a nitric oxide-mediated inhibitory pathway is not responsible for the diminished contractile response to either endothelin-1 or extracellular calcium, but contributes to the hyporesponsiveness to isoprenaline in lipopolysaccharide treated rats.

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Abbreviations: BSA, bovine serum albumin; Dex, dexamethasone; ETU, ethyl-isothiourea; LPS, lipopolysaccharide

Introduction

Lipopolysaccharide, a component of the outer layer of the gram negative bacterial cell wall, triggers a pro-inflammatory cascade, culminating in hypotension, multiple organ failure and death from septic shock (Bone, 1991; Natanson *et al*., 1994). In the early phase, cardiac output increases as systemic vascular resistance falls. However, with the progression of the disease cardiac dysfunction develops (Parker *et al*., 1984; Natanson *et al*., 1988).

Recent clinical and experimental studies have shown that the hypotension and hyporesponsiveness to vasoconstrictors may be due to excessive NO production by vascular endothelial and smooth muscle cells. NO can also be produced in cardiac tissue, as in the vasculature by the nitric oxide synthase (NOS), which catalyse the conversion of L-arginine and O_2 to L-citrulline and NO. A constitutively expressed isoform (cNOS) modulates, but is not obligatory for normal cardiac muscle function (Vandecasteele *et al*., 1999). Another isoform, iNOS is expressed under a number of pathophysiological conditions, including septic shock (Szabo & Thiemermann, 1995). The data supporting the role of NO in the pathogenesis of cardiac dysfunction associated with sepsis are controversial. Some reports show that NO is important (Brady *et al*., 1992; Finkel *et al*., 1992; Balligand *et al*., 1993), whereas others have failed to implicate NO (Yokoyama *et al*., 1993; Keller *et al*., 1995; Klabunde & Coston, 1995; Toth & Heard, 1997).

The purpose of the present study was: (i) to record the contractile properties of ventricular cardiomyocytes isolated from endotoxin treated rats, (ii) to assess the effects of the positive inotropic agents, endothelin-1, isoprenaline and calcium, which act *via* different signal transduction pathways, and (iii) to assess the *in vivo* effects of dexamethasone, a

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glucocorticoid which prevents the expression of iNOS, and the *in vitro* effects of ethyl-isothiourea, a non-selective nitric oxide synthase inhibitor, on the contractile responses to endothelin-1, isoprenaline and calcium.

Methods

Endotoxic shock

The animal experiments were performed in accordance with the Animals Scientific Procedures Act (1986). Male Wistar rats (275–325 g; $n=70$) were randomly assigned to receive either 0.9% saline (1 ml kg^{-1}, i.p.), LPS (5 mg kg^{-1}, i.p.), or dexamethasone (5 mg kg^{-1}, i.p.) administered 1 h before LPS. Eight hours later, the animals were sacrificed under terminal anaesthesia (isoflurane). The dose of LPS was set to support a model characterized by morbidity, but not lethality.

Cell preparation

The heart was removed, suspended on a Langendorff apparatus *via* the ascending aorta, and perfused with oxygenated (95% O_2, 5% CO_2) Ca^{2+}-free Krebs Ringer Buffer (KRB) containing (in mM): NaCl 110, KCl 2.6, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2 and glucose 11 (pH 7.4, 37°C). Enzymatic digestion followed using KRB supplemented with 0.04% (w v^{-1}) collagenase. The heart was then removed from the Landendorff apparatus and the atria and ventricles separated. Cells were released by mechanical chopping and gentle trituration in collagenase containing perfusate which had been supplemented with 0.1% bovine serum albumen (BSA). The dispersed cells were filtered through a nylon mesh gauze (pore size 200 μm) and washed twice. After centrifugation at 25 × *g*, calcium ions were restored by resuspension in modified KRB solutions containing 250 μM and 500 μM $CaCl_2$, respectively. Finally, the cells were layered on a solution of 4% (w v^{-1}) BSA containing 1 mM $CaCl_2$ and left to settle by gravity at 37°C. The supernatant was removed and the cells re-suspended in M199 medium with Earle's salts, supplemented with: creatine 5 mM, taurine 2 mM, streptomycin 100 i.u. ml^{-1}, penicillin 100 μg ml^{-1}, at pH 7.4 and 25°C (Kelso *et al*., 1995).

Recording technique

An aliquot of the cell suspension was placed in a transparent recording chamber ($\approx$150 μl), and allowed to settle for 10 min before being bathed with an oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution of the following composition (in mM): NaCl 125, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2, HEPES 10 and glucose 11 (pH 7.4). Cells were field stimulated at 0.5 Hz with biphasic pulses of 0.5 ms duration at 60 V *via* $Ag/AgCl_2$ wires embedded in the wall of the chamber. Cell shortening was measured by video edge detection (VED 40, Crescent Electronics, CA, U.S.A.), the signal was digitised (Digidata 1200, Axon Instruments) and recorded on computer for subsequent analysis (WCP for Windows ver. 1.8, Dr J. Dempster, Department of Pharmacology, University of Strathclyde). The video edge detection system has a time resolution of 16.7 ms and a spatial resolution of 1 in 256 (8 bit). Six consecutive contractions were signal-averaged to produce data under each discrete set of conditions. Cardiomyocyte contractile function was assessed by measuring absolute cell shortening (in μm). Viable cells were used for each experiment as defined by (1) a rod-shaped appearance without sarcolemmal blebbing; (2) quiescence when unstimulated; and (3) stable baseline contractions to electrical stimulation in Krebs-Henseleit solution. The solution bathing the cells could be changed within 30 s, using a gravity fed, multi-channel solution exchanger with eight reservoirs, a series of solenoid valves and an eight channel input manifold.

Experimental protocol

Preliminary experiments were designed to address, (1) the temporal stability of the myocyte preparation; (2) the effect of ethyl isothiourea (10 μM) on basal contractile function and contractile response to isoprenaline; and (3) the time dependent onset of action of endothelin-1 (10^{-8} M).

Following an equilibrium period to allow myocyte contraction to stabilize, concentration-response relationships were performed in a cumulative manner for endothelin-1 (10^{-11}–10^{-7} M), isoprenaline (10^{-11}–10^{-7} M) and extracellular Ca^{2+} (0.5–6 mM), for the treatment groups: (1) saline *in vivo*; (2) LPS *in vivo*; and (3) DEX + LPS *in vivo*. An additional treatment group (LPS + ETU) assessed the effects of myocyte-generated NO production *in vitro*, by exposing myocytes isolated from LPS-treated animals to Krebs-Henseleit solution containing ethyl-isothiourea (10 μM).

Reverse transcription-polymerase chain reaction (RT–PCR)

Total cellular RNA from the ventricular tissue was extracted by a modification of the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski & Sacchi (1987) using Tri Reagent, and quantified by UV spectrophotometry (Ultrospec 2000, Pharmacia Biotech). First Strand cDNA was synthesized from 2 μg total RNA in a 20 μl reaction volume using random decamers and M-MLV reverse transcriptase (Reverse-iT kit). A 2 μl aliquot of the resultant cDNA was amplified by PCR in a final volume of 25 μl, following a standard PCR protocol. *Thermus 'Icelandicus'* (Red Hot DNA polymerase) was used as the thermostable enzyme, and the reaction performed in a PTC-100 thermal cycler (MJ Research). Each PCR reaction contained 2 μl cDNA, 1× reaction buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.25 U DNA polymerase and between 0.5–1 μM of each gene specific primer.

There was an initial denaturation for 4 min at 94°C prior to a cycling profile which included denaturation for 30 s at 94°C, annealing for 40 s at each suitable temperature (57°C for iNOS, and 64°C for GAPDH), and extension for 60 s at 72°C. This was followed by a final extension at 72°C for 5 min and incubation at 15°C for 5 min. Amplification was performed over 35 cycles for iNOS and 29 cycles for GAPDH. The sequences for the gene specific primers (synthesized by the Oligonucleotide Synthesis Unit, Division of Biochemistry, The Queen's University of Belfast), were as follows: iNOS (Ishiwata *et al*., 1997), 5′-CTC TGA AGA AAT CTC TGT TC-3′; iNOS (antisense), 5′-TTG AGG TCT AGA GAC TCT GG-3′; GAPDH (sense), 5′-GCC ATC AAC GAC CCC TTC ATT G-3′; and GAPDH (antisense), 5′-TGC CAG TGA GCT TCC CGT TC-3′.

Analysis of PCR products

Following RT–PCR, a 10 μl aliquot from each PCR reaction was separated on a 2.0% agarose gel containing 10 μg 100 ml^{-1} ethidium bromide in 1× TBE buffer (89 mM Tris-borate, 2 mM EDTA) and analysed (Syngene GeneGenius Gel

documentation system; Gene Tools software). The intensity of the iNOS bands were normalized relative to the intensity of the corresponding GAPDH band.

Statistical analysis

Differences in baseline data between groups were analysed by one-way ANOVA followed by *post hoc* analysis (Bonferroni). Data from the dose response curves were baseline subtracted and differences between the saline and LPS groups analysed by one-way ANOVA. Dose response data was summarized by calculating the area under the curve (Matthews *et al*., 1990) and differences assessed by one-way ANOVA followed by *post-hoc* analysis (Dunnett's). Gene expression was analysed by a Mann-Whitney test. Data are expressed as mean ± s.e.mean. Differences are considered significant at a value of $P<0.05$.

Materials

Endothelin-1 was purchased from the American Peptide Co. (California, U.S.A.). Collagenase (type II) was acquired from Boehringer Mannheim (West Germany). Isoflurane was purchased from Abbott Laboratories (U.K.). Dexamethasone was purchased from Organon (Cambridge, U.K.). Red Hot DNA polymerase and the Reverse-iT kit was acquired from Abgene (Advanced Biotechnologies Ltd, Surrey, U.K.). Ethyl isothiourea was purchased from Aldrich (Gillingham, Dorset, U.K.). Bacterial lipopolysaccharide (*E. coli* serotype 0127:B8), and all other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Results

Animals

Rats injected with LPS were lethargic and unresponsive to external stimuli compared to those from the saline control group. No deaths occurred in any group over the treatment period.

iNOS mRNA expression

PCR amplification products were detected at 352 bp for iNOS and 590 bp for GAPDH in ventricular tissue from both the saline and LPS treated groups. The expression of iNOS mRNA was greater (2.5 fold; $P<0.001$) in the LPS (iNOS/GAPDH ratio: 0.90 ± 0.09) compared to saline (iNOS/GAPDH ratio: 0.36 ± 0.02) treated group (Figure 1).

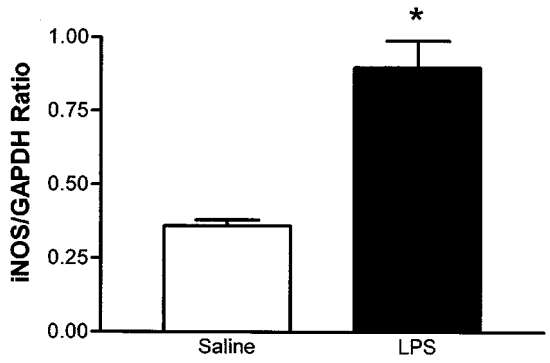

Figure 1 Ratio of iNOS mRNA to glyceraldehyde-3-phosphate dehydrogenase mRNA expression in ventricular tissue from saline and LPS-treated (5 mg kg^{-1} i.p.) rats. Values are mean ± s.e.mean of eight heart preparations. *Indicates $P<0.001$ with respect to saline.

Baseline parameters

Resting length of cells from the LPS (112.2 ± 1.7 μm) and DEX + LPS (111 ± 2.4 μm) groups were not different from the saline group (114.6 ± 1.8 μm; Figure 2a). However, the contractile amplitude was attenuated ($P<0.05$) in the LPS (7.3 ± 0.2 μm) and DEX + LPS (6.7 ± 0.3 μm) groups compared to saline (8.0 ± 0.2 μm; Figure 2b).

The contractile response of isolated ventricular cardiomyocytes to electrical field stimulation was stable under basal perfusion conditions (unmodified Krebs-Henseleit solution) over the 60 min time period studied in both the saline (0 min vs 60 min; 9.6 ± 0.6 vs 8.3 ± 0.4 μm) and LPS (0 min vs 60 min; 7.8 ± 0.4 vs 6.6 ± 0.8 μm) groups (Figure 3a). Furthermore, the addition of ethyl isothiourea (10 μM) to the Krebs-Henseleit solution did not affect the stability or modify the contractile response in either the saline or LPS groups over the 20 min period (Figure 3b).

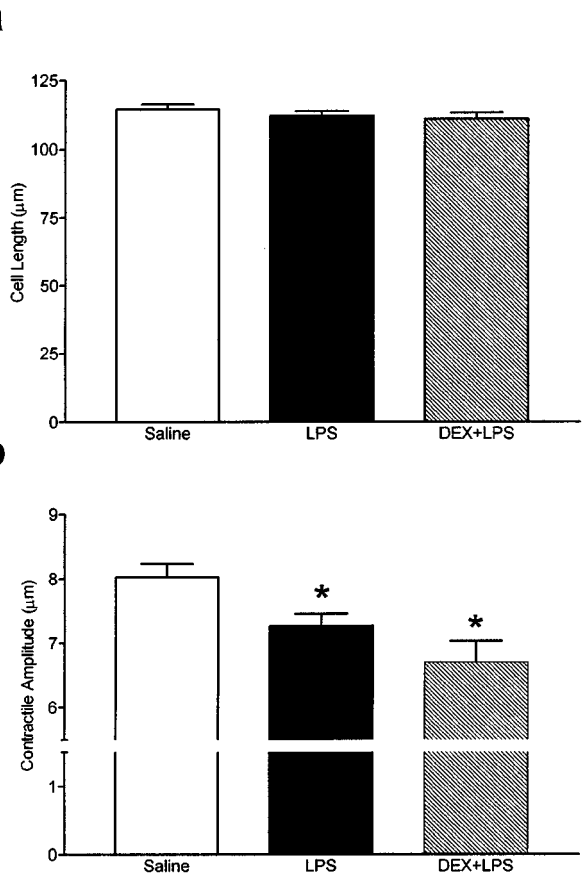

Figure 2 Initial parameters after isolation of cardiac myocytes: (a) resting cell length, and (b) basal contractile function. LPS, lipopolysaccharide (5 mg kg^{-1} i.p.) injection 8 h before cell isolation; DEX + LPS, dexamethasone (5 mg kg^{-1}; i.p.) injection 1 h before LPS (5 mg kg^{-1} i.p.). Values are mean ± s.e.mean of 105 (saline), 94 (LPS) and 28 (DEX + LPS) myocytes from 54 heart cell preparations. *Indicates $P<0.05$ with respect to saline values.

Response to endothelin-1

In myocytes isolated from either saline or LPS-treated rats, the maximum positive contractile effects attained following 8 min exposure of the cells to endothelin (10^{-8} M), remained steady for the remainder of the experiment (data not shown). When

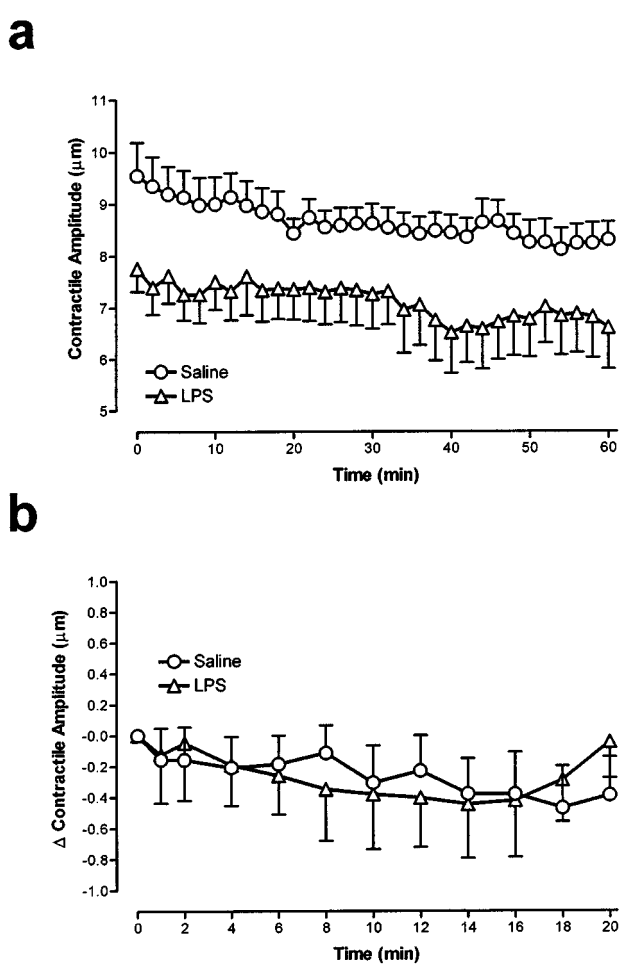

Figure 3 Temporal change in contractile function of ventricular cardiomyocytes isolated from saline and LPS-treated animals: (a) contractile amplitude in normal Krebs-Henseleit buffer and (b) change in contractile amplitude from baseline, in Krebs-Henseleit buffer containing ethyl isothiourea (ETU; 10 μM). Values are mean$\pm$s.e.mean of seven myocytes/group from seven heart cell preparations.

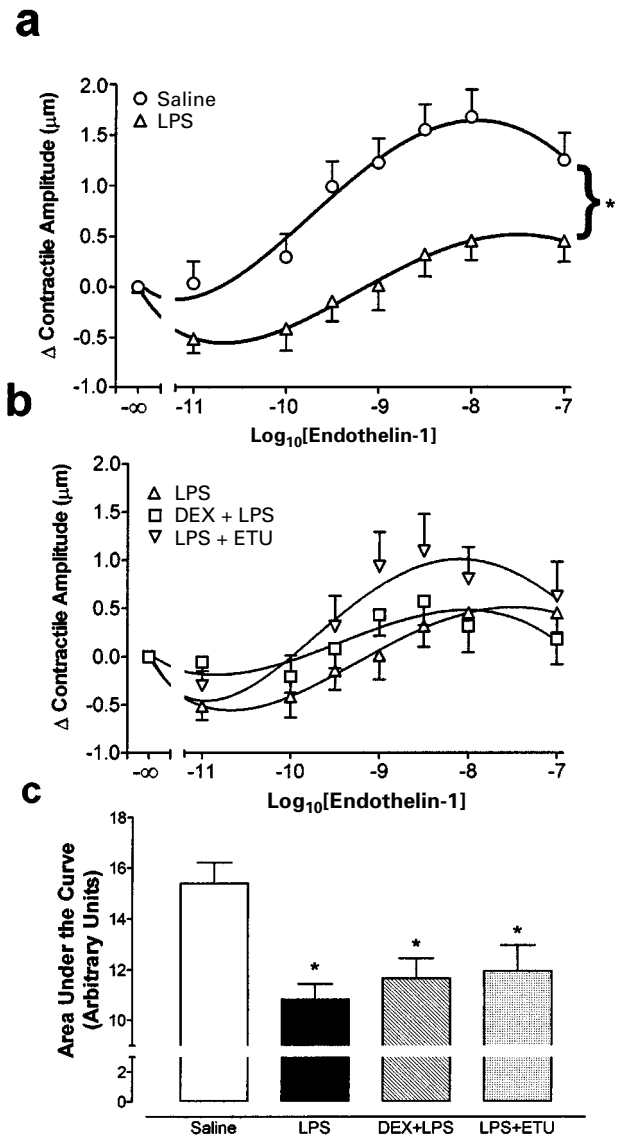

Figure 4 Effect of endothelin-1 on the contractile response of cardiac myocytes. LPS, lipopolysaccharide (5 mg kg^{-1} i.p.) injection 8 h before cell isolation; DEX+LPS, dexamethasone (5 mg kg^{-1}; i.p.) injection 1 h before LPS (5 mg kg^{-1} i.p.); LPS+ETU, *in vitro* exposure of myocytes isolated from LPS-treated animals to ethyl isothiourea (10 μM). (a) concentration-dependent changes between saline and LPS-treated animals (b) concentration-dependent changes between LPS-treated groups, (c) area under the concentration response curve. Values are mean$\pm$s.e.mean of 12–18 myocytes from 10–12 heart cell preparations. *Indicates $P<0.05$ (a,c) compared to the saline treated group.

linearly regressed, the 'steady state' portions of the endothelin-1 time courses were found to be parallel and significantly displaced ($P<0.0001$). This shows that the contractile response to endothelin was attenuated in cells from the LPS-treated animals. In subsequent experiments, cardiomyocytes were equilibrated for 8 min at each concentration of endothelin-1 prior to recording.

Endothelin-1 increased the contractile amplitude in a concentration-dependent manner, with a threshold effect observed at 3×10^{-9} M in cells from the saline group (Figure 4a). The contractile response to endothelin-1 was attenuated ($P<0.05$) in a concentration-dependent manner over the concentration range $10^{-11}-10^{-7}$ M in the LPS compared to the saline group. At lower concentrations ($10^{-11}-10^{-10}$ M) endothelin-1 depressed the inotropic effect whilst a positive inotropic effect was observed at greater than nanomolar concentrations. The maximal responses were 1.68 ± 0.3 and 0.45 ± 0.2 μm in the saline and LPS groups respectively, at a concentration of 10^{-8} M. The area under the concentration-response relationship to endothelin-1 was reduced ($P<0.05$) by 29.7 ± 4.1% in the LPS (10.8 ± 0.6 a.u.) compared to saline group (15.4 ± 0.8 a.u.). Furthermore, the area under the concentration response curve to endothelin-1 was reduced ($P<0.05$) in both the *in vivo* DEX+LPS (11.7 ± 0.8 a.u.) and the *in vitro* LPS+ETU (12.0 ± 1.0 a.u.) groups compared to saline (15.4 ± 0.8 a.u.; Figure 4b,c).

Response to isoprenaline

The contractile response to isoprenaline was attenuated ($P<0.05$) in a concentration-dependent manner over the concentration range $10^{-9}-10^{-7}$ M in the LPS compared to the saline group (Figure 5a). The maximal responses were 3.1 ± 0.4 and 1.7 ± 0.4 μm in the saline and LPS groups respectively, at a concentration of 10^{-7} M. The area under the concentration response curve of isoprenaline was reduced ($P<0.05$) by 21.2 ± 5.2% in the LPS (17.5 ± 1.2 a.u.) compared to the saline (22.2 ± 1.1 a.u.) group. However, similar areas under the concentration-response curves for isoprenaline was

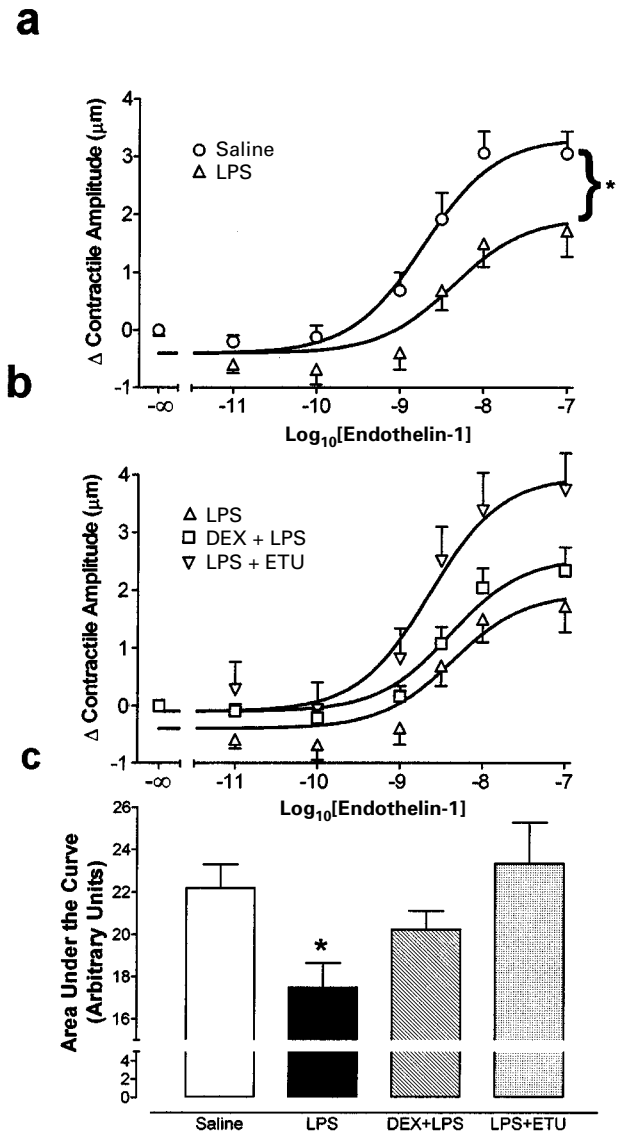

Figure 5 Effect of isoprenaline on the contractile response of cardiac myocytes. LPS, lipopolysaccharide (5 mg kg^{-1} i.p.) injection 8 h before cell isolation; DEX+LPS, dexamethasone (5 mg kg^{-1}; i.p.) injection 1 h before LPS (5 mg kg^{-1} i.p.); LPS+ETU, *in vitro* exposure of myocytes isolated from LPS-treated animals to ethyl isothiourea (10 μM). (a) Concentration-dependent changes between saline and LPS-treated animals (b) concentration-dependent changes between LPS-treated groups, (c) area under the concentration response curve. Values are mean$\pm$s.e.mean of 12–18 myocytes from 10–12 heart cell preparations. *Indicates $P<0.05$ (a,c) compared to the saline treated group.

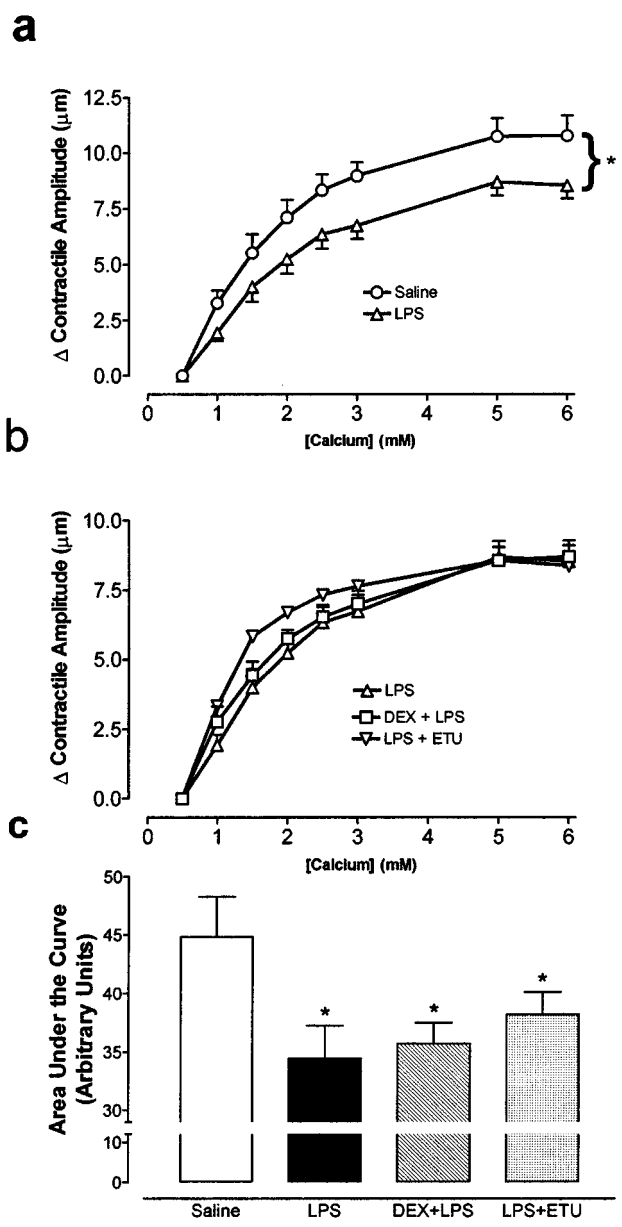

Figure 6 Effect of calcium on the contractile response of cardiac myocytes. LPS, lipopolysaccharide (5 mg kg^{-1} i.p.) injection 8 h before cell isolation; DEX+LPS, dexamethasone (5 mg kg^{-1}; i.p.) injection 1 h before LPS (5 mg kg^{-1} i.p.); LPS+ETU, *in vitro* exposure of myocytes isolated from LPS-treated animals to ethyl isothiourea (10 μM). (a) Concentration-dependent changes between saline and LPS-treated animals (b) concentration-dependent changes between LPS-treated groups, (c) area under the concentration response curve. Values are mean$\pm$s.e.mean of eight myocytes from 5–7 heat cell preparations. *Indicates $P<0.05$ (a,c) compared to the saline treated group.

seen in the DEX+LPS ($-8.8\pm3.9\%$) and LPS+ETU ($5.2\pm8.7\%$) groups as in the saline group (Figure 5b,c). Furthermore, in myocytes isolated from saline treated rats, the contractile response to isoprenaline in the presence of ETU (2.6 ± 0.8 μm) was comparable to isoprenaline alone (2.2 ± 0.6 μm).

Response to calcium

The concentration-dependent positive inotropic response to Ca^{2+} (0.5–6 mM) was attenuated in cells from the LPS compared to the saline group (Figure 6a). The area under the concentration response curve of calcium was reduced ($P<0.05$) by $23.1\pm6.2\%$ in the LPS treatment group (34.5 ± 2.8 a.u.) compared to saline treatment (44.8 ± 3.5 a.u.). The area under the concentration response curve of calcium was also reduced in both the *in vivo* DEX+LPS (35.7 ± 1.8 a.u.) and the *in vitro* LPS$\pm$ETU (38.2 ± 1.9 a.u.) groups compared to the saline group (44.8 ± 3.5 a.u.; Figure 6b,c).

Discussion

The major findings of the present study are: (i) basal contractile amplitude is reduced in isolated ventricular cardiomyocytes from LPS-treated compared to saline-treated

rats; (ii) contractile responses to endothelin-1, isoprenaline and extracellular calcium are attenuated in LPS-treated animals compared to control animals; (iii) ethyl isothiourea restores contractile responsiveness to isoprenaline, in ventricular cardiomyocytes isolated from LPS-treated rats, in contrast to an absence of effect on the endothelin-1 and calcium concentration-response relationships; and (iv) *in vivo* pre-treatment of LPS-treated rats with dexamethasone maintained contractile function in response to isoprenaline but not to endothelin-1 or calcium in LPS-treated rats.

Septic shock is a complex syndrome characterized by profound alterations to both the peripheral vasculature and myocardium. The use of isolated cardiomyocytes offers a unique opportunity to study the direct cardiac effects free from neural and mechanical factors, which are intrinsic to *in vivo* or whole tissue preparations. Compensatory mechanisms are avoided by using cells isolated from an *in vivo* model of endotoxic shock. However, the cells are still exposed to the complex pro-inflammatory milieu associated with endotoxic shock, a factor which is absent in *in vitro* models of endotoxic shock. Sepsis is associated with the release of several cytokines, which may cause myocardial dysfunction in a paracrine or humoral manner (Ungureanu-Longrois *et al*., 1995). The model of endotoxic shock in the present study is based on the administration of purified endotoxin (lipopolysaccharide). This elicits a typical septic shock-like condition in animals (Natanson *et al*., 1988). Typically used at concentrations of 5.15 mg kg^{-1} in rodent studies, we found that 5 mg kg^{-1} was sufficient to generate a model characterized by morbidity, without early lethality.

Consistent evidence of myocardial contractile dysfunction in experimental models of endotoxic shock has been reported using isolated perfused hearts (Decking *et al*., 1995), cardiac tissues (Keller *et al*., 1995; Toth & Heard, 1997) and isolated ventricular cardiomyocytes (Brady *et al*., 1992; Keller *et al*., 1995; Zhong *et al*., 1997a,b) In the present study, we found a 9% decrease in basal contractile function, with a depressed maximal response to stimulation with isoprenaline (−45%) and endothelin-1 (−75%). Studies in guinea-pigs administered 4 mg kg^{-1} LPS have shown a 29–46% decrease in basal contractile function (Brady *et al*., 1992; Zhong *et al*., 1997b). Such variance may be due to species differences, with rats being less sensitive to LPS than guinea-pigs. The contractile response to increasing extracellular calcium concentrations was also attenuated in myocytes isolated from LPS-treated rats in the present study, consistent with attenuation of either the $[Ca^{2+}]_i$ transient (Zhong *et al*., 1997b) or myofilament responsiveness to cytosolic calcium (Yasuda & Lew, 1997b).

Plasma levels of endothelin-1 are elevated several fold in endotoxic shock as reviewed by Battistini *et al*. 1996. In patients raised levels correlate positively with the severity of endotoxaemia (Pittet *et al*., 1991). In a rat model of septic shock, non-specific endothelin receptor blockade exacerbated vasodilation and vascular hyporesponsiveness (Gardiner *et al*., 1995). This suggests that increased endothelin levels may act as a counter-regulatory measures to the associated NO-mediated vasodilatation associated with endotoxic shock, and thereby likely to have a beneficial effect.

Despite endothelin being a potent positive inotropic agent in the heart (Ishikawa *et al*., 1988), the question of whether it contributes to the maintenance of cardiovascular function in septic shock is an area that has received little attention. In the present study, the positive inotropic effect of endothelin was attenuated in myocytes isolated from LPS-treated rats. Our findings are at variance with another report of enhanced contractile response to endothelin in rabbit isolated ventricular cardiomyocytes exposed to LPS *in vitro* (Yasuda & Lew, 1997a). Such differences are not unexpected as the studies differ in both the animal species used (rat vs rabbit) and type of model (our *in vitro ex vivo* model vs a completely *in vitro* model). Our isolated ventricular cardiomyocytes are exposed to the complex influences of the pro-inflammatory milieu which is generated *in vivo*, but is missing or attenuated in the *in vitro* situation of Yasuda & Lew (1997a). LPS-induced endotoxic shock involves the release of many cytokines, including endothelin and TNFα. TNFα is known to impair the effect of high extracellular calcium on contractile performance (Muller-Werdan *et al*., 1997), by decreasing the intracellular calcium transient in a NO-independent manner (Yokoyama *et al*., 1993). It is interesting to note that lower concentrations of endothelin-1 (10^{-11}–10^{-10} M) depressed the contractile response in the LPS group. Similar findings have been reported for an experimental model of heart failure and attributed to changes in the signal transduction mechanisms associated with the ET_A receptor but not in changes in receptor density or expression (Thomas *et al*., 1996).

Plasma levels of catecholamines are increased in septic shock (Hahn *et al*., 1995). In the short term, this is likely to counteract myocardial dysfunction but with a loss of efficacy over the long term. The contractile response to isoprenaline in endotoxic shock is attenuated in isolated working hearts (Sun *et al*., 1997), cardiac tissue (Sulakhe *et al*., 1996) and ventricular cardiomyocytes (Yasuda & Lew, 1997b). In the present study, we report a 45% decrease in the contractile response to isoprenaline, consistent with the 30–60% attenuation reported in other studies (Sun *et al*., 1997; Yasuda & Lew, 1997b). The mechanisms underlying the attenuation of the contractile response to β-adrenoceptor stimulation are likely to be multi-factorial with, β-adrenoceptor down regulation, uncoupling of the receptor from the associated G-protein (Gulick *et al*., 1989; Chung *et al*., 1990) and a decrease in myofilament responsiveness to intracellular Ca^{2+} (Yasuda & Lew, 1997b) being implicated.

Although NO is established as an important causative agent in hypotension in endotoxic shock (Parratt, 1998), its effects on the contractile properties of the heart are more controversial. There are reports that NO inhibition has no effect on (Decking *et al*., 1995; Toth & Heard, 1997), partially reverses (Brady *et al*., 1992) or completely reverses (Sun *et al*., 1997; Yasuda & Lew, 1997b) contractile dysfunction in experimental models of endotoxic shock. Indeed, the present study and others (Ishiwata *et al*., 1997) have shown iNOS mRNA expression to be increased several fold in the poorly contracting ventricle of LPS-treated rats compared to control hearts.

Although ETU is a potent non-selective inhibitor of the nitric oxide synthase isoenzymes (Southan *et al*., 1995), neither ETU nor dexamethasone, which prevents the expression of iNOS mRNA (Radomski *et al*., 1990), improved basal contractile function, or the inotropic responses to either endothelin-1 or extracellular calcium. The concentration of dexamethasone (5 mg kg^{-1}) used in the present study was selected from evidence provided in the literature which indicate that doses of 1–10 mg kg^{-1} inhibited the induction of iNOS (Di Rosa *et al*., 1990; Radomski *et al*., 1990; Salter *et al*., 1991). The attenuated response to isoprenaline in the LPS-treated group was prevented by dexamethasone *in vivo*, and reversed by ethyl isothiourea *in vitro*. In addition to this, ethyl isothiourea did not affect the magnitude of the contractile response to isoprenaline in myocytes isolated from saline-treated animals. Taken together, these data are consistent with other evidence that attenuation of the contractile response to

isoprenaline is NO-dependent (Sun *et al*., 1997; Yasuda & Lew, 1997b).

In conclusion, the present study provides evidence that a nitric oxide-mediated inhibitory pathway is not responsible for attenuating the contractile response to either endothelin-1 or extracellular calcium, but does underlie the hyporesponsiveness to isoprenaline in LPS-treated rats. Such a failure of the myocardial contractile response to both endothelin and sympathetic pathways may well contribute to the cardiovascular dysfunction associated with septic shock and the limited effects of NO synthase inhibition in experimental models and clinical treatment.

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