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Nitric oxide supports atrial function in sepsis: relevance to side effects of inhibitors in shock

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Abstract

The mechanisms underlying myocardial dysfunction in sepsis remain poorly understood. The theoretical benefits of nitric oxide synthase (NOS) inhibition in reversing the haemodynamic changes that characterise septic shock have not been supported by clinical trials, some of which have demonstrated detrimental myocardial effects. We have therefore assessed the effects of endotoxaemia on NOS enzyme expression as well as a number of functional responses of myocardial tissue from rats. Atrial tissue expressed high levels of mRNA for inducible (i)NOS and released increased levels of nitrite after animals were treated with endotoxin. In parallel, the inotropic response stimulated by isoprenaline was reduced in atria from endotoxin-treated animals, an effect that was reversed when endogenous release of NO was maximised. Our results suggest that myocardial contractility is maintained by NO production and that inhibitors may compromise cardiac output; this may explain the deleterious effects of NOS inhibition on cardiac function in clinical trials.

Keywords: Nitric oxide (NO); Contractility; Inotropic agent; Endotoxaemia

1. Introduction

Septic shock accounts for 100 000 deaths per annum in the USA alone, and the development of cardiovascular dysfunction is known to be of major prognostic significance, increasing mortality from 20–30% to 40–70% (Bone et al., 1987; Sprung et al., 1984). Patients with sepsis develop profound hypotension which is resistant to pressor agents and can be associated with a highly specific cardiac dysfunction characterised by tachycardia, systolic depression, increased ventricular compliance and a normal or increased cardiac index (Ognibene et al., 1988; Parker et al., 1987).

The mechanisms associated with the profound drop in blood pressure associated with septic shock have been studied extensively in blood vessels and partly explained by the induction of vasoactive enzymes. Thus, in experimental sepsis, the inducible form of nitric oxide (NO) synthase (iNOS) is rapidly expressed in the vascular smooth muscle component of arteries and veins (Mitchell et al., 1992). Under these conditions, very high levels of the vasodilator gas NO are formed locally, rendering the vessels hypo responsive to constrictor mechanisms (Szabo et al., 1993). These observations and others have led to the suggestion that inhibition of NOS may be of therapeutic benefit in the treatment of septic shock in man. In fact, a clinical trial has been performed where patients with sepsis were treated with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA; Grover et al., 1999a,b). However, this trial was terminated early because, despite correcting the drop in peripheral vascular tone, L-NMMA induced a fall in cardiac index in a significant number of patients (Grover et al., 1998, 1999a,b). The mechanisms involved in this fall are not understood, but likely to involve direct effects on cardiac tissue.

By contrast to blood vessels, less is known about the effects of endotoxin on heart tissue and even less about how NO may influence responses. Thus, in light of findings revealed in the trial of L-NMMA in human sepsis, we have performed a systematic study of the potential role of NO in several cardiac function in a model of endotoxaemia in rats.

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Specifically, we used atrial tissue to study contractility, myocardial cell death and atrial natriuretic peptide (ANP) release.

2. Methods

The investigations were performed in accordance with the Home Office Animals (Scientific Procedure) Act 1986.

2.1. Measurement of release of nitrite, ANP and LDH by atria in organ culture

Male Wistar rats (weight 250–300 g) were randomized to control or endotoxaemic (treated with lipopolysaccharide, 20 mg^{-1} kg^{-1} i.p.) groups. Four hours after treatment, the animals were anaesthetized with sodium pentabarbitone (100 mg^{-1} kg i.p.) and killed by cervical dislocation. Hearts were removed and flushed with ice-cold, gassed (95% O_2–5% CO_2) Krebs–Henseleit solution. The composition of the buffer was (in mM): NaCl 118, KCl 4.7, $MgSO4{\cdot}7H_2O$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.1, glucose 10, and $CaCl_2{\cdot}2H_2O$ 2.5. Right and left atria were dissected into equal pieces, placed into Dulbecco's modified Eagles medium (DMEM) and incubated at 37 °C for 1 h. To identify the effects of NOS inhibition, medium was then replaced, and atrial tissue incubated at 37 °C in the presence or absence of the non-specific NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM), or medium alone, for 24 h (0–24 h). In some experiments, medium was replaced at 24 h, with or without drugs, and tissues incubated for a further 24-h period (24–28 h). In a separate set of preliminary experiments, the ability of the specific iNOS inhibitor 1400W (10 μM) (Garvey et al., 1997) to inhibit iNOS activity in lipopolysaccharide-treated atria was assessed. However, unlike L-NAME, 1400W did not influence iNOS activity in this tissue, possibly as a result of accelerated metabolism or reduced uptake. Therefore, in subsequent experiments, the non-specific NOS inhibitor L-NAME was used. Medium was assayed at 24 and 48 h for nitrite by the Griess reaction adapted for a 96-well plate recorder (Bishop-Bailey et al., 1997; Schmidt et al., 1992). Briefly, at each time point, 100 μl of sample was incubated with 100 μl of Griess reagent (sulphanilamide [0.5%], orthophosphoric acid [2.5%] and *N*-(1-naphthyl)ethyl-enediamine [0.005%]) for 5 min in 96-well plates. The OD_{550} was measured by a Titertek Multiskan MCC/350 MK.II plate reader and nitrite concentration calculated with solutions of sodium nitrite in DMEM (0–100 μM). Under these conditions, the detection limit of the Griess reaction was 1 μM. Lactate dehydrogenase (LDH) was measured using a kinetic assay; 20 μl of sample medium was incubated with 20 μl of reagent (KH_2PO_4 [6.8 g/l, pH 7.5] β-NADH, sodium pyruvate), and the OD_{340} was measured by a Titertek Multiskan MCC/350 MK.II plate reader (kinetic mode), and LDH release calculated with solutions of LDH in DMEM (0–10 000 units M^{-1}). ANP was measured by ELISA (according to manufacturers' recommendations).

2.2. Measurement of eNOS, nNOS and iNOS expression in rat atria by semi-quantitative reverse transcription polymerase chain reaction (RTPCR)

Atrial tissue was obtained as above, frozen immediately and stored at −80 °C until RNA was extracted. Tissues were homogenized and RNA extracted using a Tri reagent kit (Sigma). Samples were processed and analyzed using standard RT-PCR techniques (Nicoletti and Sassy-Prignet, 1996). For the reverse transcription stage, single-stranded cDNA was synthesized from 600 ng of total RNA in the presence of Moloney murine leukemia virus reverse transcriptase and oligo $(dT)_{12-18}$ primers. Double-stranded cDNAs were synthesized and amplified using 1.25 U *Taq* polymerase, 20 mM Tris–HCl (pH 8.0), 50 mM KCl, 0.2 mM dNTP, 20 pmol of each primers of iNOS, eNOS and nNOS, 1.5 mM $MgCl_2$, 4×105 cpm of ^{33}P end-labeled primer in a 25-μl reaction final volume. The amplification was carried out in a DNA thermal cycler. Initial denaturation was done at 94 °C for 2 min followed by 16 (β-actin), 21 (iNOS), 36 [neuronal (n) NOS] or 32 [endothelial (e) NOS] cycles of amplification. Each cycle consisted of 30 s of denaturation at 94 °C, 1 min of annealing at 60 °C (62 °C, eNOS) and 1 min for enzymatic primer extension at 72 °C. Polymerase chain reaction products were then size fractionated through 2% polyacrylamide gel electrophoreses and visualized by ethidium bromide staining. The sequences for primers were selected according to the published sequences in GenBank from homologous parts of the coding region of rat genes β-actin, iNOS, eNOS and nNOS (upstream and downstream sequences respectively: β-actin: 5′ TAA AAC GCA GCT CAG TAA CAG TCC G 3′, 5′ TGG AAT CCT GTG GCA TCC ATG AAA C 3′, iNOS: 5′ CGC TAC ACT TCC AAC GCA AGA 3′, 5′ GGG TGG GAG GGG TAG TGA TGT 3′, eNOS: 5′ GGT ATT TGA TGC TCG GGA CTG 3′, 5′ GAG TAA CAG GGG CAG CAC ATC 3′, nNOS: 5′ GAA TAC CAG CCT GAT CCA TGG AA 3′, 5′ TCC TCC AGG AGG GTG TCC ACC GCA 3′).

2.3. Atrial contractility

Hearts were removed as described above and placed immediately into gassed (95% O_2/5% CO_2) ice-cold Krebs'–Henseleit solution. Right and left atria were rapidly dissected, and vertically mounted in 10 ml organ baths containing gassed (as above) Krebs'–Henseleit solution at 37 °C. The base of each atrium was fixed, and the apex attached by a size 6/0 prolene suture, to a force transducer (MacLab, ADI Instruments). Right atrial preparations were allowed to beat spontaneously. Left atrial preparations were stimulated with electrical field stimulation, specifically they were paced 100% above the threshold voltage at 0.5 Hz, stimulus duration 1 ms, by

Table 1
Release of nitrite from myocardial tissue from control animals and animals pre-treated in vivo with lipopolysaccharide

Region	Assay	Control		Lipopolysaccharide	
		0–24 h	24–48 h	0–24 h	24–48 h
LA	Nitrite (μmol/mg)	0.89 ± 0.18 (10)	3.22 ± 0.93 (12)	* 3.08 ± 0.93 (12)	* 6.18 ± 1.48 (7)
RA		0.91 ± 0.13 (12)	2.67 ± 0.51 (7)	* 3.52 ± 0.98 (9)	* 5.84 ± 0.61 (7)
LV		0.08 ± 0.03 (8)	0.08 ± 0.3 (8)	* 0.25 ± 0.062 (8)	* 0.57 ± 0.13 (8)
RV		0.13 ± 0.04 (8)	0.07 ± 0.04 (8)	* 0.17 ± 0.04 (8)	* 1.05 ± 0.24 (8)

Nitrite release was measured after 24 h ex vivo and calculated per milligram of tissue. LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle. *n* numbers are shown in parentheses. Statistical differences between the release of nitrite by control treated versus lipopolysaccharide-treated cardiac tissue were calculated by one-way ANOVA. *P* values of < 0.05 are denoted by *.

two parallel platinum electrodes, connected to a pulse generator (Grass, S4). Changes in rate (right atria) and isometric force (left atria) were recorded via a bridge amplifier into an Apple Macintosh personal computer, connected to a MacLab 4 recording and analysis system (ADI Instruments). Preparations were left to equilibrate for 5 min and then set to L_{max} (the length at which the generated tension was maximal) and all subsequent measurements were taken at L_{max}. After 20 min of equilibration, stability of each atrial preparation (as measured by stability of generated and baseline tension) was assessed. Serial measurements were made at 5-min time intervals for baseline tension and peak-generated tension (PGT, grams), and for right atrial preparations, atrial rate (beats per minute^{-1} or bpm^{-1}). In other experiments, the L-NAME (1 mM), or the substrate for NO, L-arginine (1 mM) was added after the period of equilibration. In separate experiments, similar protocols were performed on atria stimulated with the β agonist isoprenaline. After the period of equilibration, L-NAME (1 mM), D-NAME (1 mM), L-arginine (1 mM), or D-arginine (1 mM) was added for 15 min followed by increasing concentrations (100 pM to 3 μM) of isoprenaline.

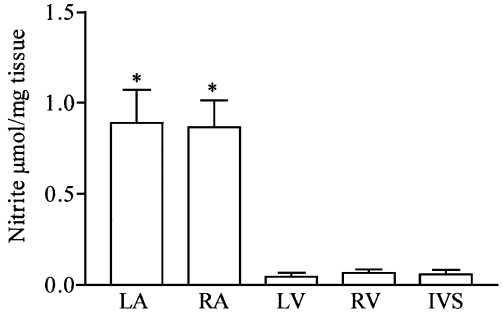

Fig. 1. Release of nitrite from myocardial tissue from control animals. Nitrite release was measured after 24 h and was calculated per milligram of cardiac tissue. LA: left atrium, RA: right atrium, LV: left ventricle, RV: right ventricle, IVS: interventricular septum. * denotes significance when nitrite release by atrial tissue is compared to LV, RV or IVS calculated by one-way ANOVA followed by Tukey–Kramer post-test. $n = 10$ in each group.

Table 2
The effect of endotoxaemia on atrial LDH release

Time	Region	Treatment	Control	L-NAME
0–24 h	LA	control	6.58 ± 4.2	7.31 ± 4.7
		lipopolysaccharide	8.89 ± 6.3	21.27 ± 9.1
	RA	control	9.95 ± 6.5	5.35 ± 2.9
		lipopolysaccharide	8.85 ± 5.7	14.4 ± 8.8
24–48 h	LA	control	0.290.03	0.23 ± 0.11
		lipopolysaccharide	0.61 ± 0.21	0.398 ± 0.12
	RA	control	0.15 ± 0.09	0.14 ± 0.08
		lipopolysaccharide	0.59 ± 0.36	0.37 ± 0.21

Atrial tissue from control animals and animals pre-treated in vivo with lipopolysaccharide was incubated ex vivo for 0–24 and 24–48 h. LDH release was calculated per milligram of tissue. LA: left atrium, RA: right atrium. $n = 4$ in each group. No statistical differences between any of the groups (one-way ANOVA) were found.

2.4. Drugs and chemicals

Lipopolysaccharide (*Salmonella enteritidis*), DMEM, L-NAME, D-NAME, L-arginine, D-arginine, LDH reagents, Greiss reagents and isoprenaline, were obtained from Sigma (Poole, Dorset, UK). 1400W was obtained from ALEXIS (Nottingham, UK), ANP was assayed by ELISA (kit from Peninsula Laboratories, S^{t} Helen's, UK). Lipopolysaccharide was dissolved in normal saline, all other drugs in distilled water or culture medium. RT buffer, dithiolthreitol, reverse transcriptase, oligo (dT), PCR buffer, magnesium chloride, *Taq* polymerase, primers (β-actin, iNOS, nNOS, eNOS) were obtained from Life Technologies (Paisley, UK), and agarose, dNTP, RNAsine, dNTP, from Promega (Southampton, UK). Chemicals for Krebs'–Henseleit buffer were obtained from Merck (Poole).

2.5. Statistics

Results are expressed as mean ± S.E.M. for *n* (separate animals) experiments. For all contractility data, measure-

Table 3
Atrial ANP release in response to endotoxaemia and NOS inhibition

Treatment	Control (ng/mg)		Lipopolysaccharide (ng/mg)	
	0–24 h	24–48 h	0–24 h	24–48 h
Control	17.97 ± 2.65 (10)	12.64 ± 2.12 (10)	* 59.92 ± 14.37 (10)	14.91 ± 3.17 (10)
L-NAME	24.97 ± 7.70 (10)	19.65 ± 3.54 (7)	* 53.84 ± 15.91 (10)	19.40 ± 4.20 (10)

Atrial tissue from control animals and animals pre-treated in vivo with lipopolysaccharide was incubated ex vivo for 0–24 and 24–48 h in the presence and absence of the NOS inhibitor L-NAME (1 mM). ANP release is calculated per milligram of tissue, and results are pooled for right and left atria. *n* numbers are shown in parentheses. Statistical differences between the release of ANP by control treated versus lipopolysaccharide-treated cardiac tissue were calculated by one-way ANOVA. *P* values of < 0.05 are denoted by *.

ments from at least eight consecutive twitches were averaged. Where indicated, the change from baseline and percentage change from baseline was calculated. Within-group comparisons were performed using a paired one-way analysis of variance (ANOVA), followed by Tukey–Kramer post-test analysis using Instat software where appropriate. Between-group comparisons were performed by two-way ANOVA using Prizm software. A P value of <0.05 was considered significant and denoted by *.

3. Results

3.1. Effects of lipopolysaccharide treatment in vivo on the release of nitrite, LDH and ANP from heart tissue

Segments of atrial but not ventricular tissue from control animals released detectable levels of nitrite over 24 h in culture (Table 1, Fig. 1). Under these conditions, there was no significant difference between the nitrite release from right versus left atria (Table 1). In vivo treatment with lipopolysaccharide increased the ability of atrial and ventricular tissue from rats to release nitrite (Table 1). Atrial tissue also released significant levels of LDH in culture (Table 2). LDH release tended to be elevated in tissue from animals treated with lipopolysaccharide, although this did not reach statistical significance ($p>0.05$, one-way ANOVA). Similarly, atrial tissue released detectable levels of ANP (Table 3), which like nitrite, was increased in tissue from animals treated with lipopolysaccharide. As expected, L-NAME significantly inhibited the release of nitrite from atrial (Fig. 2) and ventricular (by 91.2 ± .02%) tissue but had no effect on the accompanying release of either LDH (Table 2) or ANP (Table 3). It should be noted that D-NAME (1 mM) had no effect on either nitrite, LDH or ANP release by atrial tissue in culture.

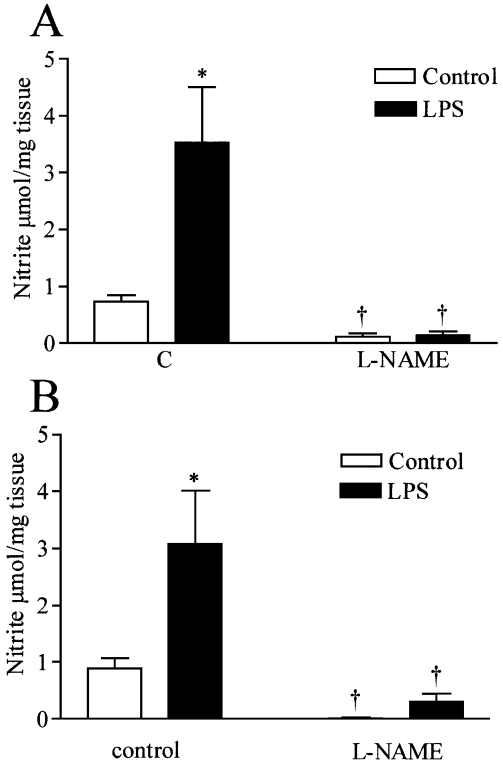

Fig. 2. Effects of lipopolysaccharide treatment in vivo on the level of iNOS mRNA expression in atrial and ventricular tissue. (A) Representative blot of iNOS expression in right and left atria (RA, LA) or right and left ventricle (RV, LV) and (B) data expressed semiquantitatively as optical densitometry (OD) measurements when compared with the OD of β-actin for the corresponding tissue. Tissue was taken from control and endotoxaemic rats. $n=4$ in each group.

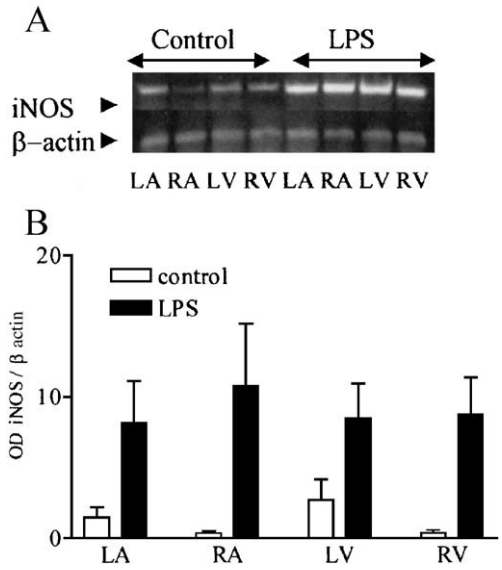

Fig. 3. The effects of L-NAME (1 mM) on nitrite release from left (A) or right (B) atrial tissue control and lipopolysaccharide-treated rats. Nitrite was measured after 24 h and was calculated per milligram of tissue. The data represent the mean ± S.E.M. for $n=7–10$. * denotes significant differences ($p<0.05$) in nitrite release when atria from control animals are compared with those from animals treated with lipopolysaccharide. † denotes significant differences in nitrite release by atrial tissue in the presence and absence of L-NAME. Statistical analysis is calculated by one-way ANOVA, followed by Tukey–Kramer post-test.

3.2. Effects of endotoxaemia on NOS mRNA expression in atrial and ventricular tissue

In freshly prepared heart tissue from control animals, mRNA for eNOS or nNOS (not shown) and iNOS (Fig. 3) were detected. nNOS mRNA expression was higher in atrial than ventricular tissue, although there was no apparent difference between nNOS expression in the right and left atria (not shown). There was no difference in iNOS (Fig. 3) and eNOS mRNA (data not shown) expression between atrial or ventricular tissue. When animals were treated with lipopolysaccharide, the levels of mRNA for iNOS (Fig. 3), but not eNOS or nNOS (not shown), were increased.

3.3. Effect of endotoxaemia on left atrial contractility induced by electrical field stimulation (baseline conditions)

There was no difference in peak-generated left atrial tension stimulated by electrical field stimulation in atria from control versus lipopolysaccharide-treated rats (control 0.19 g ± 0.04; $n=6$: lipopolysaccharide 0.18 g ± 0.05; $n=6$). Neither NOS inhibition with L-NAME (1 mM) nor substrate supplementation with L-arginine (1 mM) had any effect on baseline contractility in atria from either control or endotoxaemic animals (< 0.025% change from baseline over 40 min).

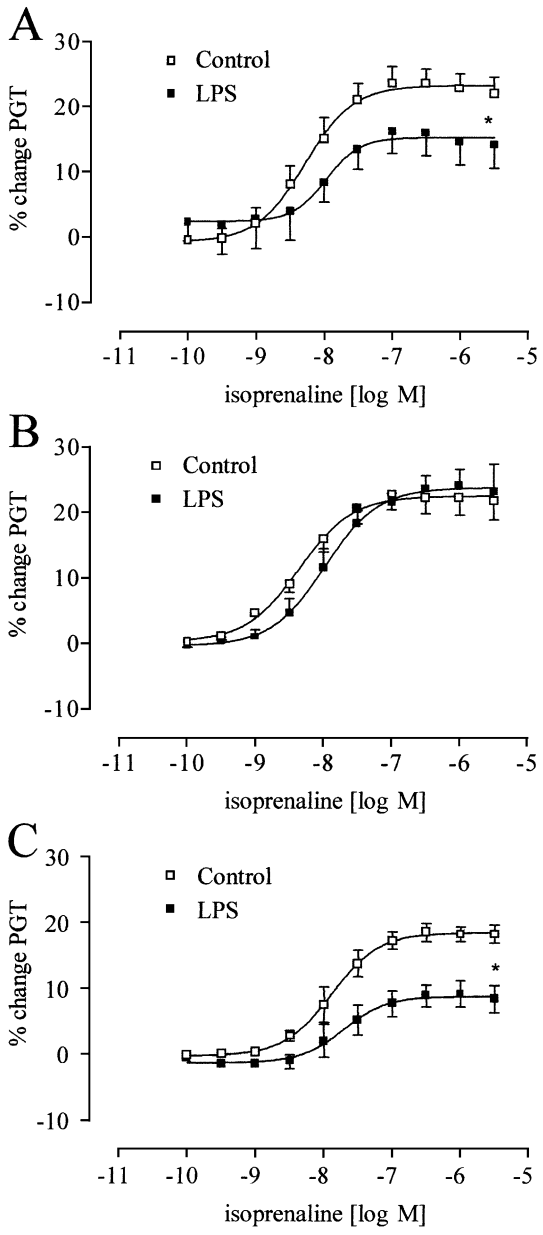

Fig. 4. The effects of lipopolysaccharide treatment in vivo on the positive inotropic response induced by isoprenaline in isolated left atria preparations. (A) In Krebs buffer; (B) in the presence of L-arginine (1 mM); (C) in the presence of L-arginine plus L-NAME (1 mM). Results are expressed as percentage change in peak-generated tension (percentage change PGT) and presented as the mean ± S.E.M. for $n=4-6$ experiments. Statistical differences were assumed at $p<0.05$ using two-way ANOVA and denoted by *.

3.4. Effect of endotoxaemia on the positive inotropic effects of isoprenaline in isolated atria

Isoprenaline induced a concentration-dependent increase in peak-generated tension in tissue from both control and endotoxaemic animals. However, the effect of isoprenaline was significantly ($p<0.05$; two-way ANOVA) depressed in atria from lipopolysaccharide-treated animals when compared to controls (Fig. 4). The addition of L-NAME (1 mM) had no affect on the response of atria from either control or lipopolysaccharide-treated animals to isoprenaline (data not shown). However, supplementation of the Krebs buffer with L-arginine (1 mM) restored the ability of atria from lipopolysaccharide-treated animals to contract to isoprenaline (Fig. 4), without effecting the response in tissue from control animals. In a separate group of experiments, the positive inotropic effects of L-arginine on the response to isoprenaline in atria from lipopolysaccharide-treated rats was blocked by presence of L-NAME (1 mM; Fig. 4).

3.5. Right atrial contractility

There was no statistically significant difference between the spontaneous rate of contraction (bpm) in atria from control versus lipopolysaccharide-treated rats (control 399 ± 5; $n=4$, lipopolysaccharide 432 ± 12; $n=5$). There was a concentration-dependent increase in atrial rate (chronotropy) in response to isoprenaline (E_{max} control 467 ± 10; $n=4$, E_{max} lipopolysaccharide 480 ± 16; $n=5$). Treatment with L-arginine, or NOS L-NAMEs had no significant effect on atrial chronotropy, or upon the chronotropic response to isoprenaline (data not shown).

4. Discussion

Overproduction of NO by stromal cells in the cardiovascular system is thought to contribute to the pathogenesis of septic shock. Thus, inhibitors of NOS have been highlighted as potential therapies to treat septic shock in man. However, despite encouraging reports using animal models of sepsis, the NOS inhibitor L-NMMA did not increase survival when given to patients with septic shock. Moreover, in a subpopulation of patients with cardiac dysfunction, L-NMMA may have been associated with increased mortality (Grover et al., 1999b). Here we present data that help to explain how NOS inhibitors can, under some circumstances, compromise cardiac function.

Under control conditions, rat atria released relatively high levels of nitrite, when compared to ventricular tissue. Some studies have shown that cardiac tissue can generate

NO non-enzymatically and that this may be important during conditions of ischaemia and reperfusion (Zweier et al., 1995). However, in our study, nitrite release by atrial tissue was greatly reduced by L-NAME, confirming that it originated from authentic NOS activity. In association with high NOS activity, atrial tissue expressed relatively high levels of nNOS mRNA. By contrast, levels of eNOS expressed by atrial and ventricular tissue were comparable. The relatively high levels of nNOS expressed in atrial tissue compared to that in the ventricle may be explained by the high density of nitrergic nerves present in the intracardiac ganglia of the atria (Schmidt et al., 1992; Tanaka et al., 1993). Despite the high levels of NOS activity present in atria constitutively, we (current study) and others (Nawrath et al., 1995) have found no evidence for a role in the modulation of myocyte contractility under baseline conditions. In addition to its contractile function, the atrium is a prime source for the generation, storage and release of the potent vasodilator peptide, ANP. Since NO modulates the synthesis of some hormones such as insulin (Southern et al., 1990), we hypothesised that the high levels formed by atrial tissue could modulate the co-release of ANP. However, we found that blocking NOS activity with L-NAME had no effect on ANP release in our model. Thus, it remains unclear what function constitutively released NO has in the atria, although others have suggested it modulates sympathetic neurotransmission (Sears et al., 1999).

Treatment of laboratory animals with bacterial lipopolysaccharide induces a pathophysiological state that has many similarities to sepsis in man. In addition, lipopolysaccharide induces the expression of iNOS in most organs tested, including the heart (Knowles et al., 1990; Mitchell et al., 1993, 2000). Further, iNOS is expressed in cardiac myocytes exposed to cytokines (Ballingand et al., 1994). In this study, we have confirmed that iNOS mRNA is expressed in cardiac tissue in experimental sepsis and that this is associated with an increase in the release of nitrite by cardiac tissue. In parallel, lipopolysaccharide treatment increased the release of ANP by atrial tissue. This observation is consistent with the hypothesis that plasma levels of ANP are raised in man during septic shock (Schneider et al., 1993). Nevertheless, the elevated levels of ANP associated with sepsis in this study were not influenced by the parallel increase in NO formation. In addition to nitrite and ANP, we also observed that cardiac tissue released LDH in culture, indicative of cell death having occurred. Again, as was the case for ANP release, NO did not appear to influence LDH, and thereby cell death in our model. Finally, the ability of atrial preparations to contract when stimulated electrically were also unaffected by lipopolysaccharide treatment in vivo. This is consistent with observations made using isolated myocytes in culture (Ungureanu-Longrois et al., 1995).

The observations described above seem to indicate that there is little role for NO in modulating the cardiac dysfunction associated with sepsis. However, in septic shock, patients have high intrinsic sympathetic drive, and often receive inotropic support with adrenergic agonists. To mimic more accurately the conditions of sepsis in man, we therefore studied the effects of endotoxaemia and NOS inhibition in atria stimulated with the beta agonist, isoprenaline. Atria from endotoxaemic animals displayed significant hyporesponsiveness to isoprenaline compared to tissue from control animals. These observations are consistent with others using isolated myocytes in culture stimulated with lipopolysaccharide or cytokines (Balligand et al., 1993; Chung et al., 1990; Ungureanu-Longrois et al., 1995), and with the cardiovascular dysfunction seen in septic shock in man.

The lipopolysaccharide-induced hyporeactivity to isoprenaline in atria was unaffected by NOS blockade with L-NAME. However, this dysfunction was entirely reversed by supplementation with the NO substrate L-arginine, an effect that was specifically associated with the NOS pathway (because it could be prevented when L-NAME was added together with L-arginine). These observations suggest that in our in vitro model using Krebs' buffer, L-arginine becomes rate limiting for the production of NO. Moreover, in the presence of plasma levels of L-arginine (0.5–1 mM), NOS inhibition has negative inotropic effects. This is in contrast to experiments using isolated ventricular myocytes, where diminished inotropic responses to isoprenaline were restored with NOS inhibition (Balligand et al., 1993; Chung et al., 1990). The differences between our observations, and others in the literature, may be explained by the levels of NO, and substrate depletion, that myocytes are exposed to in individual models. These factors may also be important at the level of response to NO. Indeed, when we performed extensive concentration responses studies with the NO donor SNP, a biphasic effect on atrial contractility was demonstrated (unpublished observations). In atria from control animals, SNP had a mild positive inotropic response followed by a profound negative response. A positive inotropic effect has been observed by others using another nitrovasodilator, SIN-1, and human atrial preparations (Nawrath et al., 1995). A negative inotropic response is consistent with several other studies using exogenously applied NO or nitrovasodilators (Brady et al., 1993; Nawrath et al., 1995). Interestingly, we found that atria from animals treated with lipopolysaccharide displayed an increased sensitivity to SNP (unpublished observations). The mechanisms involved in a lipopolysaccharide-induced 'sensitisation' to SNP are not known but may be related to either a pre-exposure to NO in vivo and/or upregulation of the guanylyl cyclase or other downstream pathways.

In conclusion, NOS inhibition has been found to be beneficial in some models of septic shock, reversing the haemodynamic effects. These promising effects in animal studies have not been reproduced in recent clinical trials of the inhibitor L-NMMA in patients with septic shock. In fact, although the adverse changes in peripheral vascular tone were reversed, in a significant number of patients, there was

a fall in the cardiac index (Grover et al., 1998, 1999a,b; Killbourne, 1999). The current study reveals an 'experimental window' where NOS inhibition induces significant negative inotropic effects which may help to explain the lack of benefit found in clinical trials, and indeed why mortality was increased by the use of L-NMMA in patients with septic shock and depressed cardiac function.

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