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Type II nitric oxide synthase activity is cardio-protective in experimental sepsis

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Abstract

Overproduction of nitric oxide (NO) via the induction of NO synthase (NOS) II is implicated in the pathogenesis of the refractory hypotension that characterizes septic shock. However, clinical trials of nonselective NOS inhibitors have failed to afford a mortality benefit in patients with sepsis, and in those with depressed left ventricular function, death rates were increased. Such observations have led to the suggestion that a selective inhibitor of NOSII would be more effective in treating septic shock, although precisely how NO modulates cardiac function in these circumstances remains unclear. We therefore used an isolated ejecting rodent heart model to study the effects of NO and experimental sepsis (endotoxin 20 mg kg i.p.) on cardiac functions. Coronary flow and cardiac output and ventricular functions were reduced by LPS, effects that were partially obviated by supplementation of perfusate with the NO substrate, L-arginine. These improvements were partially blocked by the selective NOSII inhibitor *N*-(3-(aminomethyl)benzyl)acetamidine (1400W) and further reduced by the combined NOSI, II and III inhibitor L-nitro L-arginine methyl ester (L-NAME). These findings suggest that NOSII is cardio-protective in the heart in sepsis and explain why its inhibition in man led to increased mortality in a subpopulation of patients.

Keywords: Nitric oxide synthase; Cardio-protective; Sepsis

1. Introduction

Sepsis and septic shock remain the major cause of death in the critically ill, accounting for around 250,000 deaths per annum in the USA alone. Both conditions are characterized by profound peripheral vascular dysfunction and reduced tissue oxygen extraction. Characteristic changes in myocardial performance also develop, which include systolic dysfunction, increased ventricular compliance, tachycardia and increased or normal cardiac index (Parker et al., 1984; Fink et al., 1985).

Experimental and clinical evidence suggest that the vasoactive mediator nitric oxide (NO) is involved in the pathogenesis of sepsis and septic shock. NO is produced from the semi-essential amino acid, L-arginine by NO synthases (NOS; Hobbs et al., 1999; Sessa, 1994). To date, three isoforms have been identified: two constitutive, and calcium-dependent (neuronal NOS, or NOSI, and endothelial NOS, or NOSIII; Bredt and Snyder, 1990; Pollock et al., 1991), and one inducible and calcium-independent (inducible NOS, or NOSII; Stuehr et al., 1991). NOSII is induced by a variety of stimuli implicated in the pathogenesis of sepsis, including cytokines and lipopolysaccharide (Hobbs et al., 2000).

In sepsis, NOSII is induced in the heart (Mitchell et al., 2000) where it limits vascular (Mitchell et al., 2000) and atrial (Price et al., 2002) function. Furthermore, in endotoxemia, NOSII has been identified in microvascular and vascular endothelial cells and in ventricular myocytes (Cook et al., 1994). In isolated cardiac myocytes, endotoxemia reduces contractile amplitude, an effect restored by NOS inhibition (Brady et al., 1992).

Patients with septic shock treated with NOS inhibitors display improved mean arterial pressure and systemic vascular resistance (Petros et al., 1991). However, large scale trials of the NOS inhibitor L-N^G monomethyl-L-arginine (L-NMMA) failed to show a mortality benefit (Grover et al., 1999) and may have been associated with increased death rates in patients with sepsis complicated by reduced left ventricular ejection fraction, which is known to occur in animal models (Harbrecht et al., 1992; Klabunde and Ritger, 1991a,b). Why this apparently adverse effect should occur in certain patients is unclear.

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In order to elucidate the role of NOS in modulating cardiac function in sepsis, we assessed the effects of L-nitro L-arginine methyl ester (L-NAME) and the selective NOSII inhibitor *N*-(3-(aminomethyl)benzyl)acetamidine (1400W) on the performance of isolated ejecting rodent hearts under control conditions and in endotoxemia.

2. Methods

All procedures and experiments were performed in accordance with the Home Office Animals (Scientific Procedure) Act (UK) 1986.

Male Wistar rats (weight 250–300 g) were randomized to endotoxemic (treated with lipopolysaccharide, 20 mg^{-1} kg^{-1} i.p.) and control (equivalent volume of saline i.p.) groups. Four hours after treatment, the animals were anesthetized with sodium pentobarbitone (100 mg^{-1} kg i.p) and were killed by cervical dislocation. Hearts were removed and immediately immersed in ice-cold, gassed (95% O_2/5% CO_2) Krebs–Henseleit solution (composition in mmol/l: NaCl 118, KCl 4.7, MgSO 4.7 H_2O 1.2, $NaHCO_3$ 24, KH_2PO_4 1.1, glucose 10 and $CaCl_2 \cdot 2H_2O$ 2.5), with added acebutolol 0.1 μmol/l (to prevent arrhythmias). The aorta was cannulated and the heart perfused retrogradely in Langendorff mode at a constant pressure of 80 cm H_2O with gassed Krebs–Henseleit buffer at 37 °C.

The left atrium was cannulated via the largest pulmonary vein, the others being ligated and the heart switched to ejecting mode (Grocott-Mason et al., 1994). Loading conditions were constant throughout (left atrial filling pressure, 10 cm H_2O, aortic afterload, 70 cm H_2O), and the heart was paced via an electrode applied to the right atrium, approximately 10% above its intrinsic rate. The pulmonary artery was vented to allow free drainage of the coronary effluent, permitting measurement of coronary flow. Aortic flow was measured using a flotation flowmeter (KDG Flowmeters), and coronary perfusion by 30-s timed collection of the pulmonary effluent. Cardiac output was calculated by the sum or coronary and aortic flows. Left ventricular pressure was recorded using a 3F Millar micromanometer-tipped catheter-transducer inserted into the left ventricular cavity, calibrated with a transducer control unit (TC-510, Millar Instruments) and zeroed to atmospheric pressure at the level of the left ventricle. The left ventricular pressure signal was sampled at 4 kHz and fed via a bridge amplifier into an Apple Macintosh personal computer connected to a recording and analysis system (MacLab 4, Analog Digital Instruments). Left ventricular dP/dt_{max} was obtained from the first derivative of the left ventricular pressure signal. Left ventricular end-diastolic pressure was measured as the pressure at the time of the initial upward deflection on the dP/dt trace.

Baseline left ventricular pressure and aortic and coronary flows were monitored for an equilibration period of 12 min. If these parameters were not stable, the heart was excluded from the study. Subsequently, study drugs were introduced as appropriate, and repeated measurements were taken over the next 24 min. Hearts were studied as follows ($n = 6$ for each): Group 1 control hearts; Group 2, control hearts treated with L-NAME 10^{-3} M; Group 3, control hearts treated with 1400W 10^{-5} M, then L-NAME 10^{-3} M; Group 4 control hearts in the presence of L-arginine 10^{-3} M; Group 5, control hearts in the presence of L-arginine 10^{-3} M treated L-NAME 10^{-3} M; Group 6, control hearts in the presence of L-arginine treated with 1400W 10^{-5} M, then L-NAME 10^{-3} M; Group 7, endotoxemic hearts; Group 8, endotoxemic hearts treated with L-NAME 10^{-3} M; Group 9, endotoxemic hearts treated with 1400W 10^{-5} M, then L-NAME 10^{-3} M; Group 10, endotoxemic hearts in the presence of L-arginine 10^{-3} M; Group 11, endotoxemic hearts in the presence of L-arginine 10^{-3} M treated with 1400W 10^{-5} M, then L-NAME 10^{-3} M; Group 12, endotoxemic hearts in the presence of L-arginine 10^{-3} M treated with L-NAME 10^{-3} M.

2.1. Drugs and chemicals

Lipopolysaccharide (*Salmonella enteritidis*) and L-NAME were obtained from Sigma (Poole, Dorset, UK). 1400W was obtained from ALEXIS (Nottingham, Notts, UK). Lipopolysaccharide was dissolved in normal saline, and all other drugs in distilled water. Chemicals for Krebs–Henseleit buffer were obtained from Merck (Poole, Dorset, UK).

2.2. Statistics

Data are expressed throughout as mean ± S.E.M. For all left ventricular pressure data, measurements from four consecutive beats were averaged and the percentage change from baseline was calculated. Between-groups comparisons were performed by two-way analysis of variance (ANOVA; Prism software, version 2.01) and statistically significant differences were assumed at $P < 0.05$.

3. Results

3.1. Effects of endotoxemia on cardiac function

Endotoxemia induced characteristic changes in myocardial function that were apparent 4 h after lipopolysaccharide administration and remained stable over the time course of the experiments (Fig. 1). Specifically, lipopolysaccharide administration was associated with reduction in coronary flow, aortic flow, stroke volume and cardiac output (Fig. 1). In addition, hearts from endotoxemic animals displayed a significant reduction in peak left ventricular pressure and an increase in left ventricular end-diastolic pressure (Fig. 1). There was a significant reduction in both dP/dt_{max} and dP/dtmin induced by experimental endotoxemia (Fig. 1), associated with an increase in t_{PP}, however, $R_{t_{1/2}}$ remained unchanged (data not shown).

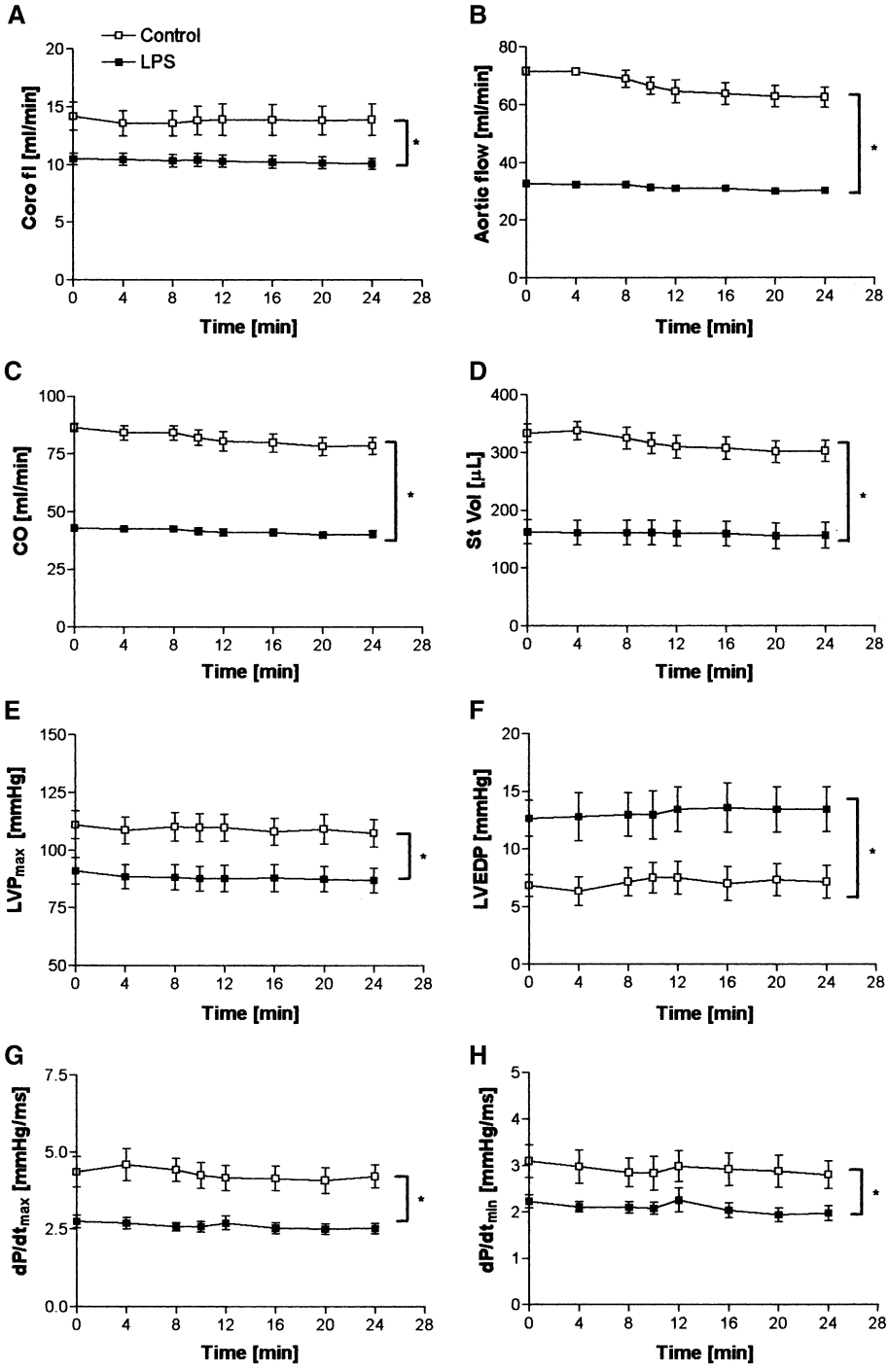

Fig. 1. Effects of in vivo administration of endotoxin (LPS) on the ex vivo coronary functions in working heart preparations. Measurements of coronary flow (Coro fl), aortic flow, cardiac output (CO), stroke volume (St Vol), left ventricular pressure (LVP), left ventricular end-diastolic pressure (LVEDP) and the integrated functions of dP/dt_{max} and dP/dt_{min} are shown for hearts from control (open squares) and endotoxemic (filled squares) rats. The data represents data collected at each time point shown and is the mean of data from $n=6$ animals ± S.E.M. Statistical differences were calculated between the two groups using a two-way ANOVA and statistical difference is assumed where $P<0.05$ (*).

3.2. Effects of 1400W or L-NAME in the absence or presence of L-arginine on cardiac function in hearts from control rats

In hearts from control animals, the selective NOSII inhibitor 1400W (10^{-5} M) had no effect on any of the cardiac parameters measured (Fig. 2). In hearts from control animals L-NAME (10^{-3} M) adversely affected myocardial function, an effect that was stable 12 min following addition of the drug. Specifically, L-NAME reduced coronary flow, aortic flow and stroke volume (Fig. 2). In addition L-NAME reduced LVP_{max} and increased in LVEDP (Fig. 2). The

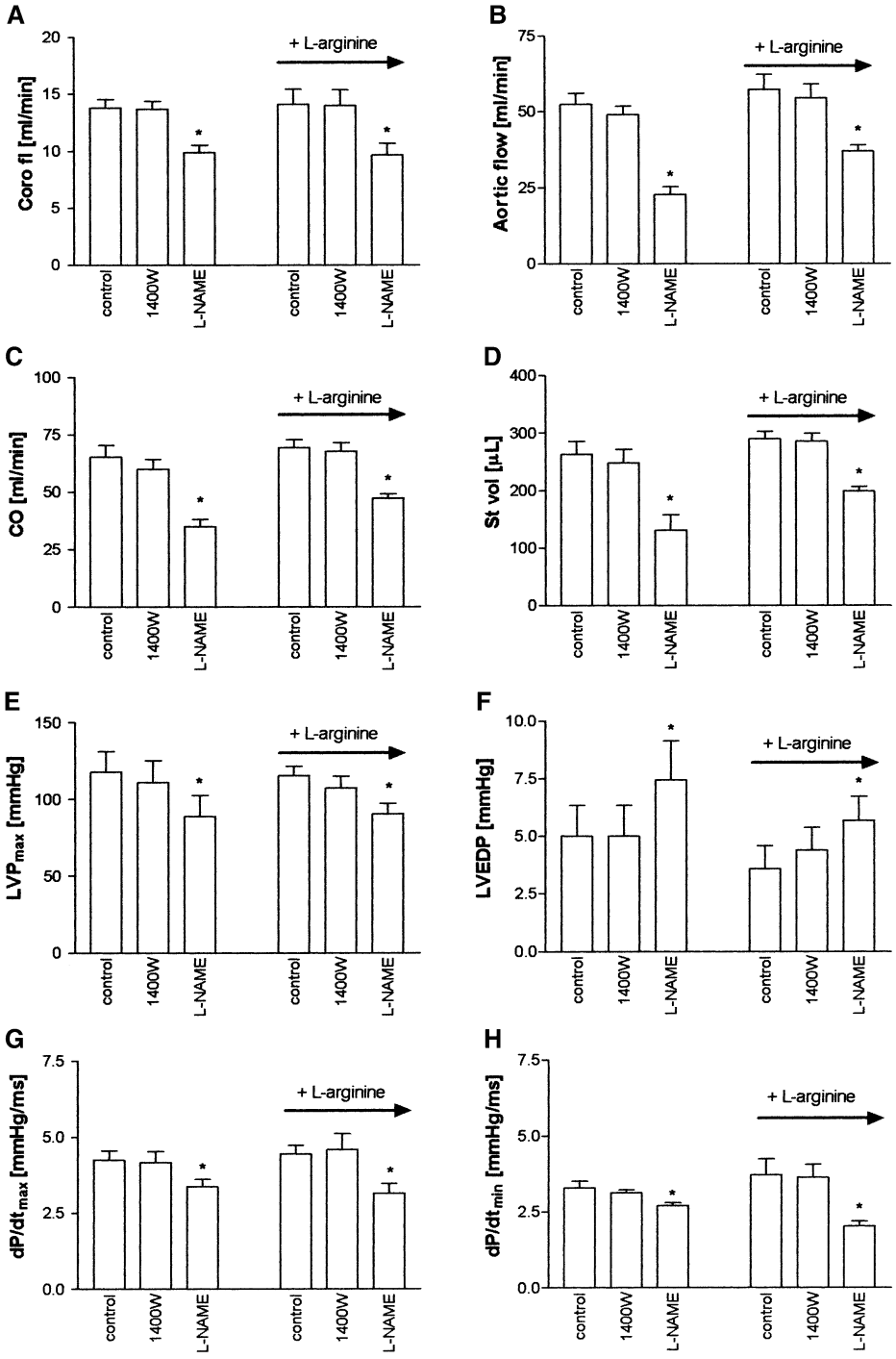

Fig. 2. Effects of 1400W, L-NAME and L-arginine on cardiac function in hearts from control rats. The figure shows parameters measured at a single time point taken 20 min after infusion of L-NAME or 1400W. Where L-arginine is shown, this was present in the Krebs buffer from the beginning of the experiment. Statistically analysis was performed on all time points collected (see Fig. 1) using a two-way ANOVA, and statistical significance between treatments and control responses was indicated in the figure by *. Where differences between parameters in the presence or absence of L-arginine were noted, statistical difference is indicated by +. The data is the mean ± S.E.M. for responses in hearts from $n = 6$ animals. Please note that responses observed in experiments where L-NAME was added together with 1400W did not differ with these where it was added alone.

changes in LV pressures in response to L-NAME were associated with a reduction in both dP/dt_{max} and dP/dt_{min}. No significant change in either t_{PP} or $R_{t_{1/2}}$ was noted (data not shown).

L-Arginine (10^{-3} M) had no effect on any of the parameters measured in control hearts and did not change the effects of 1400W or L-NAME given together with 1400W (Fig. 2) or by itself (data not shown).

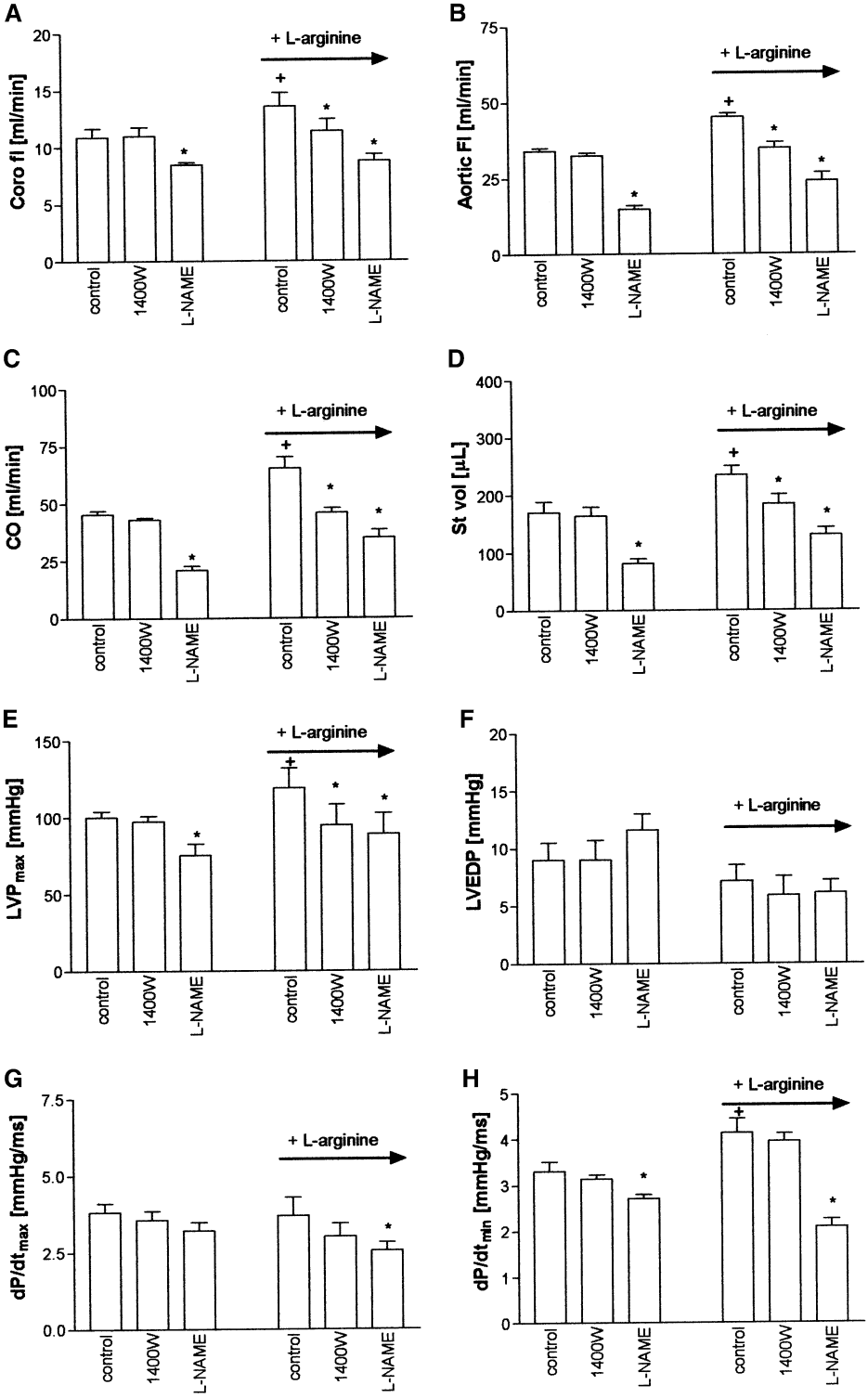

Fig. 3. Effects of 1400W, L-NAME and L-arginine on cardiac function in hearts from endotoxemic rats. The figure shows parameters measured at a single time point taken 20 min after infusion of L-NAME or 1400W. Where L-arginine is shown, this was present in the Krebs buffer from the beginning of the experiment. Statistical analysis was performed on all time points collected (see Fig. 1) using a two-way ANOVA, and statistical significance between treatments and control responses was indicated in the figure by *. Where differences between parameters in the presence or absence of L-arginine were noted, statistical difference is indicated by +. The data is the mean ± S.E.M. for responses in hearts from $n = 6$ animals. Please note that responses observed in experiments where L-NAME was added together with 1400W did not differ with these where it was added alone.

3.3. Effects of 1400W or L-NAME in the absence or presence of L-arginine on cardiac function in hearts from endotoxemic rats

As demonstrated in Figs. 1 and 2, hearts from endotoxemic rats exhibited cardiac dysfunction. When these hearts were perfused with Krebs buffer alone, 1400W had no effect on any of the cardiac parameters studied. However, addition of L-NAME reduced still further the already compromised (see Fig. 1) coronary flow, aortic flow, cardiac output and stroke volume (Fig. 3). L-NAME also increased LVEDP and reduced LVP_{max}, dP/dt_{max} and dP/dt_{min}. In contrast to the effects seen in hearts from control animals, L-arginine supplementation (10^{-3} M) in endotoxemic animals increased coronary flow, aortic flow, cardiac output and stroke volume and partially restored compromised LV function. When arginine was present in the perfusate, 1400W reduced coronary flow, cardiac output, aortic flow and stroke volume (Fig. 3). In the presence of L-arginine 1400W also tended to decrease some ventricular functions, although these effects were small (Fig. 3). Again, as discussed above, the effects of L-NAME in endotoxemic hearts were not modified by the presence of absence of 1400W (not shown).

4. Discussion

In rodents, lipopolysaccharide produces a pattern of cardiovascular injury similar to that seen in human septic shock, in association with increased NOSII expression and activity. Using the isolated, ejecting rodent heart, we have confirmed that in control animals, NO released via constitutive isoforms of NOS modulates coronary flow, aortic flow and ventricular function (Kelm and Schrader, 1990; Grocott-Mason et al., 1994). Second, we have shown that in endotoxemia, coronary flow, aortic flow and stroke volume are reduced, leading to profound ventricular dysfunction. Third, we have demonstrated for the first time that in endotoxemia, L-arginine is rate limiting and protective to cardiac function; and that under conditions of adequate substrate provision, selective NOSII inhibition, as well as nonselective inhibition of NOS, is incrementally detrimental to cardiac function already severely compromised.

In our study, the addition of L-NAME, which inhibits all NOS isoforms, caused a significant impairment in function (reduction in LVP_{max}, dP/dt_{max}, dP/dt_{min}, coronary flow and stroke volume and increase in LVEDP) in hearts from control animals, independent of alterations in preload or afterload. We and others have previously shown that under control conditions NO has no direct effect on myocardial contractility (Price et al., 1999a,b, 2002; Mitchell et al., 2000), suggesting that alterations in left ventricular contractility and reduced stroke volume induced by L-NAME occur secondarily to reduced coronary flow leading to ventricular ischemia. By contrast, the selective NOSII inhibitor 1400W had no effect on any of the measured parameters of cardiac function in hearts from control animals. These findings imply that under physiological conditions, basal production of NO via constitutive forms of NOS is necessary to maintain coronary flow and mediates cardiac function in the cardiovascular system.

In previous studies, myocardial dysfunction induced by sepsis has been attributed, at least in part, to overproduction of NO via NOSII. In our endotoxemic model, the reduction in myocardial function, readily apparent 4 h after lipopolysaccharide administration, was coincident with the induction of expression of NOSII mRNA in myocardial tissue and an increase in myocardial NOS activity and nitrite release (Price et al., 2002; Mitchell et al., 2000). As described, reduced coronary flow results in profound changes in myocardial function, independent of any direct effects of endotoxin upon myocyte contractility. In isolated blood vessels, NOSII expression and activity results in hyporesponsiveness to pressor agents (Rees et al., 1990). Thus, where NOSII is expressed in the heart, it is possible that NO production contributes to a reduction in coronary vascular tone. The reduction in coronary flow associated with this model of sepsis has been attributed to an increase in the release of the vasoconstrictor peptide endothelin-1 (Warner and Klemm, 1996). We hypothesized that in sepsis, release of NO as a result of NOSII expression in the heart acts to counterbalance this reduction in flow (or increased coronary tone) and is therefore cardio-protective. Indeed, when L-NAME was added to hearts removed from endotoxemic rats, the already impaired myocardial function was further compromised, with diminished LVP_{max}, dP/dt_{max}, dP/dt_{min} coronary flow and stroke volume and increase in left ventricular end-diastolic pressure. These results are consistent with findings in both animal and clinical studies in sepsis, where NOS inhibition has been found to be detrimental to myocardial function (Petros et al., 1994; Harbrecht et al., 1992; Klabunde and Ritger, 1991a,b). However, nonselective NOS inhibition was used in these studies, and it is possible that the detrimental effects found were due to inhibition of constitutive NOS (I and III) necessary for the maintenance of physiological function, masking the potentially beneficial effects of NOSII inhibition. We therefore addressed the effects of selective NOSII inhibition on these parameters of myocardial function using 1400W.

In hearts from control animals 1400W had no effect on any of the measured parameters. In initial experiments, in hearts from endotoxemic animals, 1400W had no effect—a surprising result as L-NAME had profound effects on myocardial function in the same group of experiments, and NOSII is known to be induced in cardiac tissue in this model of sepsis (Mitchell et al., 2000). It was thought possible that the NOSII expressed in myocardial tissue

might be inactive and/or might have no contribution to myocardial function. Alternatively, L-arginine might have been rate limiting for the production of NO under these conditions, as has been demonstrated in other systems (Hibbs et al., 1988) and as we have previously shown in the heart (Mitchell et al., 2000; Price, 2002). In view of these findings, all experiments were repeated in the presence of L-arginine. As expected, L-arginine alone had no effect on any of the measured parameters of myocardial function in tissue from control animals. In hearts from endotoxemic animals, however, L-arginine increased LVP_{max}, dP/dt_{max}, dP/dt_{min}, coronary flow and stroke volume. These beneficial effects on cardiac performance were clearly due to NOSII activity, as they were reversed by 1400W. It seems likely that NOSII may represent an important compensatory mechanism in established myocardial dysfunction in sepsis, as has been indicated by some experiments with knockout mice, where there is a reduction in the inflammatory response and hypotension in response to endotoxemia, but no reduction in mortality, and an increase in cardiac oedema (MacMicking et al., 1995; Laubach et al., 1995). We would therefore warn that even selective NOSII inhibition might be detrimental to patients with septic shock, especially those with impaired cardiac function.

4.1. Potential significance of this study

These data suggest that NO produced by NOSII is not responsible for inducing the myocardial dysfunction in endotoxemia, but rather is cardio-protective. The results of this study indicate that the use of selective NOSII inhibitors in clinical trials in sepsis is inappropriate until their effects have been more fully investigated. Finally, it should be noted, however, that differences may well exist between observations made in laboratory animals and the more complex setting of human sepsis, where overproduction of NO has recently been positively correlated to death in human sepsis (Brealey et al., 2002).

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