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Effects of nitric oxide-modulating amino acids on coronary vessels: relevance to sepsis

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Abstract

Excessive nitric oxide (NO) production in septic shock is thought to contribute to the associated profound hypotension. Here we show that despite induction of NO synthase (NOS) in the hearts of endotoxin-treated rats, coronary vascular responses to the contractile peptide endothelin-1, were not modified. This was not due to any change in the expression of endothelin receptors. However, when the substrate for NOS, L-arginine, was added to the perfusate, increases in coronary perfusion pressure stimulated by endothelin were reduced in hearts from endotoxin-treated animals compared to those from controls. In addition, L-glutamine, which blocks the generation of L-arginine from intracellular stores, enhanced the increase in perfusion pressure stimulated by endothelin-1. These data suggest that L-arginine becomes rate limiting for the production of NO in the coronary vessels during septic shock. Moreover, it suggests that vascular reactivity may be modulated positively or negatively by supplementation with the relevant amino acids.

Keywords: Arginine; Glutamine; Endothelin; Endotoxin; Nitro-arginine

1. Introduction

The continuous formation of nitric oxide (NO) by endothelial cells maintains blood pressure within the physiological range. Indeed, when NO production is inhibited, blood pressure increases (Rees et al., 1989). In health, the formation of NO by endothelial cells is mediated by a constitutive and calcium Ca^{2+}-dependent form of the enzyme NO synthase (eNOS; Pollock et al., 1991). A Ca^{2+}-independent form of NOS (iNOS; Baek et al., 1996) is expressed in many cell types after exposure to certain cytokines and/or bacterial endotoxin. Production of NO by iNOS appears unregulated and contributes to cardiovascular dysfunction. Indeed, the expression of iNOS in vascular smooth muscle cells is thought to account for the profound and unresponsive decline in blood pressure seen in patients with septic shock (see, Thiemermann, 1997).

Most patients with septic shock succumb to multiple organ failure. In these circumstances, cardiac function is also impaired, although the cellular mechanism behind these changes is not known. We (Mitchell et al., 1993) and others (Salter et al., 1991) have shown that iNOS is expressed in the heart after endotoxin treatment. In some blood vessels, the expression of iNOS results in a vascular hyporeactivity to contractile agents that is reversed by NOS inhibitors (see Thiemermann, 1997). However, in the case of coronary vessels, there appears to be a counterbalance to over production of NO with an increase in release of the constrictor peptide, endothelin-1 (see Warner and Klemm, 1996). Indeed, in a number of studies, some of us have shown that the coronary vasculature is not hyporesponsive, but actually acquires elevated tone ex vivo after treatment with endotoxin in vivo (Hohlfeld et al., 1995). Thus, it is not clear, despite a predicted induction of iNOS, why the coronary vessels in hearts from endotoxin-treated animals are more contracted than vessels from controls. Studies, using isolated cells in culture, have shown that L-arginine, the substrate for NOS, is rate limiting for the

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production of NO under certain conditions (Palmer et al., 1988; Mitchell et al., 1990a,b; Hattori et al., 1994). It is, therefore, possible that amino acid imbalances occur in sepsis, which influence the amount of NO formed in some organs. Since the heart is particularly metabolically active, amino acid depletion may occur and influence NO formation in coronary vessels. In the current study, we therefore addressed the nature of any interaction between (i) iNOS expression, (ii) L-arginine supply and the (iii) vascular effects of endothelin-1 in isolated perfused hearts from control and endotoxin-treated rats.

2. Materials and methods

2.1. Materials

The Krebs salts were from BDH (UK). All reagents were obtained from Sigma, unless otherwise stated.

2.2. Methods

2.2.1. In vivo treatments

Male Wistar rats were injected with endotoxin (20 mg kg^{-1}, i.p.) and were anaesthetised with sodium pentobarbitone (60 mg kg^{-1}) and killed by cervical dislocation 4 h later. The 4-h time point was selected for these experiments for two main reasons: (i) this time point has been used extensively by our group and others in the study of the effects of endotoxemia on cardiovascular parameters and (ii) NOS activity in the hearts at 4 h after endotoxin was clearly elevated compared to control, with little variation in the specific activity between animals (Fig. 2). Hearts were removed immediately and used for either (i) the measurement of NOS activity by the conversion of L-arginine to L-citrulline, (ii) the measurement by reverse transcriptase polymerase chain reaction (RT-PCR) of gene expression for iNOS and/or ET_A/ET_B or (iii) the study of the reactivity of coronary vessels using isolated perfused preparations.

2.2.2. Measurement of NOS activity and expression

Hearts of either control or endotoxin-treated rats were weighed, frozen in liquid nitrogen and stored at −80°C until NOS activity was measured by the ability of heart homogenate to convert [^{3}H]L-arginine to [^{3}H]L-citrulline (Amersham, UK; 37 MBq/ml; 1.3–2.6 TBq/mmol; Mitchell et al., 1993). Briefly, heart tissue was homogenised on ice in Tris (5×10^{-2} M; pH 7.4), containing phenylmethylsulfonyl fluoride (1×10^{-3} M), pepstatin (1×10^{-6} M) and leupeptin (1×10^{-6} M), in a ratio of 1:5 (w/v). Tissue homogenates were incubated at room temperature for 30 min in the presence of NADPH (1×10^{-3} M), calmodulin (300 U/ml), tetrahydrobiopterin (5×10^{-6} M), L-valine (5×10^{-5} M), L-arginine (1×10^{-5} M) and [^{3}H]-L-arginine (3×10^{-8} M; 10 kBq). Total NOS activity was measured in the presence of calcium (2×10^{-3} M). Ca^{2+}-independent NOS activity was determined by replacing calcium with EGTA (1×10^{-3} M). NOS-independent L-arginine conversion was determined by adding L-nitro-arginine methyl ester (L-NAME; 1×10^{-3} M) to separate samples measured in parallel with the other incubations. The reaction was terminated after 30 min, by the addition of HEPES buffer (2×10^{-2} M; pH 5.5 containing EGTA, 1×10^{-3} M and EDTA, 1×10^{-3} M). Newly formed L-citrulline was separated form the L-arginine by passing the reaction mixture over columns containing Dowex-50W (sodium form; Sigma), samples were eluted with 1 ml of the HEPES buffer. The samples were counted in a β-counter (Canberra Packard, Berks, UK).

2.2.3. Isolation of mRNA for endothelin ET_A, endothelin ET_B receptors and iNOS in rat heart by RT-PCR

Whole hearts were removed, frozen in liquid nitrogen and stored at −80°C until total RNA was extracted. A sample of approximately 50 mg of tissue was taken from each heart (ventricular apex) and total RNA was isolated with Micro-scale Total Separator Kit (Clontech Laboratories). Tissue (50 mg) was homogenised in a glass grinder with denaturing solution. Total RNA was isolated by a guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987) with minor modifications. RNA was precipitated with ethanol and washed with 75% ethanol. The RNA was then air-dried for 15–20 min before being redissolved in 20 μl of purified water. The yield and purity of RNA in the sample were checked by measuring the absorbency ratio, A_{260}/A_{280}. One microgram of mRNA from total RNA was converted to cDNA by reverse transcriptase using Ready-To-Go T-Primed First Strand Kit (Pharmacia Biotech). The cDNA was diluted in a 1:10 ratio, and 5 μl was used in the PCR. Each PCR reaction contained 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each dNTP (Promega), 0.4 μM of each gene-specific primer (Sevem Biotech) and 0.025 U/μl Taq polymerase (Promega).

Gene-specific primers were selected according to the published sequences in Gene Bank and were as follows.

Rat ET_A sense	5′-ATC GCT GAC AAT GCT GAG AG-3′
(226 bp) antisense	5′-CCA CGA TGA AAA TGG TAC AG-3′
Rat ET_B sense	5′-GAA AAG AGG ATT CCC ACC TG-3′
(255 bp) antisense	5′-ACG AAC ACG AGG CAT GAT AC-3′
Rat iNOS sense	5′-TCT GTC CCT TTG CTC ATG AC-3′
(304 bp) antisense	5′-CAT GGT GAA CAC GTT CTT GG-3′
GAPDH sense	5′-ACC ACA GTC CAT GCC ATC AC-3′
(452 bp) antisense	5′-TCC ACC ACC CTG TTG CGT TA-3′

Rat glyceraldehdye-3-phosphate dehydrogenase (GAPDH), a constitutively expressed gene, was chosen as a control. PCR was performed using an Omni Gene thermocycler. Initial denaturation was performed at 94°C for 2 min. Samples were annealed for 45 s at each suitable temperature and enzymatic primer extension occurred at 72°C for 1 min. The annealing temperatures were set as follows: iNOS, ET_A and ET_B receptors, 58°C and GAPDH 60°C. PCR was performed over a total of 30–35 cycles and final extension occurred at 72°C for 7 min. The PCR products were size-fractionated through 0.8% agarose gel and the bands were visualised with ethidium bromide.

2.2.4. Semi-quantitative analysis of PCR products

The amplified PCR products were electrophoresed on 0.8% agarose gels, stained with ethidium bromide, visualised by a UV transilluminator and photographed. The photographs were scanned by Kodak Biomax ID Image Analysis Software for Macintosh. The ratio of GAPDH to ET_A, ET_B, and iNOS was determined by densitometry.

2.2.5. Rat-isolated Landgendorff heart preparation

Hearts were removed immediately after death and plunged into ice-cold Krebs solution, whilst a glass cannula was inserted and fixed into the aortic stump. The cannula was attached to a perfusion system and the coronary vessels perfused through the aortic stump with oxygenated (95% O_2, 5% CO_2) Krebs buffer (in mM): NaCl 118; KCl 4; $CaCl_2 \cdot 2H_2O$ 2.5; KH_2PO_4 1.2; $MgSO_4 \cdot 7H_2O$ 1; $NaHCO_3$ 24; D-glucose 5; 37°C) at a constant flow rate of 10 ml/min. A pressure transducer (Gould Statham Instruments, Oxnard, CA, USA), attached to a side arm of the apparatus monitored coronary perfusion pressure. Left ventricular pressure was monitored throughout each experiment by a second transducer (Gould Statham Instruments) connected to a water-filled latex balloon inserted into the left ventricle via the mitral valve. Both transducers were attached to an Electro Med Multi Trace two-pen chart recorder. After a 1-h equilibration period, increasing doses of endothelin-1 (1×10^{-13} to 1×10^{-9} mol) were given by bolus injection into the perfusate solution. In parallel experiments, L-arginine, D-arginine, L-NAME, D-NAME (1×10^{-3} M for each), L-glutamine or D-glutamine (2×10^{-4} M for each) was included in the Krebs buffer at the time of attaching the hearts.

2.2.6. Statistics

Dose–response curves were compared by parametric or non-parametric two-way analysis of variance (ANOVA; GraphPad PRISM for Windows 97). Differences were considered statistically significant where p was less than 0.05 and denoted by an asterisk. EC_{50} and E_{max} values were also calculated using GraphPad PRISM.

3. Results

3.1. Characterisation of NOS isoforms in hearts from control and endotoxin-treated rats

Hearts from control rats contained low, but detectable levels of NOS activity, which were partially dependent on the presence of calcium (Fig. 1; $n = 4$). Hearts from endotoxin-treated animals contained significantly higher amounts of NOS activity, which were calcium-independent (Fig. 2; $n = 4$). In addition, hearts from endotoxin-treated, but not control, rats expressed iNOS mRNA (Fig. 2). Levels of eNOS and nNOS mRNA were very low or undetectable in hearts from either control or endotoxin-treated rats (data not shown). mRNAs for ET_A and ET_B receptors were also present in hearts from control and endotoxin-treated animals. When the bands corresponding to the relevant products were analysed by densitometry and standardised to the expression of GAPDH, no significant differences in the expression of either receptors were seen (optical density ratio), (ET_A, control 0.5 ± 0.1; endotoxin-treated, 0.49 ± 0.09: ET_B, control 0.52 ± 0.05; endotoxin-treated, 0.4 ± 0.1; $n = 4$).

3.2. Effects of endothelin-1 on coronary perfusion pressure and left ventricular pressure in hearts from control versus endotoxin-treated animals

Endothelin-1 (1×10^{-13} to 1×10^{-9} mol) caused concentration-dependent increases in coronary perfusion pressure measured in isolated hearts from both control and endotoxin-treated animals (Fig. 3). However, there were no significant differences (two-way ANOVA) between the potency or efficacy of endothelin-1 to increase coronary perfusion pressure in hearts from the two groups. In most,

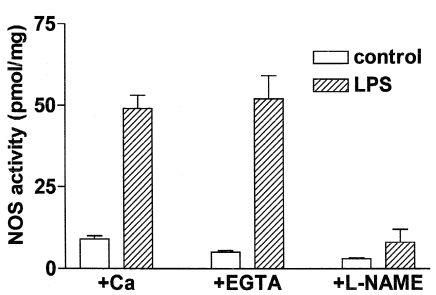

Fig. 1. Effect of endotoxin administration in vivo on NOS activity in the heart ex vivo, as determined by the conversion of L-arginine to L-citrulline. Total NOS activity was determined in the presence of calcium (2×10^{-3} M; +Ca) and calcium-independent NOS activity (iNOS) in the absence of calcium and in the presence of EGTA (1×10^{-3} M; +EGTA). The figure also shows the amount of formation of L-citrulline from L-arginine in the presence of L-NAME; this activity is representative of non-NOS conversion. The data represent the mean ± S.E.M. for four separate experiments each performed in duplicate.

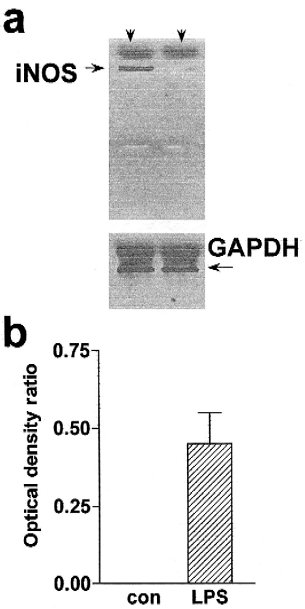

Fig. 2. Effect of endotoxin administration in vivo on the expression of iNOS mRNA in rat hearts. (a) Representative experiment showing the levels of iNOS and GAPHD in hearts taken from an endotoxin-treated rat and from a control rat. (b) Densitometric analysis of mRNA for iNOS in hearts from endotoxin-treated and control rats. Values are expressed as ratio to the expression of GAPDH as a standard. Mean ± S.E.M. from four hearts for each group is shown.

but not all, experiments, endothelin-1 caused maximum effects on coronary perfusion pressure at 1×10^{-9} mol. However, endothelin-1 as doses of 1×10^{-8} mol or above invariably caused a cessation of LV movement, and were therefore not included in the study. In parallel with the increase in coronary perfusion pressure, endothelin-1 caused dose-dependent reductions in left ventricular pressure in hearts from both control and endotoxin-treated animals (Fig. 3). Despite indistinguishable effects on coronary perfusion pressure, endothelin-1 was significantly more potent at inhibiting left ventricular pressure in hearts from endotoxin-treated animals than from control animals.

3.3. Effect of L-NAME on endothelin-1-induced response in rat-isolated heart preparations

L-NAME (Fig. 4), but not D-NAME (plus D-NAME, control heart coronary perfusion pressure, 53 ± 8 mm Hg: endotoxin heart coronary perfusion pressure, 55 ± 8 mm Hg; $n = 6$), (1×10^{-3} M for each) increased resting coronary perfusion pressure similarly in hearts from control and endotoxin-treated animals (Fig. 4). Moreover, L-NAME increased the potency, but not the E_{max} of endothelin-1 as a constrictor of coronary vessels in hearts from control rats and endotoxin-treated rats (Fig. 4). In the presence of

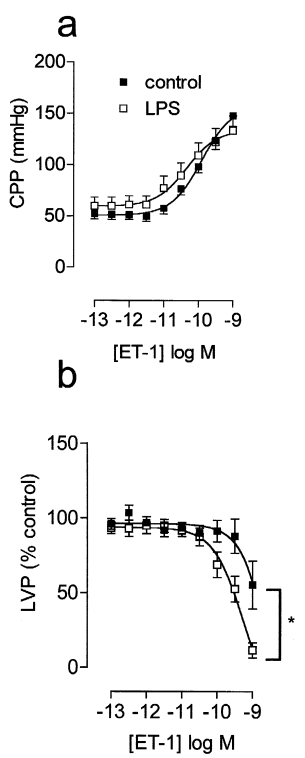

Fig. 3. Effect of endotoxin treatment on endothelin-1-induced changes in (a) coronary perfusion pressure and (b) left ventricular pressure in hearts from control and endotoxin-treated rats. The data represent the mean ± S.E.M. for five to eight separate experiments. Data were compared by two-way ANOVA and the asterisk illustrates where a significant difference was found.

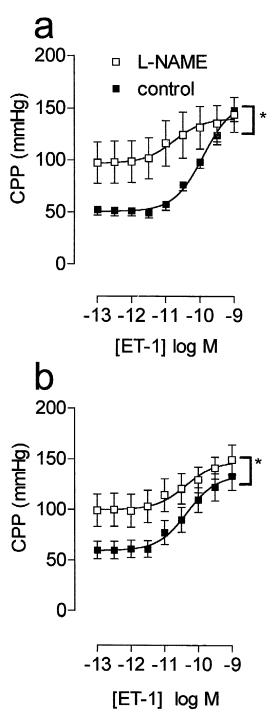

Fig. 4. Effect of L-NAME (1×10^{-3} M) on the increase in coronary perfusion pressure induced by endothelin-1 in hearts from (a) control (b) and endotoxin-treated rats. The data represent the mean ± S.E.M. for six to eight separate experiments. Data were compared by two-way ANOVA and the asterisk illustrates where a significant difference was found. In separate experiments, D-NAME was without effect on responses of hearts from either control or endotoxin-treated animals ($n = 6$).

L-NAME, no significant differences were seen in the response to endothelin-1 in hearts from control versus endotoxin-treated animals. L-NAME had no significant effects on basal left ventricular pressure or on endothelin-1-induced changes in left ventricular pressure in hearts from either control ($p = 0.13$; two-way ANOVA) or LPS-treated rats ($p = 0.997$; two-way ANOVA), ($n = 8$).

3.4. Effect of L-arginine and L-glutamine on endothelin-1-induced responses in rat-isolated heart preparations

L-arginine (1×10^{-3} M) reduced the ability of endothelin-1 to increase coronary perfusion pressure in hearts from endotoxin-treated animals (Fig. 5), but had no effect on the actions of endothelin-1 in hearts from control animals (Fig. 5). L-arginine had no significant effects on endothelin-1-induced changes in left ventricular pressure in hearts from either control ($p = 0.752$; two-way ANOVA) or endotoxin-treated rats ($p = 0.844$; two-way ANOVA), ($n = 8$).

L-glutamine potentiated the effects of endothelin-1 on coronary perfusion pressure in hearts from endotoxin-treated, but not control rats (Fig. 6). By contrast, L-glutamine had no significant effects on endothelin-1-induced changes in left ventricular pressure in hearts from either control ($p = 0.752$; two-way ANOVA) or endotoxin-treated rats ($p = 0.844$; two-way ANOVA), ($n = 6$; data not shown). In experiments carried out at the same time as the L-isomer counterparts (see Figs. 5 and 6 for control values), neither D-arginine (E_{max} for endothelin-1 in endotoxin-treated hearts, 148 ± 20 mm Hg) nor D-glutamine (E_{max} for endothelin-1 in endotoxin-treated hearts, 121 ± 14 mm Hg) had any effect on endothelin-1-induced responses in rat hearts from either group of animals ($n = 6$–8).

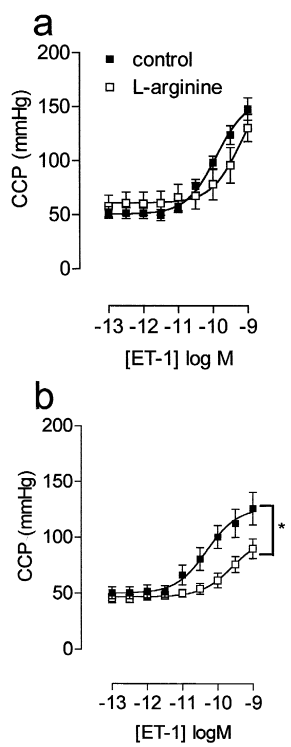

Fig. 5. Effect of L-arginine (1×10^{-3} M) on the increase in coronary perfusion pressure induced by endothelin-1 in hearts from (a) control (b) and endotoxin-treated rats. The data represent the mean ± S.E.M. for six to eight separate experiments. Data were compared by two-way ANOVA and the asterisk illustrates where a significant difference was found. In separate experiments, D-arginine was without effect on responses of hearts from either control or endotoxin-treated animals ($n = 6$).

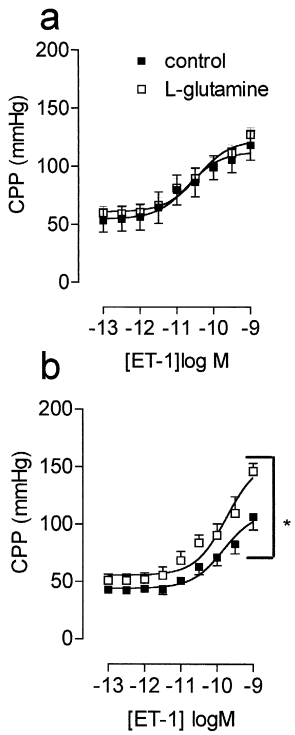

Fig. 6. Effect of L-glutamine (2×10^{-4} M) on the increase in coronary perfusion pressure induced by endothelin-1 in hearts from (a) control (b) and endotoxin-treated rats. The data represent the mean ± S.E.M. for six to eight separate experiments. Two-way ANOVA tests were performed on responses produced by the two groups of animals and the asterisk illustrates where a significant difference was found. In separate experiments, D-glutamine was without effect on responses of hearts from either control or endotoxin-treated animals ($n = 6$).

4. Discussion

iNOS expression has been demonstrated previously in hearts from rats treated with endotoxin (Schulz et al., 1992; Mitchell et al., 1993), where the peak of enzyme activity occurs at around 6 h, although mRNA expression is likely to increase much earlier. Here we confirm the observation that iNOS is expressed in the heart and show that despite this, in hearts from endotoxin-treated rats, there was no hyporeactivity to the contractile peptide, endothelin-1. These observations contrast with those of others using isolated vessels (mainly aorta) in organ baths treated, either in vitro (Rees et al., 1989) or in vivo

(Julou-Schaeffer et al., 1990), with endotoxin. However, we have previously shown that the mesenteric (Mitchell et al., 1993) or pulmonary (Griffiths et al., 1997) vascular beds of the rat are not hyporesponsive to constrictor agents, despite the induction of iNOS (Mitchell et al., 1993; Griffiths et al., 1997). In the case of the mesentery and the lung, the lack of hyporeactivity was attributed to upregulation of receptors and/or contractile elements counter acting the increased release of NO (Mitchell et al., 1993; Griffiths et al., 1997). Our current observations were not explained by an increased responsiveness of the coronary vessels to endothelin-1 since inhibition of NOS with L-NAME revealed no difference in the ability of endothelin-1 to constrict coronary vessels from control and endotoxin-treated rats. In support of this, we found no difference in the expression of endothelin A or endothelin B receptors in hearts taken from the two groups of animals.

Why then were the responses to endothelin-1 not antagonised by over production of NO in hearts taken from endotoxin-treated animals? Our data suggest that this is because L-arginine is rate limiting for the production of NO in hearts from septic animals, but not in those from controls. In health, plasma levels of L-arginine are 100 μM to 1 mM and intracellular levels of L-arginine in endothelial cells are maintained in the same range (Mitchell et al., 1990a,b; Swierkosz et al., 1990). The K_m of arginine for eNOS is in the 3 μM (Pollock et al., 1991). Thus, L-arginine is not rate limiting for the production of NO by a healthy cardiovascular system. However, the reduced ability of some blood vessels to contract after endotoxin can be potentiated by L-arginine (Julou-Schaeffer et al., 1990) and the production of nitrite by cells expressing iNOS is sensitive to the exogenous L-arginine concentration (Hattori et al., 1994). Moreover, arginase, which converts arginine to citrulline, is upregulated in vitro (Currie, 1978) and in vivo (Sonoki et al., 1997) by bacterial endotoxin. We suggest that as a combined result of arginase and iNOS induction, there is a net loss of L-arginine in sepsis that results in substrate depletion in the heart. Similar changes may also occur, to lesser or greater extents, in other organs during sepsis.

Arginine is available to NOS enzymes from extracellular and intracellular pools. Arginine enters cells via low (Winkle et al., 1985) and high (Christensen and Kilber, 1987) affinity uptake routes. Thus, while plasma L-arginine is in excess, the NOS enzymes in vascular cells are saturated by substrate. The intracellular pool of L-arginine is stored as a precursor, which may be L-citrulline (Hecker et al., 1991). Liberation of intracellular L-arginine is suppressed by L-glutamine (Sessa et al., 1990; Swierkosz et al., 1990; Hecker et al., 1991). Our study shows that L-glutamine potentiates the contractile action of endothelin-1 on coronary vessels in hearts from endotoxin-treated, but not control, animals. Thus, whole hearts, like isolated endothelial cells in culture (Mitchell et al., 1990a,b), or in situ (Swierkosz et al., 1990) express a glutamine-sensitive pathway for the intracellular generation of L-arginine. Moreover, this pathway would seem to have some relevance in septic shock. For this pathway to become apparent, intracellular levels of both L-arginine and L-glutamine must be limited. Since cellular depletion of L-glutamine and L-arginine occur in clinical and experimental septic shock (see Biolo et al., 1997), we hypothesise that this pathway may well be active in vivo.

In the current study, we showed that endotoxin treatment potentiated the depression of left ventricular pressure induced by endothelin-1. These observations were clearly not due to changes in coronary perfusion pressure, suggesting a direct effect of endotoxin on cardiac myocytes. This finding is in accordance with those of others showing that endotoxin directly depresses contractility of isolated LV myocytes (Keller et al., 1995; Toth and Heard, 1997; Yasuda and Lew, 1997), which in some cases (Brady et al., 1992; Balligand et al., 1997), but not others (Klabunde and Coston, 1995; Toth and Heard, 1997), are mediated by NO production. In our study, we found that the effects of endotoxin on left ventricular pressure were not mediated by iNOS expression, since L-NAME had no effect on these responses. Thus, endotoxin could be directly suppressing myocyte contractility or acting by the induction of one or more other genes known to be regulated in sepsis.

In conclusion, our data suggests that iNOS is expressed in rat hearts during septic shock. Under these conditions, L-arginine is rate limiting and NO release is supported by endogenous stores of substrate that can be reduced by exogenous L-glutamine. Much interest has recently been shown in the therapeutic potential of amino acid supplementation in septic shock as well as in other critical illnesses (see Alexander and Peck, 1990; Biolo et al., 1997). Our data provide further impetus for the selective or combined use of L-arginine and L-glutamine in these circumstances.

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References

Alexander, J.W., Peck, M.D., 1990. Future prospects for adjunctive therapy: pharmacological and nutritional approaches to immune system modulation. Crit. Care Med. 18, S159–S164.

Baek, K.J., Thiel, B.A., Lucas, S., Stuehr, D.J., 1996. Macrophage nitric oxide synthase subunits purification, characterisation and role of prosthetic groups and substrate. J. Biol. Chem. 268, 21120–21124.

Balligand, J.L., Ungureanu, D., Kelly, R.A., Kobizik, L., Pimental, D., Michel, T., Smith, T.W., 1997. Abnormal contractile function due to induction of nitric oxide synthase in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. J. Clin. Invest. 91, 2914–2919.

Biolo, G., Toigo, G., Stulin, R., Iscra, F., Gullo, A., Guarnieri, G., 1997. Metabolic response to injury and sepsis: changes in protein metabolism. Nutrition 13, 52S–57S.

Brady, A.J., Poole-Wilson, P.A., Harding, S.E., Warren, J.B., 1992. Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia. Am. J. Physiol. 263, H1963–H1966.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Christensen, H.N., Kilber, M.S., 1987. Amino acid transport across the plasma membrane: role of regulation in terorgan flows. In: Yudilevich, A.S., Boyd, D.P. (Eds.), Amino Acid Transport in Animal Cells. Manchester Univ. Press, UK, pp. 10–45.

Currie, G.A., 1978. Activated macrophages kill tumour cells by releasing argininase. Nature 29, 758–759.

Griffiths, M.J.D., Curzen, N., Mitchell, J.A., Evans, T.W., 1997. In vivo treatment with LPS increases rat pulmonary vascular contractility despite NOS induction. Am. Rev. Respir. Crit. Care Med. 156, 654–658.

Hattori, Y., Campbell, E.B., Gross, S.S., 1994. Argininosuccinate synthetase mRNA and activity are induced by innunostimulants in vascular smooth muscle. Role in the regeneration of arginine for nitric oxide synthase. J. Biol. Chem. 13, 9405–9408.

Hecker, M., Sessa, W.C., Mitchell, J.A., Anggard, E.E., Vane, J.R., 1991. The biosynthesis of EDRF by endothelial cells as a means of removing excess nitrogen. J. Cardiovasc. Pharmacol. 17, S19–S24, Suppl. 3.

Hohlfeld, T., Klemm, P., Thiemermann, C., Warner, T.D., Schror, K., Vane, J.R., 1995. The contribution of tumour necrosis factor alpha and endothelin-1 to the increase of coronary resistance in hearts from rats treated with endotoxin. Br. J. Pharmacol. 116, 3309–3315.

Julou-Schaeffer, G., Gray, G.A., Fleming, I., Schott, C., Parrat, J.R., Stoclet, J.C., 1990. Loss of vascular responsiveness induced by endotoxin involves L-arginine pathway. Am. J. Physiol. 259, H1038–H1043.

Keller, R.S., Jones, J.J., Kim, K.F., Myers, P.R., Adams, H.R., Parker, J.L., Rubin, L.J., 1995. Endotoxin-induced myocardial dysfunction: is there a role for nitric oxide. Shock 4, 338–344.

Klabunde, R.E., Coston, A.F., 1995. Nitric oxide synthase inhibition does not prevent cardiac depression in endotoxic. Shock 3 (205), 992–997.

Mitchell, J.A., Hecker, M., Ånggard, E., Vane, J.R., 1990a. Cultured endothelial cells maintain their L-arginine level despite the continuous release of EDRF. Eur. J. Pharmacol. 182, 573–576.

Mitchell, J.A., Hecker, M., Vane, J.R., 1990b. The generation of L-arginine in endothelial cells is linked to the release of endothelium-derived relaxing factor. Eur. J. Pharmacol. 176, 253–254.

Mitchell, J.A., Kohlhass, K.L., Sorrentino, R., Murad, F., Warner, T.D., Vane, J.R., 1993. Induction of calcium-independent NO synthase in rat mesentery: possible role in the hypotension associated with sepsis. Br. J. Pharmacol. 109, 265–271.

Palmer, R.M., Ashton, D.S., Moncada, S., 1988. Vascular endothelial cells synthesise nitric oxide from L-arginine. Nature 333, 664–666.

Pollock, J.S., Förstermann, U., Mitchell, J.A., Warner, T.D., Schmidt, H.H.H.W., Nakane, M., Murad, F., 1991. Purification and characterisation of EDRF synthase. Proc. Natl. Acad. Sci. U. S. A. 88, 10480–10485.

Rees, D.D., Palmer, R.M.J., Moncada, S., 1989. Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc. Natl. Acad. Sci. 86, 3375–3378.

Salter, M., Knowles, R.G., Moncada, S., 1991. Widespread tissue distribution, species distribution and changes in activity of calcium-dependent and calcium-independent nitric oxide synthase. FEBS 291, 145–149.

Schulz, R., Nava, E., Moncada, S., 1992. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. Br. J. Pharmacol. 150, 575–580.

Sessa, W.C., Hecker, M., Mitchell, J.A., Vane, J.R., 1990. The metabolism of L-arginine and its significance for EDRF biosynthesis: L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 87, 8607–8611.

Sonoki, T., Nagasaki, A., Gotoh, T., Takiguchi, M., Matsuzaki, H., Mori, M., 1997. Co-induction of nitric oxide and argininase I in cultured rat peritoneal macrophages and rat tissue in vivo by lipopolysaccharide. J. Biol. Chem. 272, 3989–3993.

Swierkosz, T.A., Mitchell, J.A., Sessa, W.C., Hecker, M., Vane, J.R., 1990. L-glutamine inhibits the release of endothelium-derived relaxing factor from the rabbit aorta. Biochem. Biophys. Res. Commun. 172, 143–149.

Thiemermann, C., 1997. Nitric oxide and septic shock. Gen. Pharmacol. 29, 159–160.

Toth, I., Heard, S.O., 1997. Nitric oxide does not mediate lipopolysaccharide-induced myocardial depression in guinea pigs. Crit. Care Med. 25, 684–688.

Warner, T.D., Klemm, P., 1996. What turns on the endothelins?. Inflammation Res. 5, 51–53.

Winkle, L.J.V., Christensen, H.N., Campione, A.L., 1985. Na^{+}-dependent transport of basic, zwitterionic and bycylic amino acids by a broad-scope system in mouse blastocytes. J. Biol. Chem. 260, 12118–12123.

Yasuda, S., Lew, W.Y., 1997. Lipopolysaccharide depresses cardiac contractility and beta-adrenergic contractile response by decreasing myofilament response to Ca^{2+} in cardiac myocytes. Circ. Res. 81, 1011–1020.