BACTERIAL ENDOTOXIN RAPIDLY STIMULATES PROLONGED ENDOTHELIUM-DEPENDENT VASODILATATION IN THE ISOLATED PERFUSED RAT HEART

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Running Title: **Vasodilator actions of lipopolysaccharide in the rat heart**

Summary

1. The effects of bacterial lipopolysaccharide (*Escherichia coli* 0111-B4; LPS) on coronary vascular tone were examined in the isolated perfused rat heart. The role of nitric oxide and/or prostaglandin products of the cyclooxygenase pathway in mediating the actions of LPS were also investigated.

2. Coronary vascular tone was raised and maintained by a continuous perfusion of the thromboxane mimetic U46619 (5 nM). LPS perfusion (0.1 - 100 μg ml^{-1}) caused a concentration-dependent fall in coronary tone without any significant change in the force of cardiac contractility.

3. At 5 μg ml^{-1} LPS reduced perfusion pressure by 38 ± 9 mmHg. This effect was rapid in onset, maximal within the first 5 min and sustained for 90 ± 10 min (n=6).

4. The vasodilatation induced by LPS was dependent on the presence of an intact endothelium and abolished following endothelial damage caused by air embolism.

5. N^G-nitro-L-arginine methylester (L-NAME; 50 μM) or N^G-nitro-L-arginine (L-NOARG; 50 μM) blocked the vasodilatation induced by LPS (5 μg/ml). The inhibition caused by these arginine analogues was partially reversed by 1 mM L- but not D-arginine.

6. The vasodilator action of LPS was also completely blocked by the glucocorticoid dexamethasone (10 μM) but unaffected by indomethacin (10 μM).

7. These results suggest that LPS evokes rapid release of nitric oxide (NO) in the microvasculature of the isolated rat heart presumably via activation of the constitutive L-arginine-NO pathway in the endothelium. Furthermore, the lack of effect of indomethacin suggests that prostaglandins released via the cyclooxygenase pathway are not involved in mediating this action of LPS.

Key Words: Rat Heart; Coronary microcirculation; Bacterial Lipopolysaccharide; Nitric oxide; L-arginine; N^G-nitro-L-arginine methylester; N^G-nitro-L-arginine; Dexamethasone; Indomethacin; Bradykinin

Introduction

Experimental models of endotoxin shock have shown that administration of bacterial lipopolysaccharide (LPS) to animals *in vivo* results in a characteristic biphasic fall in systemic blood pressure in which the first phase of the response occurs rapidly following administration of LPS and the second phase develops as a gradual progressive fall in blood pressure after a lag phase of about 30 min (Thiemermann & Vane, 1990; Wright *et al*., 1992). The development of the sustained hypotensive response (second phase) is currently attributed mainly to nitric oxide (NO) (Kilbourn *et al*., 1990; Thiemermann & Vane, 1990, Wright *et al*., 1992), derived from the cationic amino acid L-arginine (Palmer *et al*., 1988a; Schmidt *et al*., 1988). Synthesis of NO under these conditions involves induction of the Ca^{2+}/calmodulin-independent NO synthase previously identified in macrophages (Stuehr & Marletta, 1987; Marletta *et al*., 1988). Expression of this enzyme is time-dependent and involves *de novo* protein synthesis which can be inhibited by the protein synthesis inhibitor cyclohexamide and by the glucocorticoid dexamethasone (Knowles *et al*., 1990; Radomski *et al*., 1990; Rees *et al*., 1990a). Once induced the activity of the enzyme can be blocked by inhibitors of NO synthase including N^G-monomethyl-L-arginine (L-NMMA) (Kilbourn *et al*., 1990; Radomski *et al*., 1990; Rees *et al*., 1990a; Thiemermann & Vane, 1990, Wright *et al*., 1992).

In addition to the inducible enzyme there is also a constitutive Ca^{2+}/calmodulin dependent NO synthase which generates NO in the endothelium under normal physiological conditions. NO released via this pathway regulates blood flow and is thought to maintain the cardiovascular system in a constant state of vasodilatation (Rees *et al*., 1990b; see Moncada *et al*., 1991 for review). At present it is not clear whether, in the vasculature, this enzyme is also

activated by LPS and whether NO released via the constitutive pathway plays any role in maintaining the deleterious actions of LPS either *in vivo* or *in vitro*. In this context it is of interest that in rats both the initial and sustained phases of the vasodilator response to LPS are attenuated by L-NMMA (Thiemermann & Vane, 1990), suggesting that NO may be released acutely by LPS in the earlier stages of endotoxic shock. This hypothesis however remains to be substantiated, since in similar studies L-NMMA did not inhibit the initial action of LPS in lowering arterial blood pressure in rabbits (Wright *et al*., 1992).

In the present study we have investigated the effects of LPS in the isolated perfused rat heart with the aim of establishing whether coronary tone can be altered acutely by a direct action of LPS on the microvasculature. Using inhibitors of nitric oxide synthase, N^G-nitro-L-arginine (Moore *et al*., 1990; Ishii *et al*., 1990) and N^G-nitro-L-arginine methylester (Rees *et al*., 1990b), we have also examined whether NO is released acutely in response to LPS and whether this may mediate the effects of LPS in the microvasculature of the rat heart. Furthermore, since LPS is known to activate phospholipase A_2 (Liu & Takeda, 1982) and thereby elevate prostaglandin synthesis we have assessed whether inhibition of the cyclooxygenase pathway influenced the actions of LPS in the rat heart.

Methods

Isolation and perfusion of the rat heart

Hearts obtained from male Sprague-Dawley rats (200-350 g) were perfused by a modified Langendorff technique as described previously (Baydoun & Woodward, 1991). Briefly, hearts were perfused at a constant rate of 10 ml min^{-1} with Krebs-Henseleit buffer maintained at 37^{o}C and equilibrated with 95 % oxygen and 5 % carbon dioxide. Coronary perfusion pressure was monitored continuously using a pressure transducer attached to an aortic cannula. The force of cardiac contraction was measured with a tension transducer attached to the apex of the heart. Each heart was allowed to equilibrate for a minimum period of 20 min prior to beginning an experimental protocol.

In some experiments, coronary tone was raised and maintained using the thromboxane-mimetic, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619; 5 nM). When applied, *E. coli* lipopolysaccharide (LPS) was dissolved in Krebs buffer and perfused continuously through the heart. Maximal doses of bradykinin (BK, 1 nmol) and sodium nitroprusside (SNP; 10 nmoles), used in a previous study (Baydoun & Woodward, 1991), were applied as bolus injections into the perfusate, 3 cm proximal to the aortic cannula. All other agents, including indomethacin (10 μM), dexamethasone (10 μM), N^{G}-nitro-L-arginine (L-NOArg; 50 μM) and N^{G}-nitro-L-arginine methylester (L-NAME; 50 μM) were added to the buffer reservoir and perfused through the heart for a minimum period of 30 min before obtaining responses to LPS (5 μg ml^{-1}). In other experiments dexamethasone (10 μM) or L-NAME (50 μM) was administered after the vasodilator response to LPS (5 μg ml^{-1}) had reached a maximum. The dependency of the vasodilator action of LPS on an intact endothelium was investigated after damaging the endothelium by injecting 1 ml bolus of air into the coronary circulation as

previously described (Baydoun *et al*., 1989).

Materials

Bradykinin, sodium nitroprusside, *Escherichia coli* lipopolysaccharide (serotype: 0111 B4), indomethacin, dexamethasone, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$, L-N^G-nitro arginine, L-N^G-nitro arginine methylester, L- and D-arginine were obtained from Sigma (Pool, Dorset). All other reagents were of analytical grade and purchased from British Drug House (Poole, Dorset).

Statistical analysis

Results are representative of observations in at least three separate hearts and are expressed as mean ± S.E. of n experiments. Statistical significance was assessed using a Student's t-test and values of $P < 0.05$ were considered significant.

Results

Vasodilator action of bacterial lipopolysaccharide

Coronary tone was raised in each experiment prior to the administration of LPS by continuous perfusion of the thromboxane mimetic U46619. At 5 nM U46619 increased perfusion pressure from a basal value of 44 ± 6 mmHg to 120 ± 15 mmHg (n = 9). Cumulative application of LPS (0.1 - 100 μg ml^{-1}) caused a rapid, concentration-dependent decrease in coronary tone (Fig 1). At 5 μg ml^{-1}, the lowest concentration required to initiate a reproducible sustained response, LPS induced a maximum fall in perfusion pressure of 38 ± 9 mmHg (n = 6). This response was maximal within 5 min and reversible upon the removal of LPS from the perfusate. Subsequent administration of LPS to the same tissue, after a 50 min washout period, resulted in a marked reduction in the response compared with that previously observed (Fig. 2).

During continuous infusion of LPS (5 μg ml^{-1}; Fig. 3), the vasodilatation induced was sustained for 30 ± 2 min before recovering, requiring a further 60 ± 10 min to return to the pre-LPS level (n=3). Cardiac contractility was not altered over this time period (Fig. 3). Furthermore, continued infusion of LPS after this period did not result in any additional decrease in perfusion pressure nor did it cause a loss in the responsiveness to the vasoconstrictor U46619 (5 nM). Following recovery, coronary tone was maintained at 120 ± 14.4 mmHg (n=3) for a further 3 h in the continued presence of both LPS and U46619.

Effects of NO synthase inhibitors on LPS-induced vasodilatation

N^G-Nitro-L-arginine methylester (L-NAME; 50 μM) or N^G-nitro-L-arginine (L-NOArg; 50 μM) produced a maintained rise in perfusion pressure, increasing basal coronary tone by 81 ± 19 mmHg (n = 6) and 75 ± 5 mmHg (n = 3) respectively. The vasoconstriction caused by either

compound was partially reversed by L- but not

D-arginine (1mM), and appeared to be endothelium-dependent as it was abolished by the passage of air through the heart. Perfusion of L-arginine (1 mM) had no significant effect on coronary tone in the absence of either L-NAME or L-NOARG but respectively reduced the vasoconstriction caused by these compounds by 18 ± 3 mmHg (n = 5) and 15 ± 2 mmHg (n = 3).

Infusion of L-NAME (50 μM) through hearts 30 min prior to the application of LPS (5 μg ml^{-1}) completely blocked the vasodilatation induced by LPS (Fig 4) in four, and reduced the response by 50 and 75 % respectively in two out of six hearts. In the experiments where complete inhibition was not achieved, the vasodilator response to LPS was only short-lived in that the fall in pressure returned to control levels within 8 min. In addition, L-NAME (50 μM) reversed the vasodilatation caused by LPS when added to the perfusate after the response to LPS had reached a maximum. Similar results were also obtained using L-NOArg (50 μM) which completely blocked the vasodilator action of LPS in 3 seperate experiments. The inhibition caused by either of these arginine analogues was partially reversed by L- arginine (1 mM).

The above findings suggest that nitric oxide may be released acutely in the microvasculature of the rat heart in response to LPS. We therefore investigated whether the gradual recovery of the vasodilator action of LPS (Fig 3) was due to the depletion of L-arginine, the substrate for nitric oxide biosynthesis. In these experiments, addition of L-arginine (1 mM) to the buffer reservoir after the vasodilatation to LPS had returned to its pre-LPS level failed to elicit a drop in coronary perfusion pressure. Furthermore, inclusion of L-arginine (1mM) in the perfusate at the start of LPS infusion failed to prolong the vasodilator action of LPS (data not

shown).

Endothelium-dependent vasodilator action of LPS

Continuous perfusion of the isolated rat heart with bradykinin (BK) results in a rapid desensitization, and thus it could only be applied in bolus doses (unpublished observations). Administration of either BK (1 nmol) or sodium nitroprusside (SNP; 10 nmol) to the intact perfused rat heart, with tone raised by U46619 (5 nM), produced a marked fall in coronary tone, respectively reducing perfusion pressure by 68 ± 5 mmHg (n = 5) and 66 ± 3 mmHg (n = 5). The responses to these agents were characteristically different from that obtained with LPS in that they were rapid in onset, reaching a maximum within 30 s.

Application of air (1 ml) to the intact heart had no significant effect on the maximal vasodilator action of SNP but completely abolished the vasodilatation produced by BK and LPS. When administered to hearts after air embolism, BK (1 nmol) initiated a marked vasoconstriction response, increasing perfusion pressure by 45 ± 3 mmHg (Fig. 5; n = 4).

Effects of indomethacin on LPS-induced vasodilatation

Perfusion of isolated rat hearts with indomethacin (10 μM) caused a marked drop in coronary tone (Fig 6), reducing perfusion pressure from 102 ± 12 mmHg to 58 ± 4 mmHg (n = 3). To maintain the coronary tone at its previous elevated level the concentration of U46619 was raised to 10 nM.

In three separate experiments indomethacin (10 μM) did not significantly modify the vasodilator actions of LPS. Under these conditions both the magnitude (43 ± 9 mmHg; n =3)

and duration (94 ± 15 min; n = 3) of the LPS vasodilator response was maintained even though hearts had been perfused with indomethacin for 30 min prior to and during the LPS challenge (Fig 6).

Effects of dexamethasone on LPS-induced vasodilatation

In contrast to indomethacin, infusion of dexamethasone (10 μM) 30 min prior to LPS administration completely blocked the vasodilator action of LPS. Interestingly, when added to the perfusate after the response to LPS had reached a maximum, dexamethasone failed to reverse the vasodilatation induced by LPS (data not shown).

Discussion

The data obtained in this study show that LPS is a potent vasodilator agent in the microvasculature of the isolated rat heart, causing an acute drop in coronary tone which was rapid in onset and sustained over a prolonged period. Following recovery, perfusion pressure was maintained at its pre-LPS levels for periods of up to 3 hours without any loss of vascular tone even though hearts were continually perfused with LPS and U46619. This lack of hyporeactivity to U46619, following prolonged exposure to LPS, is in contrast to findings in isolated rat aortic rings in which exposure to LPS for 4 h results in a hyporeactive response to phenylephrine (Rees *et al*., 1990a) and norepinephrine (Julou-Schaeffer *et al*., 1991; Fleming *et al*., 1990). This effect has been attributed to induction of NO biosynthesis, as it could be potentiated by L-arginine and abolished by L-NMMA (Rees *et al*., 1990a; Julou-Schaeffer *et al*., 1991; Fleming *et al*., 1990).

Lack of a sustained loss in coronary tone under our experimental conditions indicates that induction of the Ca^{2+}/calmodulin-independent NO synthase may not occur in the microvasculature of the rat heart *in vitro*. It is likely however that under *in vivo* conditions LPS could induce NO synthase in the coronary vessels of the rat. Indeed in other studies, vasoconstrictor responses of isolated perfused rabbit hearts to U46619 were significantly reduced if the animals were preexposed to LPS for 4 h prior to isolation of the hearts (Smith *et al*., 1991). The fact that large blood vessels (aorta) can be induced *in vitro* whereas resistance vessels could not perhaps reflects a difference between macrovascular and microvascular tissues. Alternatively, it is possible that in the rat heart marked desensitization to LPS occurs following the initial vasodilator response. This would explain the recovery of vascular tone after ~ 90 min and the inability of continued LPS administration to sustain the vasodilatation. Furthermore, since L-arginine did not maintain the LPS-mediated response, this suggests that depletion of

cellular L-arginine did limit production of NO.

The inhibition of LPS-induced vasodilatation caused by L-NAME and L-NOArg, both competitive inhibitors of NO synthase (Ishii *et al*., 1990; Moore *et al*., 1990; Rees *et al*., 1990b), strongly suggests that NO is a mediator of this effect. More importantly, it indicates that LPS can cause NO release acutely in the microvasculature of the isolated rat heart. The rapidity in onset of the response suggests that under these conditions synthesis and release of NO does not involve induction of the Ca^{2+}/calmodulin-insensitive NO synthase but instead the activation of the constitutive pathway in endothelium. This finding is further substantiated by recent studies showing that exposure of cultured endothelial cells to LPS results in the rapid release (within 1 min) of a NO-like factor which was capable of inhibiting platelet aggregation (Salvemini *et al*., 1990). In addition, it also lends support to the hypothesis that acute release of NO *in vivo* may in part mediate the early hypotensive response observed following administration of LPS to rats (Thiemermann & Vane, 1990).

In our studies both L-NAME and L-NOArg caused a marked increase in perfusion pressure, in the absence of U46619, indicating that NO is released basally in the rat coronary microcirculation. This finding is consistent with studies in other tissues *in vitro* (Amezcua *et al*., 1989; Moore *et al*., 1990; Palmer *et al*., 1988b; Rees *et al*., 1989a; 1990b) and *in vivo* (Aisaka *et al*., 1989; Gardiner *et al*., 1990; Rees *et al*., 1989b, 1990b) and further strengthens the belief that basal release of NO modulates vascular tone (Amrani *et al*., 1992; Rees *et al*., 1990b; see Moncada *et al*., 1991).

In the vasculature, constitutive NO synthase is localized entirely in the endothelial layer

(Knowles *et al*., 1990). Our observation that the vasodilator action of LPS was endothelium-dependent and abolished following perfusion of air through the coronary circulation further supports the concept that LPS activates constitutive NO synthase in endothelium. Under these conditions the vasodilator action of bradykinin, an endothelium-dependent vasodilator, was abolished whereas responses to the endothelium-independent vasodilator sodium nitroprusside were maintained. Thus, following air embolism endothelial function is impaired with the smooth muscle layer remaining virtually intact. The underlying mechanism for the vasoconstrictor action of bradykinin following endothelial damage is as yet unclear but may involve a direct action of this peptide on the vascular smooth muscle.

The data from the present study implicates the constitutive, rather than the inducible, L-arginine-NO pathway in the actions of LPS in the isolated rat heart. It is therefore paradoxical that the vasodilatation caused by LPS within 5 min of administration could be inhibited by pretreatment with the glucocorticoid dexamethasone. In other studies dexamethasone blocks induction of the Ca^{2+}/calmodulin-independent NO synthase but does not affect the activity of either the constitutive or the induced enzyme (Radomski *et al*., 1990; Rees *et al*., 1990a). The mechanism by which dexamethasone exerts its inhibitory action in our study is yet to be established.

Dexamethasone has a wide spectrum of activity and, in addition to preventing the expression of inducible NO synthase, can also block metabolism of membrane phospholipid to arachidonic acid and thus inhibit production of vasoactive prostanoids including prostacyclin. If prostacyclin were involved in mediating the effects of LPS, then inhibition of its synthesis would account for the actions of dexamethasone. However, our data obtained with indomethacin

suggest that cyclooxygenase products are not released in the rat heart following administration of LPS. Indeed it is questionable whether vasodilator products from this pathway are involved in regulating vascular tone in the rat heart *in vitro*. In previous studies we could not detect significant changes in prostacyclin levels in the effluent collected from rat hearts stimulated with bradykinin (Baydoun & Woodward, 1991). The fact that perfusion with indomethacin (Fig. 6), or flurbiprofen (Baydoun et al., 1990), results in a marked fall in coronary perfusion pressure suggests that vasoconstrictor prostaglandins are released basally in the rat heart and contribute to the maintenance of vascular tone.

One possible mechanism by which dexamethasone can exert its inhibitory action would be by preventing the binding of LPS to its recognition sites on the endothelium. This hypothesis is supported by recent studies in mouse peritoneal macrophages in which binding of tritium-labelled LPS was strongly blocked by dexamethasone, over the same concentration range as used in our study (Tahri-Jouti & Chaby., 1990). The fact that dexamethasone could not reverse a vasodilatation induced by LPS suggests that LPS may avidly bind to its recognition sites on the endothelium and may not be easily displaced.

In summary, the data obtained from the present study show that LPS is a potent vasodilator agent in the microvasculature of the isolated rat heart. This action of LPS is endothelium-dependent, rapid in onset and maintained over 90 min. Furthermore, it is unaffected by indomethacin but susceptible to inhibition by NO synthase inhibitors. This inhibition by the L-arginine analogues is partially reversed by L-arginine, suggesting that the vasodilator action of LPS in the fine resistance vessels of the rat heart may be mediated by NO synthesized by the Ca^{2+}/calmodulin dependent NO synthase. More importantly, it delineates a crucial pathogenic

role for NO, synthesized via the constitutive pathway, in the initial hypotension observed in the earlier stages of endotoxic shock.

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Figure 1. Concentration response curve to bacterial lipopolysaccharide (LPS) in the isolated perfused rat heart. The graph shows the mean with s.e. of the mean indicated by the vertical bars (n = 3).

Figure 2. Representative tracing of the vasodilatation induced in the isolated rat heart by acute perfusion of bacterial lipopolysaccharide (LPS). PP denotes coronary perfusion pressure.

Figure 3. Representative tracing of the vasodilatation induced in the isolated rat heart by prolonged perfusion of bacterial lipopolysaccharide (LPS). PP denotes coronary perfusion pressure and DT the developed tension.

Figure 4. Inhibition of the vasodilator action of bacterial lipopolysaccharide (LPS) by N^G-nitro-L-arginine methylester (L-NAME) and the partial reversal of the inhibition by L-arginine (L-Arg).

Figure 5. Effect of perfusion of air (1 ml) through the coronary circulation of the isolated rat heart on the vasodilator responses to bolus injections of bradykinin (BK) and sodium nitroprusside (SNP) and to continuous perfusion of bacterial lipopolysaccharide (LPS).

Figure 6. Effects of indomethacin (Ind) on coronary perfusion pressure (PP) and LPS-induced vasodilatation in the isolated perfused rat heart.