Mechanisms of acute vasodilator response to bacterial lipopolysaccharide in the rat coronary microcirculation

Toby R Cannon, Giovanni E Mann and Anwar R Baydoun[1]

Vascular Biology Research Centre, Physiology Group, Biomedical Sciences Division, King's College, Campden Hill Road, London W8 7AH, U.K.

[1]Author for correspondence: Anwar R Baydoun
Vascular Biology Research Centre
Biomedical Sciences Division
King's College
Campden Hill Road
London W8 7AH, U.K.

Telephone 0171 333 4372
Fax 0171 333 4008
E-mail A.Baydoun@kcl.ac.uk

Running Title: Vasodilator action of lipopolysaccharide in the rat heart

Key words: Coronary microvasculature, bacterial lipopolysaccharide, nitric oxide, PLA_2, glucocorticoid, lipocortin 1, cyclooxygenase, lipoxygenase

Summary

1 This study has investigated the mechanisms of the acute vasodilator action of bacterial lipopolysaccharide (LPS) in the rat Langendorff perfused heart.

2 Infusion of LPS (5 μg/ml) caused a rapid and sustained fall in coronary perfusion pressure (PP) of 59 ± 4 mmHg (*n* = 12) and a biphasic increase in NO levels determined in the coronary effluent by chemiluminescent detection. Both the fall in PP and the increase in NO release were completely abolished (*n* = 3) by pre-treatment of hearts with the NO synthase inhibitor L-NAME (50 μM).

3 LPS-induced vasodilatation was markedly attenuated to 5 ± 4 mmHg (*n* = 3) by pre-treatment of hearts with the B_2 kinin receptor antagonist HOE 140 (100 nM).

4 Vasodilator responses to LPS were also blocked by brief pre-treatment with mepacrine (0.5 μM, *n* = 3) or nordihydroguaiaretic acid (0.1 μM, *n* = 4) and markedly attenuated by WEB 2086 (3 μM, n=4).

5 30 min pre-treatment of hearts with dexamethasone (1 nM), but not progesterone (1 μM), significantly modified responses to LPS. The action of dexamethasone was time dependent, having no effect when applied either simultaneously with or pre-perfused for 5 min prior to administration of LPS but inhibiting the response to LPS by 91 ± 1% (*n* = 4) when pre-perfused for 15 min. The inhibition caused by dexamethasone was blocked by 15 min pre-treatment with the glucocorticoid receptor antagonist RU-486 (100 nM) or by 2 min pre-perfusion of a 1:200 dilution of LCPS1, a selective anti-LC 1 neutralising antibody.

6 Treatment with the protein synthesis inhibitor, cycloheximide (10 μM, for 15 min)

selectively blunted LPS-induced vasodilatation, reducing the latter to 3 ± 5 mmHg (n = 3) while having no effect on vasodilator responses to either bradykinin or sodium nitroprusside.

7 These results indicate that LPS-induced vasodilatation in the rat heart is dependent on activation of kinin B_2 receptors and synthesis of NO. In addition, PLA_2 is activated by LPS resulting in the release of PAF and lipoxygenase but not cyclo-oxygenase products. These effects are dependent on *de novo* synthesis of an intermediate protein which remains to be identified.

Introduction

Nitric oxide (NO) synthesised by inducible NO synthase has been identified as an important mediator of the marked hypotension and hyporeactivity to vasoconstrictor agents induced by bacterial lipopolysaccharide (LPS) in experimental models of endotoxemia (Kilbourn *et al.,* 1990; Thiemermann & Vane, 1990). Although the role of constitutive NO synthase in this disease state still remains to be established, it has become apparent that LPS may also activate this pathway (Thiemermann & Vane, 1990) with the released NO accounting, at least in part, for the initial fall in mean arterial pressure observed *in vivo*. In this regard we have previously demonstrated that infusion of LPS through the rat isolated perfused heart causes a rapid fall in coronary perfusion pressure (Baydoun *et al.,* 1993). This acute vasodilator response was endothelium-dependent and blocked by inhibitors of nitric oxide (NO) synthase including L-N^G-nitro-L-arginine methylester (L-NAME), strongly suggesting that LPS acutely releases NO, presumably via the constitutive L-arginine-NO pathway.

We have now extended these studies by examining whether LPS activates constitutive NO synthase directly or via intermediate pathways. It is worth noting that both bradykinin and platelet activating factor (PAF) may be released acutely following administration of LPS *in vivo* (Doebber *et al.*, 1985; Katori *et al.*, 1989). Furthermore, PAF produces a similar *in vivo* hypotensive response to that caused by LPS (Bessin *et al.*, 1983; Doebber *et al.,* 1985; Mulder *et al.*, 1993) and both bradykinin and PAF antagonists have been reported to attenuate the acute hypotensive response to LPS (Doebber *et al.,* 1985; Wilson *et al.*, 1989). We have therefore examined the effects of the potent and selective kinin B_2 receptor antagonist D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8] bradykinin (HOE-140) (Lembeck *et al.,* 1991) and the PAF antagonist WEB 2086 (Casals-Stenzel *et al.*, 1986) on LPS-induced

vasodilatation in the rat isolated perfused heart. We have also examined the role of PLA_2-derived mediators other than PAF in the response to LPS.

In our previous study (Baydoun *et al.*, 1993) dexamethasone (10 μM) completely abolished the acute coronary vasodilatation caused by LPS. This finding was of interest because dexamethasone has previously only been shown to inhibit expression of the inducible isoform of NO synthase whilst having no effect on the constitutive enzyme or the activity of inducible NO synthase once expressed (Radomski *et al.*, 1990). We have therefore further examined this finding by investigating both the concentration and time dependency of the action of dexamethasone and its antagonism by the glucocorticoid receptor antagonist, RU-486. Using a selective lipocortin 1 (LC1)-neutralising antibody, LCPS1 (Perretti *et al.*, 1996), we have explored whether the actions of dexamethasone are mediated by LC1, a member of the annexin family which is strongly induced by glucocorticoids and accounts, at least in part, for the pharmacological actions of dexamethasone (Flower & Rothwell, 1994).

Methods

Isolation and perfusion of rat heart

Hearts from male Sprague-Dawley rats (≥ 250g) were perfused as described previously (Baydoun *et al.*, 1993) by a modified Langendorff technique similar to that described by Broadley (1979) at a constant flow of 10 $ml.min^{-1}$ with Krebs-Henseleit buffer (composition (mM): NaCl, 118; $NaHCO_3$, 25; D-glucose, 11; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.1) maintained at 37 ºC and gassed with 95 % O_2/5% CO_2. Following an equilibration period of at least 20 min, basal coronary vascular tone (56 ± 4 mmHg; n = 12) was elevated (113 ± 8 mmHg; *n*=12) by infusion of the thromboxane mimetic 9,11-dideoxy-11α,9α-epoxymethano-

prostaglandin $F_{2\alpha}$ (U46619; 5 nM). Bolus injections of bradykinin (0.1 nmol) or sodium nitroprusside (SNP; 10 nmol) were administered into the perfusion line 3 cm proximal to the heart. All other agents, including LPS, were added to the buffer reservoir and perfused through the coronary vasculature.

Chemiluminescent detection of nitric oxide

Release of NO into the coronary effluent was monitored by chemiluminescent detection using a Sievers NO analyser (model 280A, Sievers instruments Inc. Boulder CO, USA), as previously described (Menon *et al.*, 1989). In these studies, coronary flow was reduced to 1 ml min^{-1} in order to concentrate amounts of nitrite/nitrate released into the effluent. This was carried out in a stepwise manner by reducing flow from 10 to 8, 5, 3 and 1 $ml.min^{-1}$, allowing the heart to equilibrate for 10 min at each reduced flow rate. This protocol did not result in any impairment of myocardial function, since when flow was restored to 10 $ml.min^{-1}$, cardiac contractility, vascular tone and heart rate returned to their normal values prior to reducing flow.

After equilibration at 1 ml min^{-1} aliquots of effluent (0.5 ml) were collected prior to (control sample) and at approximately 3 min intervals following perfusion of LPS (5 μg ml^{-1}) over a period of 60 min. In time-matched controls hearts were perfused with Krebs buffer alone. Samples were immediately frozen at -20 ºC until analysed. Nitrite and nitrate, the stable oxidation products of NO in the heart (Kelm & Schrader, 1990), were converted to NO by injecting 2-10 μl of effluent into a vessel containing a reducing solution of vanadium(III) chloride in HCl. Liberated NO was then purged from solution by bubbling with nitrogen under reduced pressure and analysed by reaction with ozone. This reaction yields light, the intensity of which was recorded using a cooled red-sensitive photomultiplier tube. The

integral of light intensity with respect to time is proportional to the amount of NO yielded from the sample injection. A calibration curve was constructed using standard solutions of sodium nitrate in water and used to determine NO concentration in coronary effluent samples. Each sample was analysed in triplicate.

Materials

Salts for Krebs buffer and hydrochloric acid were obtained from British Drug House Ltd. (Poole, Dorset). U46619, bacterial lipopolysaccharide (from *E. coli*, serotype 0111:B4), bradykinin, cycloheximide, L-N^G-nitro-L-arginine methyl ester, nordihydroguaiaretic acid and sodium nitroprusside were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Vanadium(III)chloride was from Fluka Ltd. (Gillingham, Dorset, UK). RU-486 and anti-LC1 antibodies (LCPS1 and LCPS2) were gifts from Professor R. Flower (William Harvey Institute, London, UK). HOE-140 and WEB 2086 were respective gifts from Hoechst (Mannheim, Germany) and Dr. G. Cirino (University of Naples; Italy).

Statistical analysis

Results are expressed as mean ± s.e. mean for *n* experiments. Statistical analyses were performed using a multiple means comparison test (Harper, 1984) validated by comparison with the Newman-Keuls multiple range test in the statistical package SPP (Royston, 1984) with the overall confidence levels set at 99% (0.01).

Results

Involvement of nitric oxide in LPS-induced coronary vasodilatation

Consistent with our previous studies (Baydoun *et al.*, 1993), LPS (5 μg.ml^{-1}) caused a rapid fall in perfusion pressure (PP) of 59 ± 4 mmHg (*n*=12). This response was maximal within 5 minutes and maintained over a prolonged period (> 90 min) during continued LPS infusion. In parallel experiments, chemiluminescent analysis of coronary effluent revealed a marked increase in NO release 3 min after introducing LPS and by 6 min this had declined again, but was still higher than basal values (Fig 1). Moreover, NO production was maintained at the lower plateau level over the 60 min period of LPS perfusion. No significant change above basal was detected in effluent collected from hearts perfused with Krebs buffer alone.

Termination of LPS infusion resulted in rapid reversal of the vasodilator response, with vascular tone returning to the pre-LPS level within 3 min after cessation of LPS perfusion. Stimulated NO production also decreased rapidly to basal levels following LPS washout. In addition, perfusion of hearts with L-NAME (50 μM) abolished the vasodilator response and release of NO caused by LPS, but did not alter the response to SNP and reduced the duration but not the magnitude of the response to bradykinin (Table 1).

Effects of HOE-140 on LPS-induced vasodilatation

Pre-perfusion of hearts with the selective kinin B_2 receptor antagonist HOE-140 (100 nM) caused a small but significant increase in PP of 19 ± 8 mmHg ($n = 3$; $p < 0.05$). More importantly, HOE pre-treatment completely abolished the vasodilator response to a maximal concentration of bradykinin (0.1 nmol) and markedly attenuated LPS-induced vasodilatation, reducing the latter by 92 ± 6 % (n=3). In contrast, HOE-140 had no effect on the

vasodilatation induced by SNP (10 nmol) confirming its selectivity of action (Fig 2).

Role of PLA_2, PAF and lipoxygenase products in LPS-induced vasodilatation

Experiments were carried out employing mepacrine, an effective but non-selective inhibitor of PLA_2, to ascertain whether activation of PLA_2 also contributes to the response to LPS. In these studies, mepacrine (0.5 μM, 30 min pre-perfusion) selectively blocked LPS-induced vasodilatation but had no direct effect on coronary tone or on the magnitude and duration of responses to either bradykinin (0.1 nmol) or SNP (10 nmol) (Table 2).

We have previously shown that indomethacin has no significant effect on the acute actions of LPS in the rat heart (Baydoun *et al.*, 1993), current studies were therefore focused on other PLA_2-dependent pathways. Studies involving PAF itself proved difficult to interpret for several reasons. Firstly, responses to PAF were quite variable. Secondly, a single administration of PAF, even at very low doses (10^{-21} to 10^{-7} mol), resulted in marked and rapid desensitisation to subsequent applications (data not shown). However, experiments carried out using the PAF receptor antagonist WEB 2086 revealed that LPS-induced vasodilatation was at least in part PAF dependent, since it was attenuated by WEB 2086. A 30 min pre-perfusion of hearts with WEB 2086 (3 μM) reduced the LPS-induced fall in PP by 70 ± 8% (n = 4) whilst having no effect on coronary tone or on either bradykinin (0.1 nmol) or SNP (10 nmol) induced vasodilatation (Table 2).

In contrast to indomethacin, pre-perfusion of hearts with nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase enzymes (Slapke *et al.*, 1983), at a concentration of 0.1 μM for 30 min, significantly decreased the vasodilator action of LPS in the rat heart. Under these conditions, NDGA reduced the LPS-induced fall in PP by 86 ± 5% (n = 4) but

failed to modify either bradykinin (0.1 nmol) or SNP (10 nmol) induced changes in vascular tone (Table 2). Coronary perfusion pressure was also unaffected by NDGA.

Inhibition of LPS-induced vasodilatation by dexamethasone

Pre-treatment of hearts with dexamethasone caused marked attenuation of LPS-induced vasodilatation at 1 nM and complete inhibition at concentrations of 0.1, 1 and 10 μM. This effect was time-dependent: when administered simultaneously with or for 5 min prior to LPS, dexamethasone (1 nM) failed to significantly alter the response to LPS; in contrast, pre-treatment of hearts for 15 or 30 min attenuated the vasodilatation induced by LPS by 92 ± 1 % (n=3) and 89 ± 3 % (n=3), respectively (Fig. 3). Under similar conditions progesterone was without effect even at 1 μM (data not shown).

The inhibition caused by dexamethasone (1 nM, 15 min perfusion) was completely blocked when hearts were pre-treated for 15 minutes with the glucocorticoid receptor antagonist RU-486 (100 nM) prior to administration of dexamethasone (1 nM). In these studies, responses to LPS (5 $\mu g\ ml^{-1}$) were not significantly different from those observed in controls (Fig 4).

The role of lipocortin 1 in the inhibitory action of dexamethasone

To establish whether the action of dexamethasone involves LC1, experiments were conducted with a selective polyclonal neutralising anti-LC1 antibody (LCPS1) raised in sheep against the N-terminus of LC1 (Perretti *et al.*, 1996). Hearts were pre-perfused for 2 min with a 1:200 dilution of LCPS1 prior to administration of dexamethasone (1 nM; 15 min) and subsequently LPS ($5\mu g.ml^{-1}$). Another sheep anti-lipocortin 1 antibody (LCPS2), which recognises the protein but does not neutralise it (Perretti *et al.*, 1996) was used as a control.

LCPS1 pre-treatment did not alter vascular tone but markedly attenuated the inhibitory action of dexamethasone, restoring LPS-induced vasodilatation to 66 ± 12 % of the control response. The non-neutralising antibody, LCPS2, was without effect (Fig 4).

Selective inhibition of LPS-induced vasodilatation by cycloheximide

Since the actions of dexamethasone apparently involved induction of synthesis and/or translocation of LC1, we tested the effects of cycloheximide on the LPS-induced fall in coronary perfusion pressure. Surprisingly, pre-perfusion of cycloheximide (10μM, 15 min) alone completely abolished responses to LPS (5 μg ml^{-1}, Fig.5). Thus, further experiments involving cycloheximide and dexamethasone could not be carried out. At 10 μM, cycloheximide had no effect on basal tone nor did it cause any statistically significant change in the responses to either bradykinin (0.1 nmol) or SNP (10 nmol).

Discussion

These data confirm that perfusion of LPS through rat isolated hearts elicits a rapid and maintained vasodilatation. As reported previously (Baydoun *et al.*, 1993) this response is blocked by L-NAME, implicating NO as a potential mediator. However, substituted arginine analogues such as L-NAME and N^G-monomethyl-L-arginine (L-NMMA) can have effects other than inhibition of nitric oxide synthase (Cocks & Angus, 1991), and may even cause increased release of NO (Archer & Hampi, 1992). To confirm unequivocally whether LPS causes NO released from the rat isolated perfused heart we monitored NO production by measuring levels of nitrite and nitrate, stable end products of NO metabolism, using chemiluminescence detection. These experiments revealed that LPS infusion was accompanied by a biphasic increase in release of nitrate and nitrite of which the amounts produced over the first 3 min of perfusion are at least six times higher than those maintaining

the plateau phase. The reason for the biphasic nature of LPS-induced NO release is not clear. There is no correlating biphasic vasodilatation to LPS. However it may be that both the initial peak and the plateau of NO production are sufficient to cause maximal and maintained dilatation. Since both coronary vasodilatation and NO release were blocked by L-NAME it can be concluded that the action of L-NAME in reducing LPS-induced vasodilatation is due to inhibition of NO synthesis.

In addition to NO release our study has also implicated bradykinin production in LPS-induced coronary vasodilatation. This effect is mediated by kinin B_2-receptors, since it was abolished by HOE 140. These findings are supported by other studies demonstrating that bradykinin antagonists block the acute phase of LPS-induced hypotension *in vivo* (Katori *et al.,* 1989; Wilson *et al.,* 1989), and inhibit the rapid release of NO from cultured endothelial cells exposed to LPS (Fleming *et al.,* 1992). Further studies in rats deficient in kininogens, the physiological precursor of bradykinin, have revealed that the hypotensive response to exogenous bradykinin was unchanged, but the biphasic response to LPS was markedly altered in that the second, phase (70-80 min after LPS) was conserved, but the first phase (~15 min after LPS) was abolished. The same profile of inhibition was seen following HOE 140-treatment in kininogen-replete rats (Ueno *et al.,* 1995). Thus bradykinin is an important mediator of the acute vasodilator response to LPS and may be released locally following stimulation.

Although the precise mechanism of action of bradykinin still remains to be established, it is evident that its vasodilator action in the rat heart is mediated, at least in part, via NO synthesis following activation of endothelial constitutive NO synthase (Baydoun & Woodward, 1991). Thus, LPS-induced NO production in this study may be secondary to the

release of bradykinin since both responses are abolished by HOE 140. Interestingly, L-NAME completely abolished vasodilatation to LPS but only partially inhibited responses to exogenous bradykinin, reducing the duration while having no significant effect on the magnitude of the response. This observation is consistent with the hypothesis that exogenous bradykinin exerts a biphasic effect in the rat heart of which only the second phase is NO mediated (Baydoun & Woodward, 1991). Furthermore, our findings raise the possibility that the effects of endogenous and exogenous bradykinin may be mediated differently. In this regard, Hecker *et al.* (1993; 1995) have reported that in endothelium-intact rings of bovine coronary artery, another inhibitor of NO synthase L-N^{ω}-nitro-L-arginine (L-NOARG) only partially blocked dilator responses to exogenous bradykinin. However in the same experiments, the vasodilator effects of moexiprilat and ramiprilat, which act by preventing the breakdown of endogenous bradykinin and thus unmasking its dilator effects, were completely blocked by L-NOARG. Further studies are clearly required to elucidate the mechanism(s) associated with the actions of both exogenous and endogenous kinins.

In addition to the established involvement of NO, additional data obtained with mepacrine, WEB 2086 and NDGA suggest that the acute vasodilator response to LPS is complex and dependent on multiple mediators. Although the precise link remains to be established, it is evident from our data that both the NO synthase and PLA_2 pathways are involved. The inhibition of LPS-induced vasodilatation by relatively low concentrations of mepacrine strongly suggests that PLA_2 activation contributes to this response. This is further supported by data obtained in the presence of dexamethasone which inhibits PLA_2 activation in a variety of cells and tissues (Burch, 1990; Lilja *et al.,* 1994). In our study pre-perfusion of hearts with dexamethasone for as little as 15 min virtually abolished the vasodilator response to LPS. Furthermore this effect was observed with concentrations of dexamethasone well

below the standard pharmacologically concentrations routinely used. The low concentrations required together with the facts that progesterone was without effect and RU 486 blocked the actions of dexamethasone strongly suggests that dexamethasone acts via glucocorticoid receptors and was not acting non-selectively via changes in membrane fluidity (Gerritsen *et al.,* 1991), or simply by competition for LPS binding sites (TahriJouti & Chaby, 1990).

Further studies established that dexamethasone acted via the induction of LC1, since pre-perfusion of hearts with the anti-LC1 neutralising antibody LCPS1 markedly attenuated the inhibitory actions of dexamethasone. This finding supports the hypothesis that LC1 is a mediator of the action of glucocorticoids (Flower & Rothwell, 1994) and is in agreement with several reports demonstrating that neutralising antibodies to lipocortin reverse many effects of glucocorticoids including their ability to inhibit cell trafficking (Perretti *et al.,* 1996), paw oedema (Duncan *et al.,* 1993), cell division and differentiation (Croxtall & Flower, 1992) as well as the inhibitory action exerted by these steroids on hormone release from the anterior pituitary (Taylor *et al.,* 1995). The rapid onset of the response to dexamethasone may indicate that, in the rat heart, dexamethasone may either induce the synthesis and/or rapid translocation of pre-formed intracellular LC1 to the membrane. Additionally, dexamethasone may also increase release of LC1 from plasma membrane bound pools. Such rapid changes in cellular LC1 disposition have indeed been reported in other systems (Solito *et al.,* 1994; Kaufman *et al.,* 1996).

We have previously shown that indomethacin has no effect on the response to LPS in the rat heart (Baydoun *et al.,* 1993) suggesting that vasodilator cyclo-oxygenase products are not involved in this response. In this study we have examined the involvement of other PLA_2 -derived mediators, including PAF, in the response to LPS. Administration of PAF to animals

causes a similar shock syndrome to LPS (Bessin *et al.,* 1983; Doebber *et al*., 1985; Mulder *et al.,* 1993). Furthermore, PAF release is increased in animals following LPS-administration, and PAF antagonism attenuates endotoxin-induced vasodilatation in experimental models of shock (Doebber *et al.,* 1985; Redl *et al.*, 1990).

Our data suggest that PAF is a mediator of LPS-induced vasodilatation in the rat isolated perfused heart since responses to LPS were significantly reduced by WEB 2086. However, LPS may bind directly to and activate PAF receptors without the need for intermediate PAF release (Nakamura *et al,*1992), an effect that presumably would also be prevented by WEB 2086.

We could not characterise the effects of exogenous PAF in our system. However, other authors have reported that exogenous PAF exerts two different effects with low doses (10 fmol - 1 pmol) initiating a vasodilator response while higher doses (≥1 pmol) induce vasoconstriction (Man *et al.,* 1990). In our limited studies, administration of PAF to freshly isolated hearts did cause coronary vasodilatation. However, these responses were not reproducible or dose-dependent.

We investigated the role of the lipoxygenase pathway in mediating responses to LPS by examining the effect of NDGA on LPS-induced coronary vasodilatation. NDGA selectively inhibited the effect of LPS but failed to modify responses to either SNP or bradykinin. However, NDGA has been reported to have several other actions including inhibition of PLA_2 and of PAF release (Lanni & Becker, 1985). Thus we cannot discount the possibility that the effects of NDGA in our studies are due to inhibition of PLA_2 rather than lipoxygenase.

Although further studies utilising more selective inhibitors of lipoxygenases are required it is worth noting that 5-lipoxygenase has been implicated in the vasodilator action of PAF in the rat isolated perfused heart (Hu *et al.,* 1991). The 5-lipoxygenase inhibitor MK-886 (Gillard *et al.* 1989; Rouzer *et al.,* 1990) was found to inhibit the response to PAF as was the leukotriene (LT) antagonist L-649,923 (Jones *et al.,* 1986) suggesting that the effects of exogenous PAF may be mediated via the release of LT, presumably the vasodilator LT, LTB_4. Thus, it would not be unreasonable to speculate that in our system, LPS acting independently on PAF receptors or via the release of PAF itself may activate 5- lipoxygenase pathway and presumably release LTB_4. Whether products of the lipoxygenase pathway interact with constitutive NO synthase is a fascinating question which remains to be investigated. Cross-talk between these two pathways would clearly explain the findings that inhibitors of either lipoxygenase or NO synthase virtually abolish LPS-induced vasodilatation in the rat heart.

One other potential mechanism of action of PAF is the release of NO, via the constitutive endothelial L-arginine-NO pathway, which has been shown to contribute to the vasodilator actions of PAF in the mesenteric vasculature of the rat (Chiba *et al.,* 1990). Clearly an action of PAF via this pathway would account for the abrogation of the LPS response by NOS inhibitors as well as PLA_2 and PAF antagonists. Thus the release of NO may be the final end point of a multifactorial cascade activated by LPS in the coronary microvasculature of the rat. This cascade is dependent on rapid synthesis and/or turnover of an intermediate protein which may be the coupling link between the LPS receptor and the initiation of bradykinin release and/or PLA_2 activation. A comparable dependence on protein synthesis has been reported by Petitclerc *et al.* (1994) who showed that rapid interleukin-1-induced

vasodilatation in the rabbit isolated mesenteric artery was blocked within 15 minutes by three functionally distinct inhibitors of protein synthesis including cycloheximide. The response to interleukin-1, like the response to LPS described here, was dependent on activation of PLA_2.

In conclusion, our data confirm that endothelial constitutive NO synthase (eNOS) can be activated acutely by LPS resulting in the release of NO in sufficient quantities to initiate coronary vasodilatation in the rat heart. This end effect also involves other intracellular events including activation of PLA_2, which may in turn interact with eNOS via the release of PAF. These findings implicate eNOS in the vasodilatation which occurs in LPS-induced shock in both experimental models and man. However, these results may have other important implications with the acute activation of coronary vasodilatory pathways having beneficial cardioprotective rather than the deleterious effects normally associated with inflammatory responses induced by endotoxin. This applies in particular to the phenomenon of "cross tolerance" induced by endotoxin against ischaemia-reperfusion injury (Song *et al.,* 1996).

Acknowledgements.

We gratefully acknowledge the support of the MRC and the British Heart Foundation. We thank Professors Jeremy Pearson and Rod Flower and Drs Mauro Perretti and Guiseppe Cirino for their valuable discussion of this work.

References

ARCHER, S.L. & HAMPI, V. (1992). N^G-monomethyl L-arginine causes nitric oxide synthesis in isolated arterial rings: Trouble in paradise. *Biochem. Biophys. Res. Commun.,* **188**, 590-596.

BAYDOUN, A.R., FOALE, R.D. & MANN, G.E. (1993). Bacterial endotoxin rapidly stimulates prolonged endothelium-dependent vasodilation in the rat isolated perfused heart. *Br. J. Pharmacol.,* **109**, 987-991.

BAYDOUN, A.R. & WOODWARD, B. (1991). Effects of bradykinin in the rat isolated perfused heart; Role of kinin receptors and endothelium-derived relaxing factor. *Br. J. Pharmacol.,* **103**, 1829-1833.

BESSIN, P., BONNET, J., APFFEL, D., SOULARD, C., DESGROUX, L., PELAS, I. & BENVENISTE, J. (1983). Acute circulatory collapse caused by platelet-activating factor (PAF-acether) in dogs. *Eur. J. Pharmacol.,* **86**, 403-413.

BROADLEY, K.J. (1979). The Langendorff heart preparation-reappraisal of its role as a research and teaching model for coronary vasoactive drugs. *J. Pharmacol. Meth.,* **2**, 143-156.

BURCH, R.M. (1990). Kinin signal transduction: Role of phosphoinositides and eicosanoids. *J. Cardiovasc. Pharmacol.,* **15**, S 44-S 45.

CASALS-STENZEL, J., MUACEVIC, G. & WEBER, K.. (1986). WEB 2086, a new and specific antagonist of platelet activating factor (PAF) in vitro and in vivo. *Naunyn-Schmiedeberg's Arch. Parmacol.,* **334**, R44

CHIBA, Y., MIKODA, N., KAWASAKI, H. & ITO, K. (1990). Endothelium-dependent relaxant action of platelet activating factor in the rat mesenteric artery. *Naunyn-Schmiedeberg's Arch. Parmacol.,* **341**, 68-73.

COCKS, T.M. & ANGUS, J.A. (1991). Evidence that contraction of isolated arteries by L-NMMA and NOLA are not due to inhibition of basal EDRF release. *J. Cardiovasc. Pharmacol.,* **17**, S159-S164.

CROXTALL, J.D. & FLOWER, R.J. (1992). Lipocortin 1 mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line. *Proc. Natl. Acad. Sci. (USA),* **89**, 3571-3575.

DOEBBER, T.W., WU, M.S., ROBBINS, J.C., CHOY, B.M., CHANG, M.N. & SHEN, T.Y. (1985). Platelet activating factor (PAF) involvement in endotoxin-induced hypotension in rats. Studies with PAF-receptor antagonist kadsurenone. *Biochem. Biophys. Res. Commun.,* **127**, 799-808.

DUNCAN, G.S., PEERS, S.H., CAREY, F., FORDER, R. & FLOWER, R.J. (1993). The local anti-inflammatory action of dexamethasone in the rat carrageenin oedema model is reversed by an antiserum to lipocortin 1. *Br. J. Pharmacol.,* **108**, 62-65.

FLEMING, I., DAMBACHER, T. & BUSSE, R. (1992). Endothelium-derived kinins account for the immediate response of endothelial cells to bacterial lipopolysaccharide. *J. Cardiovasc. Pharmacol.,* **20(suppl. 12**), s135-s138.

FLOWER, R.J. & ROTHWELL, N.J. (1994). Lipocortin-1: Cellular mechanisms and clinical relevance. *Trends in Pharmacol. Sci.,* **15**, 71-76.

GERRITSEN, M.E., SCHWARZ, S.M. & MEDOW, M.S. (1991). Glucocorticoid-mediated alterations in fluidity of rabit cardiac muscle microvessel endothelial cell membranes: influences on eicosanoid release. *Biochim. Biophys. Acta.,* **1065**, 63-68.

GILLARD, J., FORDHUTCHINSON, A.W., CHAN, C., CHARLESON, S., DENIS, D., FOSTER, A., FORTIN, R., LEGER, S., MCFARLANE, C.S., MORTON, H., PIECHUTA, H., RIENDEAU, D., ROUZER, C.A., ROKACH, J., YOUNG, R., MACINTYRE, D.E., PETERSON, L., BACH, T. & EIERMANN, G. (1989). L-663,536 (MK-886)(3-(1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl)- ,2-dimethylpropanoic acid), a novel, orally active leukotriene biosynthesis inhibitor. *Can. J. Physiol. Pharmacol.,* **67**, 456-464.

HARPER, J.F. (1984). BASIC programme of a robust multiple comparison test for statistical analysis of all differences among group means. *Comput. Biol. Med.,* **14**, 437-444.

HECKER, M., BARA, A.T. & BUSSE, R. (1993). Relaxation of isolated coronary arteries by angiotensin-converting enzyme inhibitors: Role of endothelium-derived kinins. *J. Vasc. Res.,* **30**, 257-262.

HECKER, M., BARA, A.T. & BUSSE, R. (1995). Angiotensin-converting enzyme inhibitors unmask endogenous kinin production by bovine coronary artery endothelium. *Eur. Heart J.,* **14 (suppl. 1),**161-163

HU, W., KINNAIRD, A.A.A. & MAN, R.Y.K. (1991). Mechanisms of the coronary vascular effects of platelet-activating factor in the rat perfused heart. *Br. J. Pharmacol.,* **103**, 1097-1102.

JONES, T.R., YOUNG, R., CHAMPION, E., CHARETTE, L., DENIS, D., FORD-HUTCHINSON, A.W., FRENETTE, R., GAUTHIER, J.Y., GUINDON, Y., KAKUSHIMA, M., MASSON, P., MCFARLANE, C., PIECHUTA, H., ROKACH, J., ZAMBOUI, R., DEHAVEN, R.N., MAYCOCK, A. AND PONG, S.S. (1986) L-649,923, Sodium (betaS*,gammaR*)-4-(3-(4-acetyl-3-hydroxy-2-propylphenoxy)-propylthio)-gamma-hydroxy-beta-methylbenzenebutanoate, a selective, orally active leukotriene receptor antagonist. *Can. J. Physiol. Pharmacol.,* **64**, 1068-1075.

KATORI, M., MAJIMA, M., ODOI-ADOME, R., SUNAHARA, N., UCHIDA, Y. & ODOIADOME, R. (1989). Evidence for the involvement of a plasma kallikrein -kinin system in the immediate hypotension produced by endotoxin in the anaesthetized rat. *Br. J. Pharmacol.,* **98**, 1383-1391.

KAUFMAN, M., LETO, T. & LEVY, R. (1996). Translocation of annexin I to plasma membranes and phagosomes in human neutrophils upon stimulation with opsonized zymosan: Possible role in phagosome function. *Biochemical Journal.,* **316**, 35-42.

KELM, M. & SCHRADER, J. (1990). Nitric oxide release from isolated guinea-pig heart. *Circ. Res.,* **66**, 1561-1575.

KILBOURN, R.G., JUBRAN, A., GROSS, S.S., GRIFFITH, O.W., LEVI, R. & ADAMS, J. (1990). Reversal of endotoxin-mediated shock by N^G-methyl-l-arginine, an inhibitor of nitric oxide synthase. *Biochem. Biophys. Res. Commun.,* **172**, 1132-1138.

LANNI, C. & BECKER, E.L. (1985). Inhibition of neutrophil phospholipase A2 by p-bromophenylacyl bromide, nordihydroguaiaretic acid 5,8,11,14-eicotetrayenoic acid and quercetin. *Int. Arch. All. Appl. Immunol.,* **76**, 214-217.

LEMBECK, F., GREISBACHER, T., ECKHART, M., HENKE, S., BREIPOHL, G. & KNOLLE, J. (1991). New, long-acting, potent bradykinin antagonists. *Br. J. Pharmacol.,* **102**, 207-304.

LILJA, .IA., DIMBERG, J., SJODAHL, R., TAGESSON, C. & GUSTAFSONSVARD, C. (1994). Effects of endotoxin and dexamethasone on group I and II phospholipase A2 in rat ileum and stomach. *Gut*, **35**, 40-45.

MAN, R.Y.K., HU, W. & KINNAIRD, A.A.A. (1990). Coronary vascular response to platelet activating factor in the perfused rat heart. *J. Lipid Mediat.*, **2**, 75-83.

MENON, M.K., WOLF, A., ZEHETGRUBER, M. & BING, R.J. (1989). An improved chemiluminescence assay suggests non nitric oxide-mediated action of lysophosphatidylcholine and acetylcholine. *Proc. Soc. Exp. Med. Biol.*, **191**, 316-319.

MULDER, M.F., VAN LAMBALGEN, A.A., VAN KRAATS, A.A., SCHEFFER, P.G., BOUMANN, A.A., VAN DEN BOS, G.C. & THIJS, L.G. (1993). Systemic and regional hemodynamic changes during endotoxin or platelet-activating factor (PAF)-induced shock in rats. *Circ. Shock*, **41**, 221-229.

NAKAMURA, M., HONDA, Z., WAGA, I., MATSUMOTO, T., NOMA, M. & SHIMIZU, T. (1992). Endotoxin transduces Ca^{2+} signalling via platelet-activating factor receptor. *FEBS Lett.*, **314**,125-129.

PETITCLERC, E., POUBELLE, P.E. & MARCEAU, F. (1994). Rapid protein synthesis and turnover is involved in interleukin-1-induced relaxation of the rabbit isolated mesenteric artery. Analysis of the arachidonate cascade. *J. Pharmacol. Exp. Ther.*, **268,**1419-1425

PERRETTI, M., AHLUWALIA, A., HARRIS, J.G., HARRIS, H.J., WHELLER, S.K. & FLOWER, R.J. (1996). Acute inflammatory response in the mouse: Exacerbation by immunoneutralization of lipocortin 1. *Br. J. Pharmacol.*, **117**, 1145-1154.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. (USA)*, **87**, 10043-10047.

REDL, H., VOGL, C. & BAHRAMI, S. (1990). Effect of PAF antagonist BN 52021 in an ovine endotoxin shock. *J. Lipid Mediat.,* **2**, s195-s202.

ROUZER, C.A., FORDHUTCHINSON, A.W., MORTON, H.E. & GILLARD, J.W. (1990). MK886, a potent and specific leukotriene biosynthesis inhibitor blocks and reverses the membrane association of 5-lipoxygenase in ionophore-challenged leukocytes. *J. Biol. Chem.,* **265**, 1436-1442.

ROYSTON, J.P. (1984). SPP - statistical package for personal computers. TCL., 40B Royal Hill, London. SE10 8RT. UK.

SLAPKE, J., SCHEWE, T., HUMMEL, S., WINKLER, J. & KOPF, M. (1983). Lung strips from guinea pigs as test system for lipoxygenase inhibitors. Inhibition of arachidonic acid-induced contractions by 3-t-butyl-4-hydroyanisole and nordihydroguaiaretic acid. *Biomed. Biochim. Acta*, **42**, 1309-1318.

SOLITO, E., NUTI, S. & PARENTE, L. (1994). Dexamethasone-induced translocation of lipocortin (annexin) 1 to the cell membrane of U-937 cells. *Br. J. Pharmacol.,* **112**, 347-348.

SONG, W., FURMAN, B.L. & PARRATT, J.R. (1996). Delayed protection agianst ischaemia-induced ventricular arrhythmias and infarct size limitation by the prior administration of *Eschericia coli* endotoxin. *Br. J. Pharmacol.*, **118**, 2157-2163.

TAHRIJOUTI, M.A. & CHABY, R. (1990). Specific binding of lipopolysaccharides to mouse macrophages-I. Characteristics of the interaction and inefficiency of the polysaccharide region. *Mol. Immunol.,* **27**, 751-761.

TAYLOR, A.D., COWELL, A.M., FLOWER, R.J. & BUCKINGHAM, J.C. (1995). Dexamethasone suppresses the release of prolactin from the rat anterior pituitary gland by lipocortin 1 dependent and independent mechanisms. *Neuroendocrinol.,* **62**, 530-542.

THIEMERMANN, C. & VANE, J.R. (1990). Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat *in vivo*. *Eur. J. Pharmacol.*, **182**, 591-595.

UENO, A., ISHIDA, H. & OHISHI, S. (1995). Comparative study of endotoxin-induced hypotension in kininogen-deficient rats with that in normal rats. *Br. J. Pharmacol.*, **114**, 1250-1256.

WILSON, D.D., DE GARAVILLA, L., KUHN, W., TOGO, J., BURCH, R.M. & STERANKA, L.R. (1989). D-Arg-(Hyp3-D-Phe7)-bradykinin antagonist, reduces mortality in a rat model of endotoxic shock. *Circ. Shock*, **27**, 93-101.

Fig 1. LPS induced NO release from the rat isolated perfused heart. Hearts were isolated and perfused as described in methods. Following treatment with U46619, flow was reduced sequentially to 1 $ml.min^{-1}$. Coronary effluent was collected in 0.5 ml aliquots prior to and at 3 min intervals throughout infusion of LPS. Nitrite and nitrate levels in samples were determined by chemiluminescent detection using a Sievers NO analyser (model 280A, Sievers instruments Inc. Boulder CO, USA) as described in methods. Bars represents NO levels detected prior to (basal), 3 min (peak) and 6 min (plateau) after LPS perfusion and 30 min after LPS washout. The data are the mean ± s.e.mean from 5 separate experiments . * $P<0.01$ relative to basal levels.

Fig 2. Effect of HOE-140 on vasodilator responses to bradykinin, SNP and LPS. Hearts were perfused as described in methods. Responses to bolus injection of bradykinin (0.1 nmol) and SNP (10 nmol) were obtained prior to (control) and following a 30 min treatment of hearts with 100 $nmol.l^{-1}$ HOE-140. LPS (5 $\mu g.ml^{-1}$) was administered after pretreatment with HOE-140 (100 $nmol.l^{-1}$) and compared to responses observed in separate hearts in the absence of the antagonist. Each bar graph represents the mean ± s.e.mean from 3 experiments. * denotes statistical significance at $P<0.01$ compared to respective controls.

Fig 3. Time-course of dexamethasone-induced inhibition of LPS-induced vasodilatation. Hearts were perfused as in methods. Dexamethasone (1 nM) was administered either simultaneously with or pre-perfused for 5, 15 or 30 min prior to administration of LPS (5 $\mu g.ml^{-1}$). The data represents mean ± s.e.mean from 3 experiments. * denotes statistical significance at $P<0.01$ compared to the control LPS response.

Fig 4. Effects of RU-486 and anti lipocortin antibodies on dexamethasone-induced inhibition of LPS-initiated vasodilatation. Hearts were perfused as in methods. RU486 was administered for 15 min prior to perfusion of dexamethasone (Dex; 1nM, 15 min) and subsequently LPS (5 $\mu g.ml^{-1}$). LCPS1 or LCPS2 (1:200 dilution) were applied for 2 min prior to administration of dexamethasone and then LPS. Each bar graph represents mean ± s.e.mean from 3 separate experiments. * denotes statistical significance at $P<0.01$ compared to the control LPS response.

Fig 5. Effect of cycloheximide on vasodilator responses to bradykinin, SNP and LPS. Hearts were perfused as in methods. Responses to bolus injection of bradykinin (0.1 nmol) and SNP (10 nmol) were obtained prior to (controls) and following a 15 min treatment of hearts with cycloheximide (10 µM). LPS (5 $\mu g.ml^{-1}$) was administered after pretreatment with cycloheximide and compared to responses observed in separate hearts in the absence of the inhibitor. Each bar graph represents mean + s.e.mean from 3 experiments. * denotes statistical significance at $P<0.01$ compared to respective controls.

Table 1. Effects of L-NAME treatment on responses to bradykinin, sodium nitroprusside and LPS.

	Fall in coronary perfusion pressure		NO release
	Magnitude (mmHg)	Duration (min)	NO ($pmol.g^{-1}.min^{-1}$)
Control:			
LPS	34 ± 3	>120	11.6 ± 0.8
BK	43 ± 2	8 ± 1	ND
SNP	43 ± 2	8 ± 0.3	ND
L-NAME:			
LPS	0 ± 5*	0	<MDL
BK	30 ± 3	3 ± 2*	ND
SNP	37 ± 3	11 ± 2	ND

Hearts were perfused as described in the methods and PP elevated with 5 nM U46619 (control) or 50 μM L-NAME (n=3). Responses were recorded to 10 μl bolus injections of 0.1 nmol BK (n=3) and 10 nmol SNP (n=3), or infusion of 5 $\mu g.ml^{-1}$ LPS (n=3). In parallel studies, flow was reduced to 1 $ml.min^{-1}$ and NO release due to LPS-treatment measured by chemiluminescent detection. ND denotes not determined, <MDL denotes below minimum detection limit, * denotes statistical significance at $P<0.01$ compared to respective controls.

Table 2. Effects of mepacrine, WEB 2086 and NDGA on vasodilator responses to bradykinin, sodium nitroprusside and LPS.

	Fall in coronary perfusion pressure (mmHg)		
	Bradykinin (0.1 nmol)	sodium nitroprussside (10 nmol)	LPS (5 μg.ml^{-1})
Control	39 ± 3	29 ± 4	59 ± 4
Mepacrine (0.5 μM)	30 ± 2	31 ± 3	3 ± 4*
Control	45 ± 9	34 ± 8	59 ± 4
WEB 2086 (3 μM)	52 ± 7	25 ± 4	17 ± 5*
Control	53 ± 10	61 ± 11	59 ± 4
NDGA (0.1 μM)	52 ± 10	51 ± 10	9 ± 3*

Hearts were perfused as in methods. Responses to bradykinin (BK; 0.1 nmol), sodium nitroprusside (SNP; 10 nmol) or LPS (5 μg.ml^{-1}) were obtained prior to (control) and following a 30 min pre-perfusion of hearts with either mepacrine (0.5 μM; n=3), WEB 2086 (3 μM; n=4) or NDGA (0.1 μM; n=4). *denotes statistical significance at $P<0.01$ compared to respective controls.