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Activation of soluble guanylyl cyclase by a factor other than nitric oxide or carbon monoxide contributes to the vascular hyporeactivity to vasoconstrictor agents in the aorta of rats treated with endotoxin

Chin-Chen Wu, Csaba Szabó, Shiu-Jen Chen,
Christoph Thiemermann[1] and John R. Vane

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London, EC1M 6BQ, United Kingdom

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SUMMARY. We have examined the role of soluble guanylyl cyclase and possible mediators of its activation in the vascular hyporeactivity caused by bacterial endotoxin (lipopolysaccharide, LPS) *ex vivo*. Treatment of rats with E. coli LPS (10 mg/kg, i.v. for 3h) resulted in a significant reduction in the contractions elicited by norepinephrine (NE; 10^{-9}-10^{-6} M) in endothelium-denuded aortic rings *ex vivo*. Methylene blue or LY-83583, inhibitors of soluble guanylyl cyclase, completely restored contractions to NE, whereas the nitric oxide synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), caused only a partial restoration. Zinc protoporphyrin-IX, an inhibitor of heme oxygenase, did not enhance NE-induced contraction in rings from LPS-treated rats,indicating that the production of carbon monoxide (CO) does not contribute to this vascular hyporeactivity. Indomethacin, an inhibitor of cyclooxygenase, further suppressed the contractions in rings from LPS-treated rats. These results suggest that hyporesponsiveness to NE caused by LPS is due to the activation of soluble guanylyl cyclase, which is partially mediated by NO, but not by CO. Moreover, LPS may induce the production of another mediator(s) that activate soluble guanyyl cyclase in the vascular smooth muscle.

Nitric oxide (NO) is produced from L-arginine by a family of isoenzymes, collectively termed NO synthase (NOS) in a variety of cells and tissues. NO activates soluble guanylyl cyclase thus producing relaxation of vascular and non-vascular smooth muscle through a rise in intracellular cyclic GMP (cGMP) (1). More recently, carbon monoxide (CO) has also been postulated as an endogenous stimulant of cGMP formation (2-4).

Endotoxin *in vivo* or *in vitro* suppresses the contractile responses to various vasoconstrictors and causes an increase in cGMP within the vascular smooth muscle

[1]Author for correspondence.

Abbreviations used: CO, carbon monoxide, L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, bacterial lipopolysaccharide; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase.

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(5-7). This has been attributed to the induction of a calcium-independent isoform of NOS in the vascular smooth muscle cells, that produces large amounts of NO, which, in turn increases cGMP (5,6). However, the vascular hyporeactivity of endothelium-denuded aortic rings from rats treated with LPS is only partially overcome by N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS activity (8-10), indicating that mediators or mechanisms other than NO or activation of soluble guanylyl cyclase may contribute to this hyporeactivity.

Here we have (i) studied the role of the activation of soluble guanylyl cyclase in the vascular hyporeactivity caused by endotoxin *ex vivo*, and (ii) evaluated the relative contribution made by NO and CO to its activation. As NO has been reported to activate cyclooxygenase (COX) and LPS induces a novel isoform of COX *in vivo* and *in vitro* (10,11) we have also investigated the potential role of COX metabolites in this vascular hyporeactivity. We demonstrate that activation of guanylyl cyclase in the vascular smooth muscle accounts for the vascular hyporeactivity to norepinephrine (NE) in the aorta of LPS-treated rats. We also clearly show, however, that NO and CO cannot fully account for the activation of soluble guanylyl cyclase. Therefore, other stimulants of cGMP formation also contribute to the vascular hyporeactivity in septic shock.

MATERIALS AND METHODS

In vivo experiments: Male Wistar rats (240-300g; Glaxo Laboratories Ltd., Greenford, Middx.) were anesthetized with thiopentone sodium (Trapanal; 120 mg/kg, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min. Animals then received *E. coli* LPS (10 mg/kg i.v.) as a slow injection over 10 min; and 3h thereafter they were killed by exsanguination under anesthesia.

Organ bath experiments: Thoracic aortae were cleared of adhering periadventitial fat, and then cut into rings of 3-4 mm width. In some rings, endothelium was removed by gently rubbing the intimal surface. Lack of an acetylcholine-induced relaxation was taken as evidence that endothelial cells had been removed. The rings were mounted in organ baths (10 ml) filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs' solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Isometric force was measured with Grass FT03 type transducers (Grass Instruments, Quincy, Mass., U.S.A.) and recorded on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, U.S.A.). A tension of 2 g was applied and the rings were equilibrated for 60 min, changing the Krebs' solution every 15 min.

Experimental protocols: In endothelium-denuded aortic rings from control rats and from LPS-treated rats, concentration-response curves to NE (10^{-9}-10^{-6} M) were obtained before and after the inhibitors of guanylyl cyclase (13,14) methylene blue (10^{-5} M for 20 min) or LY-83583 (10^{-5} M for 20 min), the NOS inhibitor (15) N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M for 20 min) or the heme oxygenase inhibitor (2-4) zinc protoporphyrin-IX (10^{-5} M for 20 min). To study the contribution of cyclooxygenase metabolites in the vascular hyporeactivity, in a separate set of studies concentration-response curves to NE (10^{-9}-10^{-6} M) were obtained before and after indomethacin (5×10^{-6} M for 30 min) in endothelium-denuded rings from control and LPS-treated rats. To study the effect of methylene blue, LY-83583, L-NAME, zinc protoporphyrin-IX and indomethacin on the production of NO by the constitutive NOS present in the endothelium, the effect of pretreatment with these agents (as above) on

the endothelium-dependent relaxations elicited by acetylcholine (10^{-6} M) was also investigated in intact aortic rings.

Materials: Bacterial lipopolysaccharide (*E. coli* serotype 0127:B8), N^{ω}-nitro-L-arginine methyl ester, methylene blue, indomethacin, acetylcholine chloride, and norepinephrine bitartrate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). LY-83583 was purchased from Calbiochem Co. (Nottingham, U.K.) and protoporphyrin IX zinc (II) was purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). All solutions were made in saline or distilled water except for indomethacin, which was dissolved in 5% Na_2CO_3.

Statistics: All values in the figures and text are expressed as mean $\pm$ standard error of the mean of *n* observations, where n represents the number of animals studied. Student's paired or unpaired *t* -test was used to compare means among or between groups, respectively. A P-value less than 0.05 was considered to be statistically significant.

RESULTS

NE caused a concentration-dependent increase in vascular tone in endothelium-denuded rat aortic rings obtained from vehicle-treated rats (control) and rats treated with LPS for 3 h (LPS-rats). Aortic rings obtained from rats subjected to a 3 h period of endotoxaemia, however, showed a significant reduction of the contractile response to NE ($P<0.05$ at 10^{-9}-10^{-6} M; Figure 1). *In vitro* treatment of control rings with methylene blue (10^{-5} M), LY-83583 (10^{-5} M), L-NAME ($3x10^{-4}$ M) or zinc protoporphyrin-IX (10^{-5} M) did not enhance the contractions induced by NE (Fig. 1). In contrast, in rings from LPS-treated rats the contractile responses to NE were completely restored by methylene blue or by LY-83583, but only partially restored by L-NAME ($P<0.05$ at 10^{-9}-10^{-6} M; Figure 1). In contrast to L-NAME, zinc protoporphyrin-IX did not affect the contractile responses to NE in rings from LPS-treated rats (Fig. 1). Incubation of rings from LPS-treated rats with indomethacin ($5x10^{-6}$ M) modestly, but significantly reduced the contractions elicited by NE, without significantly affecting these responses in aortic rings from control rats (Fig. 1).

Endothelium-dependent relaxations to acetylcholine (10^{-6} M) were unaffected by zinc protoporphyrin-IX or indomethacin, but they were substantially inhibited by L-NAME, methylene blue or LY-83583 (Fig. 2)

DISCUSSION

The contractions induced by NE in various blood vessels such as the rat thoracic aorta *ex vivo*, are substantially reduced by LPS treatment. This has been attributed to enhanced formation of NO by the inducible NOS (16-18). This study demonstrates that the LPS-induced vascular hyporeactivity to NE is due to activation of soluble guanylyl cyclase which is only partially triggered by enhanced formation of NO. Thus, our results demonstrate (i) that an additional factor which activates guanylyl cyclase is formed in the vascular smooth muscle of animals with septic shock and (ii) that this, as yet unidentified factor, contributes to the vascular hyporeactivity associated with sepsis.

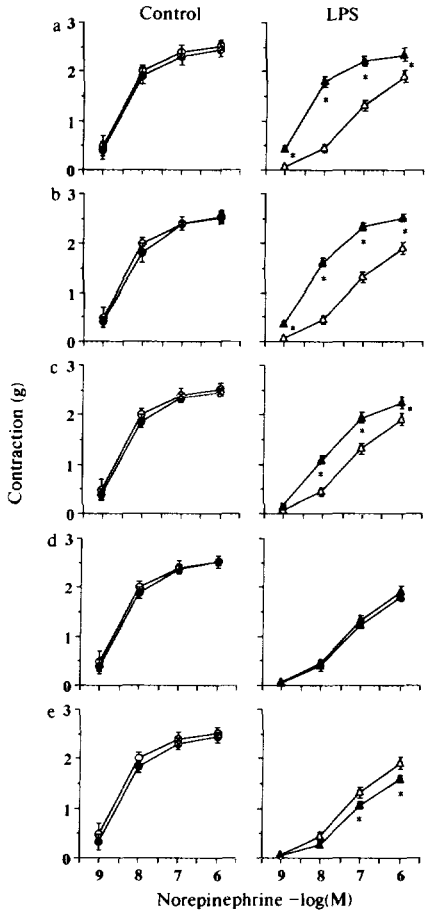

Figure 1. Effects of (a) methylene blue (10^{-5} M), (b) LY-83583 (10^{-5} M), (c) N^{ω}-nitro-L-arginine methyl ester ($3x10^{-4}$ M), (d) zinc protoporphyrin-IX (10^{-5} M) or (e) indomethacin ($5x10^{-6}$) on norepinephrine (NE; 10^{-9}-10^{-6} M) induced contractions in aotric rings without endothelium obtained from rats treated with lipopolysaccharide (LPS, 10 mg/kg, i.v.; Δ) or vehicle (saline; O) for 3 h. The NE-induced contraction was restored by methylene blue or LY-83583 and enhanced by N^{ω}-nitro-L-arginine methyl ester in rings from LPS-rats ($P<0.05$, n=8), whereas zinc protoporphyrin-IX or indomethacin did not affect that response. None of the inhibitors affected NE-induced contractions in rings obtained from control rats. Data are expressed as means±s.e.mean of n=6-8 individual experiments. Open symbols represent contractions in the absence, solid symbols in the presence of inhibitors in control rings (left panels) and in rings from LPS-treated animals (right panels). *$P<0.05$ represents significant effect of the inhibitor.

Recently, carbon monoxide (CO), formed by the action of haem oxygenase on haemoglobin, has been identified as an endogenous activator of soluble guanylyl cyclase in the brain (2,3) and in some peripheral tissues such as the internal anal spincter of the opossum (4). Thus, we have investigated the role of CO as a potential guanylyl cyclase activating factor in the aorta of endotoxin-treated animals. Zinc protoporphyrin-IX, a potent selective inhibitor of heme oxygenase (2,3), did not modify the vascular hyporeactivity to NE seen in aortic rings of LPS treated rats, so the formation of CO does not play a part in this hyporeactivity.

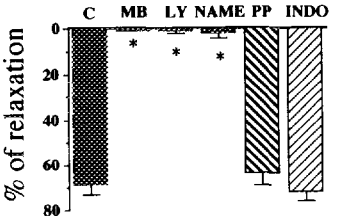

Figure 2. Effects of (a) methylene blue (10^{-5} M), (b) LY-83583 (10^{-5} M), (c) N^{ω}-nitro-L-arginine methyl ester ($3x10^{-4}$ M), (d) zinc protoporphyrin-IX (10^{-5} M) or (e) indomethacin ($5x10^{-6}$) on acetylcholine (ACh; 10^{-6} M) induced relaxation in intact aortic rings precontracted with norepinephrine ($3x10^{-7}$ M). Data are expressed as means±s.e.mean of n=6-8 individual experiments. *P<0.05 represents significant effect of the inhibitor.

Cytokines and endotoxin also induce COX (COX-2); and the expression of COX-2 protein can be prevented by glucocorticoids (21-23). Interestingly, NO enhances the activity of COX-2 through a cGMP-independent mechanism (11,12). In order to explore whether prostanoids contribute to the vascular hyporeactivity to in endotoxin shock, we studied the effect of indomethacin in endothelium-denuded aortic rings from LPS-rats. Suprisingly, indomethacin did not restore, but further suppressed the NE-induced contractions in rings from LPS-treated rats. This finding demonstrates that vasodilator prostaglandins (e.g. prostaglandin E_2 and prostacyclin) do not contribute to the LPS-induced hyporeactivity to NE in vascular smooth muscle cells. Moreover, this result suggests that vasoconstrictor prostaglandins (e.g. thromboxane A_2 or endoperoxides) may be produced in the vascular smooth muscle of septic animals, which, in turn, would result in an increase in the intracellular calcium that counteracts the cGMP-mediated vascular hyporeactivity. In this respect it is noteworthy that the aorta of the rat is relatively insensitive to the vasodilator effects of prostacyclin and other prostaglandins (unpublished data, 24). However, we cannot exclude that the formation of vasodilator prostaglandins by COX-2 in the vascular smooth muscle of the microcirculation contributes to the development of vascular hyporeactivity in septic shock.

Activation of soluble guanylyl cyclase by endogenous CO may account for the neurogenic relaxation of the internal anal sphincter (4). The present results clearly demonstrate, however, that inhibition of any endogenous production of CO does not affect the relaxations to acetylcholine in the rat aorta. Thus, in the rat aorta, endogenous CO neither contributes to the vasodilator effects of acetylcholine nor to the vascular hyporeactivity caused by LPS.

The results of the present study suggest that in addition to NO, additional, as yet unidentified factor activates guanylyl cyclase in the vascular smooth muscle of septic animals. It is noteworthy that (i) there is an NO-independent activation of soluble guanylyl cyclase by interleukin-1 (IL-1) in vascular smooth muscle cells *in vitro* (25) and (ii) treatment of rats with the endogenous IL-1 receptor antagonist (IL-1ra) *in vivo* prior to endotoxin treatment reduced the vascular hyporeactivity of the thoracic

aorta *ex vivo* (6). The latter effect, however, is only in part due to prevention of the induction of NOS by IL-1ra (6). Thus, we propose that the production of IL-1 by LPS *in vivo* is involved in the NO-independent activation of soluble guanylyl cyclase reported in the present study.

In addition, there is some evidence that some oxygen-free radicals, e.g. hydroxyl radical also activates guanylyl cyclase (19). As an enhanced formation of oxygen-free radicals in endotoxin shock is well documented (20), such radicals may also well contribute to the activation of guanylyl cyclase in rings from rats treated with endotoxin.

Methylene blue restored the reduction in peripheral vascular resistance in two patients with hyperdynamic septic shock (26) and completely restored both vascular cyclic GMP and pressor responses to NE in endotoxemic rats (27). As methylene blue is a potent inhibitor of guanylyl cyclase, it would inhibit the activation of guanylyl cyclase elicited by either NO or any novel guanylyl cyclase-activating factor produced in the vascular smooth muscle of animals with septic shock.

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