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Short communication

Low dose of lipopolysaccharide induces a delayed enhanced nitric oxide-mediated relaxation in rat aorta

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Abstract

Delayed effect on vascular reactivity of isolated aorta was studied after injection of a single low dose of lipopolysaccharide (0.5 mg/kg i.p.). The maximal vascular effect was observed 72 h after lipopolysaccharide administration with an increase in maximal endothelium-dependent relaxing response to acetylcholine and parallelly a decrease in contractile response to phenylephrine. The change in contractile response was nullified by endothelium removal as well by in vitro aortic rings incubation with N^{ω}-monomethyl-L-arginine but not with indomethacin. A low dose of lipopolysaccharide induces a delayed enhanced nitric oxide-mediated vascular relaxation which could contribute to its delayed anti-ischemic properties in ischemic tolerance phenomenon.

Keywords: Lipopolysaccharide; Aortic ring; Endothelium; Vasorelaxation; N^{ω}-monomethyl-L-arginine

1. Introduction

Lipopolysaccharide, a component of the cell wall of Gram-negative bacteria, plays a crucial role in the pathogenesis of endotoxin shock. Administration of high doses (10 to 20 mg/kg in rat) of lipopolysaccharide is responsible for endothelium-dependent relaxation impairment (Myers et al., 1995). Despite this deleterious effect, a beneficial role has been more recently recognized to lipopolysaccharide. About 5- to 20-fold lower doses of lipopolysaccharide are able to induce a delayed ischemic tolerance against the ischaemia-reperfusion injury in heart (Song et al., 1996) as well in brain (Tasaki et al., 1997).

Ischemic tolerance is a general process by which a tissue becomes more resistant to ischemic insult after a number of procedures given few days previously. A such ''delayed protection'' can result from ischemic preconditioning (i.e., brief ischemic periods protecting against injury induced by a more prolonged ischemic period), cardiac pacing or cortical spreading depression, administration of catecholamines or prostacyclin analogues, heat stress, administration of lipopolysaccharide or its non-toxic derivate, monophosphoryl lipid A (for review, see Parratt and Szekeres, 1995; Chen and Simon, 1997). Many mechanisms have been proposed to explain occurrence of ischemic tolerance: activation of K^+-ATP dependent channels; stimulation of adenosine A_1, muscarinic M_2 or opioid receptors; synthesis of protein such as heat shock proteins, anti-oxidant enzymes or anti-apoptotic proteins.

It has been reported that vascular endothelium could also play a role in the ischemic tolerance. A release of nitric oxide by endothelium has been suggested to be a trigger of the protective effect of late preconditioning in heart (Bolli et al., 1997; Kim et al., 1997). Ischemic preconditioning prevents also coronary endothelial dysfunction induced by ischemia and reperfusion, adding to the beneficial effect of preconditioning on ischemic myocytes (Richard et al., 1994). Moreover, a preliminary report suggested that heat stress, another procedure inducing ischemic tolerance, increased endothelium-dependent relaxation of rat isolated aorta, mesenteric and coronary arteries, likely contributing to its anti-ischemic properties (Richard et al., 1997).

Therefore, the present study was designed to assess whether a low dose of lipopolysaccharide, inducing a

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delayed anti-ischemic protective effect in heart or brain, improves endothelium-dependent relaxation as heat stress and whether this improvement involves NO pathway.

2. Methods

2.1. Experimental protocol

Male Wistar rats (Iffa Credo, France) weighing 280–300 g were given, by intraperitoneal injection, lipopolysaccharide derived from *Escherichia coli* (serotype 055:B5), in a dose of 0.5 mg kg^{-1}, 12, 24, 72 or 168 h prior to in vitro vascular reactivity ($n = 6$–10 rats per group). This dose of lipopolysaccharide was chosen in accordance with previous studies showing induction of ischemic tolerance in heart (Song et al., 1996) and in brain (Deplanque et al., 1997), with such a dose, which fails to induce immediate or delayed changes in blood pressure or blood gases. Some animals also received an equivalent volume (0.5 ml) of 0.9% w/v normal saline at the same time ($n = 2$ per group) and were pooled to constitute a control group.

2.2. In vitro vascular reactivity

Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and were killed by bleeding from the carotid artery. The thoracic aorta was rapidly removed and immersed in iced oxygenated Krebs–Henseleit (KH) solution of the following composition (mM): NaCl 118, KCl 4.6, $NaHCO_3$ 27.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.75, Na_2EDTA 0.03 and D-glucose 11.1 (pH 7.35 to 7.45). Vessels were cleaned of surrounding fat and connective tissue and cut into rings 3 to 4 mm long. One ring of each aorta was functionally denuded of endothelium by lightly rubbing the luminal wall with wooden applicator. All rings (three to four per animal) were mounted under 1.5 g resting tension on stainless hooks in organ chambers (Radnoti Glass Technology) filled with 40 ml warmed (37°C) and oxygenated (95% 0_2, 5% $C0_2$) KH solution (Meurice et al., 1996). Rings were connected to force transducers, and changes in isometric force were recorded continuously. The output from the transducers was amplified by signal conditioners and sent to an Intel 486-based computer (Kenitec) for analog-to-digital conversion. After an equilibration period of 1 h, the contractile response to a depolarizing concentration of KCl (60 mM) was recorded for a measure of maximal contraction to KCl in each ring. The presence or absence of functional endothelium was verified by addition of acetylcholine (3×10^{-5} M) to rings precontracted with phenylephrine (10^{-6} M).

The contractile response of aorta rings was determined by addition of cumulative concentrations of phenylephrine (10^{-9} to 10^{-5} M), alone or after a 30-min in vitro incubation of rings with N^{ω}-monomethyl-L-arginine (3×10^{-6} M) or with indomethacin (10^{-5} M), to assess respective involvement of nitric oxide pathway or prostanoids pathway in lipopolysaccharide effect on vascular contractile response. The receptor-dependent endothelium-dependent relaxing response was assessed by application of acetylcholine (10^{-9} to 3×10^{-5} M), the receptor-independent endothelium-dependent relaxing response by application of calcium ionophore calcimycin (A 23187; 10^{-9} to 3×10^{-6} M) and the endothelium-independent relaxing

Table 1
Contractile responses to phenylephrine (PE) with (E +) or without (E −) endothelium and relaxing responses to acetylcholine (Ach), A 23187 or sodium nitroprusside (SNP) of aorta rings of rats after saline administration (control; $n = 8$) or 12, 24, 72 or 168 h after LPS (0.5 mg kg^{-1}) administration ($n = 6$–10 per group). Results (means ± S.E.M.) are expressed as maximal response (E_{max} in percent) and EC_{50}

	Control	LPS-12 h	LPS-24 h	LPS-72 h	LPS-168 h
PE (E +)					
EC_{50} (nM)	65 ± 12	86 ± 23	41 ± 7	277 ± 44[b]	49 ± 10
E_{max} (%)	175.4 ± 7.4	196.7 ± 39.9	182.2 ± 1.7	108.8 ± 15.9[b]	199.6 ± 7.5
PE (E −)					
EC_{50} (nM)	10 ± 3	11 ± 3	11 ± 5	19 ± 6	13 ± 2
E_{max} (%)	207.8 ± 12.1	232.8 ± 15.1	230.5 ± 16.3	193.0 ± 8.4	223.3 ± 13.6
ACh					
EC_{50} (nM)	61 ± 13	20 ± 7[a]	19 ± 5[a]	9 ± 4[a]	88 ± 25
E_{max} (%)	90.9 ± 1.0	90.8 ± 3.6	111.5 ± 1.9[b]	107.7 ± 1.9[a]	87.9 ± 41.7
A 23187					
EC_{50} (nM)	42 ± 9	18 ± 7	27 ± 12	9 ± 4[a]	32 ± 12
E_{max} (%)	90.1 ± 1.0	85.4 ± 2.7	97.0 ± 1.4	95.7 ± 2.5	85.7 ± 4.3
SNP					
EC_{50} (nM)	39 ± 8	17 ± 6	24 ± 6	48 ± 10	36 ± 9
E_{max} (%)	108.2 ± 1.4	105.7 ± 2.1	106.5 ± 1.7	111.7 ± 1.5	106.8 ± 2.4

[a] $P < 0.05$.
[b] $P < 0.01$ as compared to control by ANOVA and PLSD Fischer post hoc test.

response by application of sodium nitroprusside (10^{-9} to 3×10^{-5} M).

2.3. Statistical analysis

Results were expressed as means ± S.E.M. Dose–response curves of relaxant agents were constructed by expressing relaxations as a reduction percentage of the maximal response to phenylephrine (10^{-5} M). Each dose–response curve was characterized by determining the maximal response to the drug (E_{max}) and by determining the EC_{50} value (concentration of drug required to elicit 50% of effect). Contractile responses were expressed as EC_{50} and percentages of maximal response to KCl (60 mM). Intergroup differences were tested by one-way analysis of variance (ANOVA), followed by a post hoc protected least significant difference (PLSD) Fisher test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Lipopolysaccharide-induced changes in relaxing responses

Responses of aorta rings to acetylcholine, A 23187 and sodium nitroprusside were summarized in Table 1. Maximal relaxing responses as well as sensitivity to acetylcholine were significantly and time-dependently increased between 12 and 72 h after lipopolysaccharide administration, with a maximum at 72 h, whereas, these effects have disappeared 168 h later. Lipopolysaccharide failed to induce any change in maximal relaxing response to A 23187 while there was only an increase in sensitivity to A 23187, 72 h after lipopolysaccharide administration, as proven by the significant decrease of EC_{50} at this time. There was no significant change in maximal relaxing response or sensitivity to sodium nitroprusside after lipopolysaccharide administration.

3.2. Lipopolysaccharide-induced changes in contractile responses

There was no difference in maximal contraction to KCl between control and animals receiving lipopolysaccharide. Lipopolysaccharide had no significant effect on maximal contractile response and sensitivity to phenylephrine, when administered 12, 24 or 168 h prior to in vitro experiment. Lipopolysaccharide induced markedly, 72 h after injection, a significant decrease in contractile response to phenylephrine of aorta rings in presence of endothelium, as proved the decrease of E_{max} (Fig. 1a) and increase of EC_{50} (Table 1). This effect was abolished by removal of endothelium (Table 1). The lipopolysaccharide-induced decrease of E_{max} was nullified after ring incubation with N^{ω}-monomethyl-L-arginine but not with indomethacin (Fig. 1b) as well as the decrease in sensitivity to phenylephrine with N^{ω}-monomethyl-L-arginine ($EC_{50} = 40 \pm 9$ nM; $P < 0.001$) but not with indomethacin ($EC_{50} = 180 \pm 40$ nM; N.S.). The E_{max} and EC_{50} to phenylephrine of rings of lipopolysaccharide-treated rats in presence of N^{ω}-monomethyl-L-arginine were not significantly different from those of control rings incubated with N^{ω}-monomethyl-L-arginine.

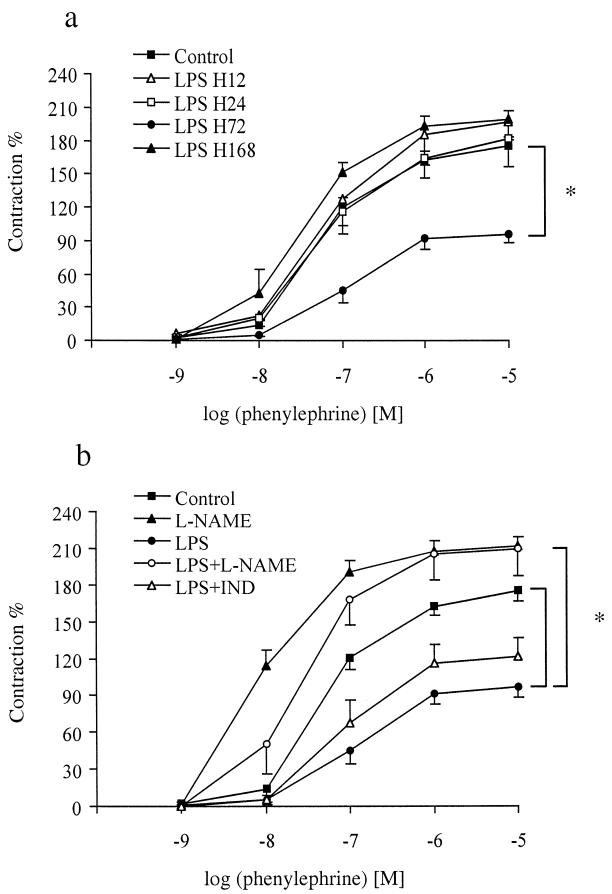

Fig. 1. (a) Phenylephrine concentration–response curves in aortic rings of rats after saline injection (control) or 12, 24, 72 or 168 h after lipopolysaccharide (LPS; 0.5 mg kg^{-1}) administration. (b) Effect of rings incubation with L-NAME (3×10^{-6} M) or indomethacin (10^{-5} M) on phenylephrine concentration–response results observed in control rats or 72 after LPS administration. All results (means ± S.E.M.) represent percentage of response to KCl (60 mM). $^{*}P < 0.05$ as compared to LPS group by ANOVA and PLSD fisher post hoc test.

4. Discussion

We show here that a low dose of lipopolysaccharide is able to induce a delayed improvement of endothelium-dependent relaxation of aorta. This effect is maximal 72 h after lipopolysaccharide administration and interests mainly the receptor-dependent component of endothelium-dependent relaxation while effect on receptor-independent component, induced by calcium ionophore A 23187, is less clear. Moreover, the endothelium-independent response to

sodium nitroprusside was unaffected by lipopolysaccharide pretreatment. This effect on relaxation obtained maximally 72 h later lipopolysaccharide injection is parallelly accompanied by a decrease in contractile response to phenylephrine, suggesting that contractile effect of phenylephrine is alleviated by increase in endothelium-dependent relaxing tone. This hypothesis is confirmed by disappearance of contractile effect when endothelium has been removed.

This beneficial effect of a low dose of lipopolysaccharide is contrasting with vascular deleterious effect induced by 5- to 20-fold higher doses of lipopolysaccharide, which induce endothelium-dependent relaxation impairment (Myers et al., 1995). A decrease in vasoconstrictive response to phenylephrine is also parallel to relaxation impairment but persists after that endothelium has been removed suggesting that this effect is endothelium-independent, contrary to effect of a low dose (Julou-Schaeffer et al., 1990). These differences suggest a dual effect of lipopolysaccharide, confirmed by clinical response to low and high doses. A low dose fails to induce any immediate or delayed change in main physiological parameters whereas high doses induce rapidly shock with decrease in blood pressure and acidosis.

The vascular effect obtained with a low dose of lipopolysaccharide confirms the possibility to improve endothelium-dependent relaxation previously demonstrated after heat stress (Richard et al., 1997) or preconditioning (Kim et al., 1997). Heat stress has been found to improve 24 h later, endothelium-dependent relaxation in three vascular bed (aorta, mesenteric artery, coronary artery) (Richard et al., 1997). At the studied time (24 h), there was no effect on contractile response to phenylephrine as well as after 24 h lipopolysaccharide administration, but authors fail to study more delayed vascular effect of heat stress. It has also been recently reported that a brief ischemia period enhanced in a delayed manner the nitric oxide-mediated coronary vasodilatation, as proved by an increase of in vivo relaxing response to acetylcholine or bradykinin 1 to 2 days after a brief coronary artery occlusion (Kim et al., 1997).

The time-course of improvement of endothelium-dependent relaxation induced by preconditioning, heat stress or low dose of lipopolysaccharide appears closely paralell to occurrence of the delayed protective induced by these procedures. It has been suggested that delayed enhanced endothelium-mediated coronary vasodilatation could be involved in the mechanism of the delayed window of preconditioning, likely by preventive effect on coronary endothelial dysfunction induced by ischemia and reperfusion (Richard et al., 1994). It has been also suggested that endothelial effect of heat stress might contribute to its delayed anti-ischemic properties (Richard et al., 1997). Low dose of lipopolysaccharide has been also found to induce a delayed protective effect in heart as well as in brain. In heart, a protective effect has been found 8 or 24 h after lipopolysaccharide administration (Song et al., 1996). A such early protective effect is not incompatible with involvement of lipopolysaccharide-induced endothelium-dependent relaxation improvement because we find that 12 or 24 h after lipopolysaccharide administration, there was already vascular effect which becomes maximal 72 h after lipopolysaccharide administration, a time when cardioprotective effect has not been yet evaluated. Moreover, the time-course of lipopolysaccharide vascular effect is parallel to the time-course of its delayed neuroprotective which has been demonstrated to be maximal between 2 and 4 days after lipopolysaccharide administration (Deplanque et al., 1997; Tasaki et al., 1997).

The mechanism underlying this enhancement of endothelium-dependent relaxation by lipopolysaccharide remains to be discussed. We demonstrated here that lipopolysaccharide-induced effect involves nitric oxide pathway because decrease in contractile response to phenylephrine observed 72 h after lipopolysaccharide administration was suppressed by blockade of nitric oxide pathway whereas blockade of cyclo-oxygenase pathway has no effect. NO pathway could be involved by two possible mechanisms: (i) an increase in basal NO level; (ii) an increase in expression and/or activity of NO synthase, resulting in a stimulated NO release. Nevertheless, the lack of difference between contractile response of lipopolysaccharide-pretreated and control rats after NO synthase blockade is not in favor of an increase in basal aortic NO content induced by lipopolysaccharide. In contrast, a stimulation of basal vascular nitric oxide release has been suggested following heat stress (Richard et al., 1997). The delayed enhanced coronary endothelial function has been correlated to an increase in production of nitric oxide metabolites (Kim et al., 1997).

Modulation in aortic smooth muscle tone involves more likely changes in expression or activity of NO synthases. Nitric oxide can be generated by three isoforms of NO synthase: constitutive endothelial NO synthase (type III); neuronal NO synthase (type I); inducible NO synthase (type II) (Furchgott and Zawadzki, 1980; Moncada et al., 1991). In spite of the role of high doses of lipopolysaccharide in NO synthase type II induction, responsible for hypotension and vascular hyporeactivity in sepsis (Hom et al., 1995), involvement of this isoform in the delayed enhanced nitric oxide-mediated relaxation is unlikely. The discrepancy between the delayed effect of low dose of lipopolysaccharide in our study and the early induction of NO synthase type II previously described (Liu et al., 1996) is not in favor of NO synthase type II hypothesis. In contrast, the disappearance of changes in contractile response when endothelium has been removed is in favor of involvement of NO synthase type III. In preconditioning-induced delayed enhancement of coronary vasodilation, NO synthase type III has been also considered as the most probable mechanism responsible for the enhanced NO production (Kim et al., 1997). Lipopolysaccharide could contribute to regulate NO synthase type III both transcrip-

tionally or posttranscriptionally in endothelial cells as other agents such as vascular endothelial growth factors (Kroll and Waltenberger, 1998) or physical stimuli such as shear stress (Busse et al., 1993). The effect of lipopolysaccharide on NO release from endothelial cell remains largely controversial. It has been reported that high doses of lipopolysaccharide inhibit endothelium-dependent relaxation and attenuate the release of endothelial NO from isolated blood vessels (Myers et al., 1995) and cultured endothelial cells (Lu et al., 1996). However, several lines of evidence indicate that synthesis of NO synthase III-derived NO is increased by lipopolysaccharide, by activation of NO synthase type III through protein tyrosine kinase (Salvemini et al., 1990; Dudek et al., 1992; Huang et al., 1998). Nevertheless, it remains to demonstrate directly that a low dose is able to up-regulate or activate in a delayed manner NO synthase type III.

The delay necessary to observe the maximal effect of lipopolysaccharide could also suggest the necessity of new protein synthesis. The anti-oxidant enzymes, that have been also involved as ischemic tolerance mechanism, could be one of the candidate proteins because increase in anti-oxidant enzymes could result in a less degradation of NO, explaining its increased level (Brown et al., 1989). It could be opposed that a such mechanism would also apply to NO derived from NO-donors and that this is not consistent with the observation that the effect of sodium nitroprusside is not changed by lipopolysaccharide pretreatment. Nevertheless, it has been also previously reported that decrease in superoxide dismutase reduced endothelium-dependent relaxation rather than endothelium-independent relaxation (Mugge et al., 1991). Another hypothesis is the heat shock proteins induction, which have been involved in ischemic tolerance and which could improve coupling between receptor and NO synthase since the receptor-dependent endothelium-dependent relaxation is mainly enhanced (Amrani et al., 1994).

In conclusion, the delayed NO-mediated enhanced vasorelaxation induced by lipopolysaccharide could contribute to its delayed anti-ischemic properties. This effect could be reproduced by monophosphoryl lipid A, the non-toxic component of lipopolysaccharide, which has been demonstrated to be safe in human (Elliott, 1998) and to be experimentally responsible for a delayed cardioprotection (Zhao et al., 1997). Moreover, involvement of NO pathway opens also a new field of search in pharmacological targets susceptible to induce ischemic tolerance.

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