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Comparison of L-arginine with Sodium Nitroprusside on Relaxant Responses in Lipopolysaccharide-treated Rat Blood Vessels

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There are regional differences in vascular hyporesponsiveness during sepsis. To determine whether nitric oxide (NO) was responsible for these regional differences, we investigated L-arginine (a substrate of NO synthase) -induced relaxation, which reflects the functional activity of NO synthase, in rat vessels treated with lipopolysaccharide (LPS). For in vivo LPS treatment, LPS (20 mg/kg) was administered intraperitoneally. The following vessels were isolated after six hours : the carotid, pulmonary, mesenteric, renal, and femoral arteries, and the mesenteric and femoral veins. For in vitro LPS treatment, vessels isolated from LPS-untreated rats were incubated with LPS (1 μg/ml) for six hours in an organ chamber. The vascular relaxant responses to L-arginine or sodium nitroprusside (SNP ; an NO-releasing drug) were measured by functional examination. All of the vessels from LPS-treated rats responded well to L-arginine, resulting in different relaxant amplitudes among the vessels ($P<0.05$). The relaxant amplitudes by L-arginine were not correlated with those by SNP ($R=0.08$, $P>0.05$). In the vessels treated with LPS in vitro, on the other hand, the different relaxant amplitudes by L-arginine ($P<0.05$) were significant linearly correlated with those by SNP ($R=0.70$, $P<0.05$). These results suggested that the different relaxant amplitudes by L-arginine among the vessels isolated from LPS-treated rats were caused not only by different responses to NO, but also by different activity of inducible NO synthase among the vessels. During sepsis, the regional heterogeneity of the activation of inducible NO synthase may contribute to heterogeneous redistribution of blood flow.

Key words : L-arginine, Sodium nitroprusside, Nitric oxide, Sepsis, Lipopolysaccharide

INTRODUCTION

Nitric oxide (NO), produced from L-arginine by NO synthase, induces many physiological and pathological effects.[1] Two major isoforms of NO synthase have been identified : one is a constitutive enzyme and is dependent on Ca^{2+} for activity, while the other enzyme is induced by bacterial endotoxin and/or inflammatory cytokines and is Ca^{2+}-independent. The former has been localized to the membrane of endothelium or to the cytosol of central and peripheral neurons, as well as to cells in extraneuronal sites such as the skeletal muscle, pancreas and kidney. On the other hand, the latter has been induced in immune cells such as macrophages and other cells such as hepatocytes, vascular smooth muscle cells, endothelial cells and cardiac myocytes.[2] NO produced by the Ca^{2+}-independent NO synthase (inducible NO synthase) in vascular smooth muscle cells particularly causes relaxation and results in sepsis-associated hypotension and hyporesponsiveness to vasoconstrictors.[3]

Sepsis shows prototypical circulatory changes characterized by a high cardiac output and low vascular resistance, with redistribution of blood flow among organs.[4,5] The changes are accompanied by impairment of oxygen delivery and utilization, which increases tissue injury and mortality.[6] As mentioned above, NO produced by inducible NO synthase causes potent vascular relaxation during sepsis. Therefore, if different activation of inducible NO syn-

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thase occurs among vessels, it may cause different vascular relaxation and vascular resistance, and consequent redistribution of cardiac output. This hypothesis may be supported by a recent report that NO synthase inhibitor caused an increase in blood flow to the colon and a simultaneous decrease to the pancreas, in ovine sepsis.[7]

Recently, we reported that L-arginine (a substrate of NO synthase) caused different relaxation among vessels treated with lipopolysaccharide (LPS).[8] In the present study, to determine whether the different relaxation was due to heterogeneous activation of inducible NO synthase among smooth muscles of vessels during sepsis, the relaxant amplitudes produced by L-arginine were measured and compared with those produced by sodium nitroprusside (SNP, an NO-releasing drug), in various rat vessels treated with LPS *in vivo* or *in vitro*.

MATERIAL AND METHODS

This study was approved by the animal care committee of Fukui Medical University.

LPS treatment in vivo and in vitro

Male Wistar rats (250-300 g) were used. For the *in vivo* treatment, LPS (lipopolysaccharide B from Escherichia coli 026 ; B6, Difco Lab, 20 mg/kg diluted in physiological saline) was administered intraperitoneally. Control rats received only physiological saline. After six hours, the rats were sacrificed by decapitation under isoflurane anesthesia, and the carotid, pulmonary, mesenteric, renal and femoral arteries and the mesenteric and femoral veins were isolated for the functional experiments. For the *in vitro* treatment, the same kind of vessels isolated from the normal rats were mounted in the organ chambers mentioned below, and treated with LPS (1 μg/ml) for six hours, during which the bath medium was exchanged with fresh medium containing the same concentration of LPS every 30 min. All responses to drugs were obtained in medium without the addition of LPS.[9,10]

Measurement of heart rate and blood pressure

Heart rate and blood pressure were measured with sphygmomanometry (Muromachi-Kikai, MK-1000, Osaka, Japan) six hours after administration of LPS or saline.[11]

Functional experiments

The vessels were placed in modified Krebs Henseleit solution (mM ; NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2 and glucose 10 ; pH 7.4). Helical strips were carefully prepared under a dissecting microscope. To avoid the possible involvement of NO derived from endothelium in the mechanical response, the endothelium of all vessels was rubbed off with filter paper,[12] and its removal was confirmed by the loss of the relaxant response to 10 μM acetylcholine in strips pre-contracted with 1 μM phenylephrine.[13] The strips were carefully suspended in an organ chamber containing 20 ml of modified Krebs Henseleit solution bubbled with 95% O_2 - 5% CO_2 at 37°C, and the tension changes were recorded isometrically. Resting tensions of 0.25 g in the arteries or 0.1 g in the veins were applied to all strips during a one-hour equilibration period.

The percent relaxation to L-arginine or SNP applied cumulatively was obtained in strips pre-contracted with phenylephrine. Percent relaxation is dependent on the agonist-induced contractile tension,[14,15] therefore, relaxation was examined in strips pre-contracted to an equivalent tension. The concentrations of phenylephrine for producing the equivalent tension were 0.1 μM in the carotid arteries, 1 μM in the renal, femoral, mesenteric and pulmonary arteries, and 10 μM in the femoral and mesenteric veins.

Statistics

The results are expressed as mean ± SD in the text and tables and as mean ± SEM in the figure. The maximal response (Emax) and the concentration producing a half-maximal response (EC_{50}) were determined graphically by Finley's probit analysis from individual concentration-response curves. Statistical analyses of heart rate and blood pressure were performed with Mann-Whitney test, and analyses of relaxant amplitudes among vessels were performed with ANOVA-factorial (Scheffe F-test) or simple regression. The statistical test of EC_{50} was based on $-\log EC_{50}$.[16] A P value of less than 0.05 was taken as significant. These analyses were performed on a personal computer using Stat View II 4.0 software (Aba-

cus Concepts, Berkeley, CA, USA).

RESULTS

Heart rate and blood pressure

The blood pressure of the rats treated with LPS for six hours was not different from that of the control rats (Table 1). However, heart rate was significantly increased by LPS treatment.

L-arginine-or SNP-induced relaxation of the vessels treated with LPS in vivo

L-arginine caused different relaxant amplitudes among the seven vessels isolated from the LPS-treated rats ($P<0.05$, Table 2). L-arginine caused no relaxation of the vessels from the saline-treated control rats. Also, SNP produced different relaxant amplitudes among the LPS-treated vessels, however, the maximal relaxant amplitudes by SNP were not correlated with those by L-arginine (R=0.08, $P>0.05$, Fig. 1A). Figure 2 shows the representative results obtained in the mesenteric and femoral arteries. Although SNP produced the same concentration-dependent relaxation in both arteries, 100 μM L-arginine produced more relaxation in the femoral than in the mesenteric arteries ($P<0.05$). The relaxation by L-arginine, but not that by SNP, was inhibited by 100 μM N^G-nitro-L-arginine (data not shown).

L-arginine-or SNP-induced relaxation of the vessels treated with LPS in vitro

The addition of L-arginine (1-100 μM) caused no relaxation in the seven vessels isolated from the LPS-untreated rats. However, the six-hours incubation with LPS (1 μg/ml) produced different relaxant amplitudes by L-arginine (Table 3). The maximal relaxant amplitudes by L-arginine in the carotid and mesenteric arteries were greater than those in the same arteries obtained from the rats treated *in vivo* ($P < 0.05$, for each vessel). Relaxation by L-arginine was not observed in the strips which were incu-

Table 1 Effect of LPS on heart rate and blood pressure in rats.

Treatment	*HR (bpm)*	*sBP (mmHg)*	*mBP (mmHg)*	*dBP (mmHg)*
Saline	382 ± 37	129 ± 13	105 ± 8	91 ± 7
LPS	519 ± 48[a]	127 ± 16	105 ± 7	93 ± 10

Heart rate (HR) and blood pressure (BP) were measured 6 h after intraperitoneal administration of lipopolysaccharide (LPS ; 20 mg/kg). Values are mean ± SD of 6 animals. sBP, mBP and dBP ; systolic, mean and diastolic blood pressure. a ; Significantly different from control (saline treatment) ($P < 0.05$).

Table 2 EC_{50} values and Emax of relaxation induced by L-arginine or sodium nitroprusside (SNP) in the vessels treated with LPS *in vivo* for 6 h.

	L-arginine		***SNP***	
	-logEC$_{50}$ (μM)	***Emax (%)***	***-logEC$_{50}$ (μM)***	***Emax (%)***
carotid artery	5.4 ± 0.2	54.5 ± 22.7	8.1 ± 0.3[a]	99.8 ± 0.4[a,c]
pulmonary artery	5.4 ± 0.1[d]	70.9 ± 9.1[b]	7.6 ± 0.1[b,c,d]	95.2 ±1.7[b,d]
mesenteric artery	5.4 ± 0.1	53.6 ± 12.0[d]	8.2 ± 0.2[c]	99.6 ± 1.0[c]
renal artery	5.4 ± 0.1[d]	63.8 ± 8.4	7.9 ± 0.2[d]	90.9 ± 7.0[d]
femoral artery	5.5 ± 0.1	71.2 ± 9.5	8.3 ± 0.1	100.0 ± 0.0
mesenteric vein	5.5 ± 0.3[e]	60.0 ± 13.2	8.2 ± 0.3	82.4 ± 17.0
femoral vein	5.0 ± 0.3	53.5 ± 10.8	8.1 ± 0.6	90.2 ± 7.1

The prepalations were pre-contracted with phenylephrine. The complete relaxation of phenylephrine-induced contraction was taken as 100 %. Values are mean ± SD of 6 experiments. EC_{50} : the concentration producing a half-maximal response, Emax : the maximal response. a ; different from pulmonary artery, b ; different from mesenteric artery, c ; different from renal artery, d ; different from femoral artery, e ; different from femoral vein ($P < 0.05$).

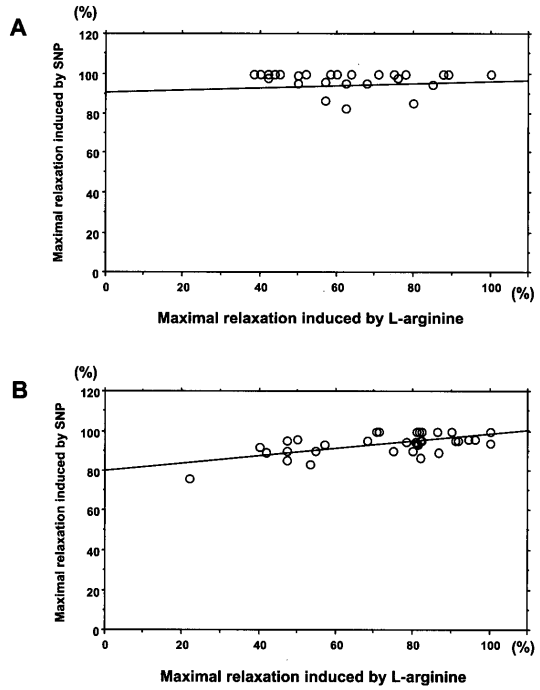

Fig. 1 Plot showing whether the maximal relaxation induced by L-arginine was correlated with that induced by sodium nitroprusside (SNP) in the carotid, renal, femoral, mesenteric and pulmonary arteries. A : In the strips treated with lipopolysaccharide (LPS) *in vivo* for 6 h, the maximal relaxation induced by L-arginine was not correlated to that induced by SNP ($R=0.21$, $P>0.05$). B : In the strips treated with LPS *in vitro* for 6 h, there was a good linear correlation between maximal relaxation induced by L-arginine and that by SNP ($y = 0.27x + 73.8$, $R = 0.81$, $P<0.05$).

bated for six hours without LPS or simultaneously with LPS (1μg/ml) and cycloheximide (10 μM) (n=4 in each vessel). Also, SNP provoked different relaxation among the LPS-treated vessels. There was a good linear correlation between the maximal relaxant amplitudes by L-arginine and those by SNP ($R=0.70$, P

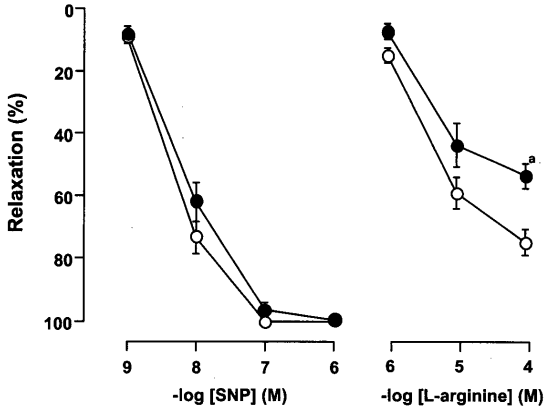

Fig. 2 Relaxation induced by L-arginine or sodium nitroprusside (SNP) in the femoral or mesenteric arteries, which had been treated with lipopolysaccharide *in vivo* for 6 h. Open circles : the mesenteric arteries, Closed circles : the femoral arteries. The preparations were pre-contracted with phenylephrine to approximately 80% of the maximal contraction. The complete relaxation of phenylephrine-induced contraction was taken as 100%. Values are mean ± SEM of 6 experiments. a : Significantly different from the relaxation by L-arginine in the mesenteric arteries ($P<0.05$).

Table 3 EC_{50} values and Emax of relaxation induced by L-arginine or sodium nitroprusside (SNP) in the vessels treated with LPS *in vitro* for 6 h.

	L-arginine		*SNP*	
	-logEC_{50} (μM)	*Emax (%)*	*-logEC_{50} (μM)*	*Emax (%)*
carotid artery	$5.6 \pm 0.1^{a,b,c}$	$100.0 \pm 0.0^{a,b,c}$	8.3 ± 0.3^{a}	$99.0 \pm 2.4^{a,c}$
pulmonary artery	5.5 ± 0.1	81.0 ± 0.7^{d}	$7.7 \pm 0.1^{b,d}$	$93.3 \pm 4.6^{b,d}$
mesenteric artery	5.4 ± 0.1	82.2 ± 6.5	8.1 ± 0.1^{d}	99.2 ± 2.0^{c}
renal artery	5.2 ± 0.2	67.2 ± 28.3	8.0 ± 0.3^{d}	90.3 ± 8.0^{d}
femoral artery	5.6 ± 0.3	91.1 ± 10.7	8.5 ± 0.1	98.6 ± 2.3
mesenteric vein	5.5 ± 0.0^{e}	47.2 ± 6.1^{e}	7.7 ± 0.4	92.5 ± 2.7
femoral vein	5.1 ± 0.1	71.0 ± 13.9	7.7 ± 0.3	89.4 ± 3.3

The prepalations were pre-contracted with phenylephrine. The complete relaxation of phenylephrine-induced contraction was taken as 100 %. Values are mean ± SD of 6 experiments. EC_{50} : the concentration producing a half-maximal response, Emax : the maximal response. a ; different from pulmonary artery, b ; different from mesenteric artery, c ; different from renal artery, d ; different from femoral artery, e ; different from femoral vein ($P < 0.05$).

<0.05, Fig. 1B).

DISCUSSION

Depression in vascular contractile responsiveness has been suggested to be involved in sepsis.[3,6] Also, the degree of depression seems to be different among vessels.[17] Recently, it has been proposed that NO released from inducible NO synthase, which is induced by LPS and/or inflammatory cytokines in vascular smooth muscle cells,[18,19] may be a major factor responsible for endotoxin-induced vascular hyporesponsiveness.[3] In the present study, induction of NO synthase was strongly suggested to cause L-arginine (a substrate of NO synthase) -induced and N^G -nitro-L-arginine (a NO synthase inhibitor)-sensitive relaxation in endothelium-denuded blood vessels from rats treated with LPS. Moreover, it was found that the relaxant amplitudes by L-arginine varied among the vessels. There are two possible explainations for the various relaxant amplitudes : (1) the amount of NO from inducible NO synthase was different among the vessels, and (2) sensitivities to NO were different among the vessels. To investigate these possibilities, the relaxant amplitudes by L-arginine were compared with those by SNP in the vessels. SNP is considered to produce its vasorelaxant action by releasing NO. Although the mechanisms of NO liberation from SNP remain undefined, it is often assumed that spontaneous chemical degradation of SNP to NO mediates vasorelaxation.[20] Therefore, the different relaxant amplitudes by SNP among vessels were interpreted as showing that the sensitivity to NO was different among vessels. However, the amplitudes were not correlated with those produced by L-arginine (Fig. 1-A). These results suggest that there may be a regional difference not only in sensitivity to NO but also in the amount of NO produced from inducible NO synthase among vessels. For example, L-arginine relaxed the femoral arteries more powerfully than the mesenteric arteries, although SNP relaxed both arteries in the same concentration -dependent manner (Fig. 2). That is, the amount of NO from inducible NO synthase was greater in the femoral arteries than in the mesenteric arteries, since the sensitivities to NO were same between both arteries.

Unlike those in *in vivo* LPS treatment, there was a correlation in the amplitudes of vascular relaxation between those by L-arginine and by SNP in *in vitro* LPS treatment. This indicates that the different relaxant amplitudes by L-arginine were due to different sensitivity to NO among the vessels and that the amount of NO from inducible NO synthase was similar in all vessels. It is easily understood that LPS induces more complex effects *in vivo* compared to those *in vitro*, because LPS activates many systems such as adrenal steroids and arachidonic acid metabolites *in vivo*.[6] These must influence the regional differences in activities of inducible NO synthase. Szabo et al. demonstrated that *in vivo* NO synthase induction is inhibited by elevation of endogenous adrenal steroids such as glucocorticoid, resulting in a bell-shaped induction curve.[21] The relatively small relaxation responses to L-arginine in the *in vivo*-treated strips in this study may imply the reduced induction of NO synthase *in vivo*, as compared with that *in vitro*.

Administration of 20 mg/kg LPS did not change arterial blood pressure, but it increased heart rate (Table 1). This change is obviously a systemic response to LPS and may correspond to the hyperdynamic state (high cardiac output and low systemic vascular resistance) in sepsis. As circulating L-arginine is sufficient for maximally activating the vascular L-arginine/NO pathway in sepsis rats,[22] the low systemic vascular resistance elicited by LPS *in vivo* may reflect the summation of the L-arginine-induced relaxation of various vessels.

In conclusion, the present study confirmed the different relaxation produced by L-arginine and SNP among various vessels treated with LPS *in vivo* and *in vitro*. By comparison of the relaxation induced by the two drugs, it was found that the different relaxation produced by L-arginine may be due to the heterogeneous activation of inducible NO synthase *in vivo*, but not *in vitro*, resulting in heterogeneous redistribution of blood flow during sepsis.

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LPS処置血管平滑筋におけるL-arginineとNitroprussideによる弛緩反応の比較

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溝上真樹

キーワード：L-arginine, Nitroprusside，一酸化窒素(NO)，敗血症，細菌内毒素

細菌内毒素(LPS)処置胸部大動脈，大腿動静脈，腸管膜動静脈，頚動脈，肺動脈及び腎動脈を用い，L-arginine(NO合成酵素基質)とnitroprusside (SNP, NO遊離薬)の血管弛緩反応性を比較検討した．LPS処置した全ての血管はL‐アルギニン及びSNPにより弛緩反応を惹起したが，その程度は血管部位だけでなく薬剤によっても異なった．この結果からL-アルギニンによる血管弛緩反応の血管の部位差がNOに対する反応性の差だけではなく細菌内毒素により誘導されるNO合成酵素活性の差にも関係していることが示唆された．

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