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Vasodilation by Intrathecal Lipopolysaccharide of the Cerebral Arteries after Subarachnoid Haemorrhage in Dogs

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Summary

To investigate the influence of inducible nitric oxide synthase on cerebral arteries after subarachnoid haemorrhage (SAH) in vivo, lipopolysaccharide (LPS), a major inducer of inducible nitric oxide synthase, was injected intracisternally into control and SAH model dogs.

Intracisternal injection of LPS (0.5 mg) produced a long-lasting, submaximal vasodilation of the basilar artery of control dogs on angiography. This effect became significant at 4 hours after LPS injection and plateaued after 6 hours. This vasodilation was reduced by N^G-monomethyl-L-arginine. Vasopressin slightly suppressed the vasodilation, while bradykinin increased it. The concentration of L-arginine in CSF decreased after LPS injection, while that of L-citrulline increased. In cytokines, the concentration of tumour necrosis factor-α (TNF-α) in CSF increased transiently at 4 hours after LPS injection, while interleukin-1β, interleukin-6, interferon-γ did not change. These data suggest that vasodilation by LPS is mainly due to nitric oxide predominantly synthesized by an inducible nitric oxide synthase, proximally induced by TNF-α.

Our data make it unlikely that SAH itself induces the inducible nitric oxide synthase in vascular tissue, since isolated endothelium-denuded basilar artery from SAH model dogs did not respond to L-arginine. In SAH model dogs, the degree of vasodilation by LPS differed with the severity of vasospasm. Vasodilation was much greater in mild than in severe vasospasm in dogs, and was increased by superoxide dismutase. These findings suggest that the induction of inducible nitric oxide synthase or its activity may be less effective in severe vasospasm.

Keywords: Angiography; dog; inducible nitric oxide synthase; lipopolysaccharide; subarachnoid haemorrhage; tumour necrosis factor-α.

Introduction

Endothelium derived relaxing factor (EDRF), now identified as nitric oxide or a closely related substance synthesized from L-arginine by the vascular endothelium, plays an important role in the physiological regulation of vascular tone [1, 9, 25, 43]. Nitric oxide is derived from the guanidine nitrogen atom(s) of L-arginine in a reaction catalysed by nitric oxide synthase [19, 26]. The expanding family of nitric oxide synthase isoforms generally divide into two categories: constitutive forms regulated by Ca^{2+} and calmodulin in vascular endothelium or neuronal cells, and cytokine-inducible forms, that are less sensitive to Ca^{2+}, but are induced in various cell types including vascular smooth muscle cells, macrophages, neutrophils, and liver cells, under pathological conditions [14, 20].

Lipopolysaccharide (LPS), which causes severe hypotension in endotoxin shock, has been proven to activate the synthesis and release of cytokines by smooth muscle cells and endothelial cells, as well as by tissue macrophages [16, 21, 44]. Several cytokines, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interferon-γ (IFN-γ) have been shown to mediate the hypotensive effect of endotoxin by producing excessive nitric oxide through the induction of inducible nitric oxide synthase [13, 28, 30]. Compared with constitutive nitric oxide synthase, cytokine-induced nitric oxide synthase is capable of producing larger amounts of nitric oxide, causing potent vasodilation and hyporesponsiveness against various hypertensive agents [7, 29, 37, 38, 42]. Once cytokine-inducible synthase is generated in vascular smooth muscle cells, its vasodilator effect on arteries seems to be mainly determined by the available concentration of L-arginine, the substrate of nitric oxide synthase [31].

In this study, we examined angiographically the vasodilator effects of intracisternal injection of LPS on control and spastic basilar arteries in dogs. We also

studied alterations in the concentration of cytokines and amino acids in cerebrospinal fluid (CSF) after intracisternal injection of LPS. We carried out these experiments in control and single or double haemorrhage subarachnoid haemorrhage (SAH) model dogs.

Materials and Methods

All protocols and surgical procedures used in this study followed the Guidelines of the Institute for Laboratory Animal Research, Nagoya University School of Medicine. Mature mongrel dogs of both sexes, 8 to 14 kg, were anaesthetized with intravenous pentobarbital (25 mg/kg). Dogs were intubated and their respiration was controlled through tracheal tubes with room air delivered by respirator (Model B2, Igarashi Ika Kogyo Co., Tokyo, Japan). The ventilation rate (approximately 12 cycles/min) and tidal volume (20 ml/kg) were adjusted to maintain arterial blood gases and pH with physiologic limits.

Angiography

A catheter for angiography was inserted into the right vertebral artery just caudal to the foramen in the transverse process of the sixth cervical vertebra. A second catheter was placed in the left femoral artery to monitor mean arterial blood pressure and heart rate. A control angiogram was performed with 3 ml of 65% meglumine iothalamate at a fixed magnification before injection of control or LPS solutions. Angiograms were then obtained periodically (1, 2, 3, 4, 5, 6, 7 hours) after LPS solution (0.5 mg/2 ml saline) or saline was injected into the cisterna magna through a 22 G spinal needle. In some experiments, L-arginine (100 µmol), N^G-monomethyl-L-arginine (L-NMMA, 10 µmol), vasopressin (10 nmol) or bradykinin (10 nmol) in 2.0 ml of saline were injected by the same route 6 hours after LPS injection, when submaximal dilation of the basilar artery had been obtained, and angiography was then performed over the next hour (5, 10, 15, 20, 25, 30, 45, 60 min).

Except for L-arginine, an alkaline amino-acid, the pH of all test solutions was almost the same as that of the control solution. The pH of L-arginine solution was neutralized with HCl to about 7.4. Solutions were injected into the cisterna magna only after the same volume of CSF had been gently withdrawn, to maintain the intracranial pressure as constant as possible.

Measurement of Amino Acids and Cytokines in CSF

CSF concentrations of L-arginine, L-citrulline, TNF-α, IL-1β, IL-6, and IFN-γ were measured after intracisternal injection of LPS (0.5 mg). Samples of CSF were withdrawn before and after LPS injection (0, 2, 4, 6 hours) and kept at –80°C until assayed. Amino acid concentrations were measured by HPLC (Hitachi Co., Tokyo, Japan). The concentration of IL-1β was measured with a human IL-1β immunoradiometric assay kit (Medgenix Diagnostics S.A., Brussels, Belgium). The concentration of IL-6 was determined with a human IL-6 ELISA kit (Fujirebio Co., Tokyo, Japan). The concentration of IFN-γ was measured with a radio-immunoassay (SRL Co., Tokyo, Japan). The concentration of TNF-α was measured with an ultra-sensitive highly specific, enzyme amplified, immunometric assay kit for human TNF-α given by Asahi Chemical Industry Co. (Tokyo, Japan).

Histological Changes in Basilar Arteries after LPS Administration

In order to compare arterial morphology between control and LPS-treated dogs, the basilar arteries were removed from dogs and fixed for study by electron microscopy (Nihon Denshi, JEM-1200EX, Tokyo, Japan). They were fixed for 3 hours in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Postfixation was performed for 2 hours in 1% O_5O_4 using the same buffer. The specimens were dehydrated with ethanol in concentrations increasing to 100%. Embedding in Epon was performed by using graded additions of Epon-propylenoxide mixtures. The final polymerization was carried out at 60 °C for 2 days. Semi-thin (0.5 µm) sections were cut on pyramitome and stained with toluidine blue.

Experimental Subarachnoid Haemorrhage

After a control angiogram, the cisterna magna was punctured with a 22 G spinal needle, and 5 ml of CSF was withdrawn. An equal volume of fresh autologous blood was then injected into the cisterna magna, and the animal was kept in a head-down position for 30 min to maintain contact of autologous blood with the basilar artery. We made both one- and two- haemorrhage models due to obtaining various rates of vasospasm. On Day 8, another angiogram was performed to determine the degree of chronic cerebral vasospasm. Angiograms were then performed periodically after injection of LPS (0.5 mg) as in the control animals.

The internal diameter of the middle third of the basilar arteries as measured using a computerized image analysis system (Macintosh 2 cx image 1.27, Apple Computer Inc., Cupertino, CA, USA), as described previously [12, 25, 35, 36]. The data were expressed as a percentage of the diameter of the arterial segment before injection.

Effect of L-arginine on the Spastic Basilar Artery in vitro

We also examined the vasodilator effect of L-arginine on isolated spastic basilar artery to determine whether inducible nitric oxide synthase had been induced by SAH. Intracranial arteries were rapidly removed from experimental SAH dogs on Day 8, and were cut into annular 5 mm segments after dissection of adhering clot and arachnoid membrane under a microscope. After stripping the endothelium, the vasodilator effect of L-arginine (100 µM) was monitored by measuring isometric tension.

Materials

LPS (E. Coli Serotype 055 : B5) was purchased from Sigma Chemical Company (St. Louis, MO, USA), and L-NMMA from Calbiochem (La Jolla, CA, USA) Vasopressin and bradykinin were obtained from Peptide Institute (Osaka, Japan). Recombinant human superoxide dismutase (SOD) was a generous gift from Asahi Chemical Industry Co. (Tokyo, Japan). L-arginine and all other chemicals were reagent grade or the best commercially available grade.

Statistical Analysis

Data are expressed as mean ± standard error of the mean. Differences were analysed either by analysis of variance, followed by Fischer's protected least significant difference multiple-range test, or Student's paired and unpaired t-test. Values of p less than 0.05 were considered statistically significant.

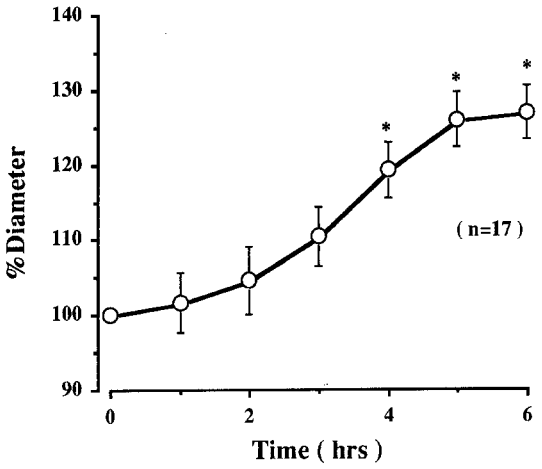

Fig. 1. Percentage change in diameter of basilar artery in response to intracisternal injection of LPS (0.5 mg) in intact dogs. Values are mean ± S.E. Number of animals is indicated in parentheses. * $P < 0.05$

Results

Effects of LPS on the Diameter of the Basilar Artery in Intact Dogs

Intracisternal injection of LPS (0.5 mg) produced a marked increase in the internal diameter of the basilar artery on angiograms. This vasodilator effect of LPS was significant from 4 hours after intracisternal injection (119.3 ± 3.7%, n = 17) and reached an asymptote after 6 hours (126.9 ± 3.6%, n = 17) (Fig. 1). This vasodilation persisted and was still recognizable until 72 hours after LPS injection. Neither mean blood pressure nor heart rate showed any change through 6 hours after LPS injection. Neither mean blood pressure nor heart rate showed any change through 6 hours after LPS injection.

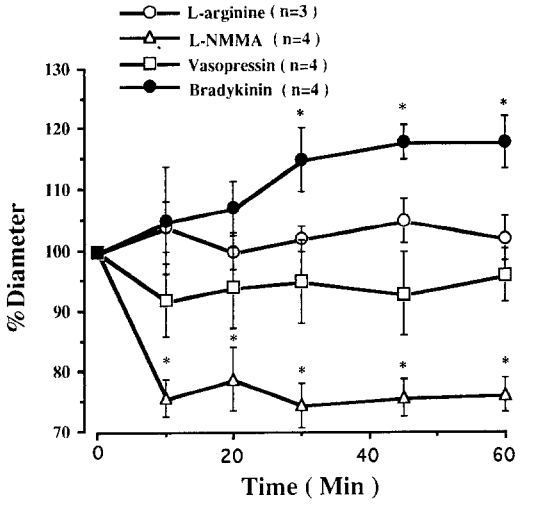

Fig. 2. Percentage change in diameter of the basilar artery by intracisternal administration of L-arginine, L-NMMA, vasopressin, and bradykinin 6 hours after submaximum vasodilation due to intracisternal injection of LPS. Values are mean ± S.E. Number of animals treated is indicated in parentheses. * $P < 0.05$

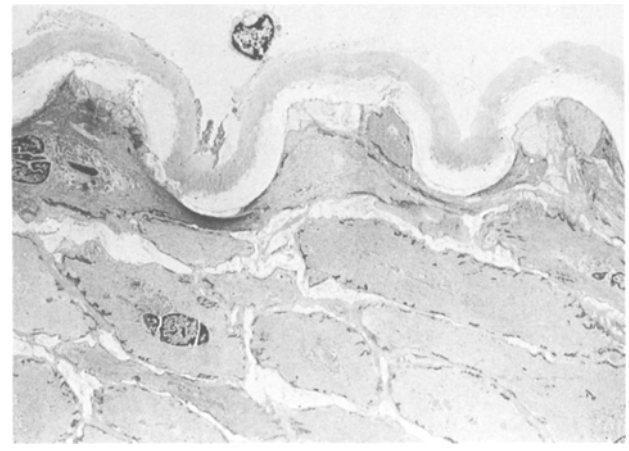

Fig. 3. Morphological changes in the basilar artery under influence of intracisternal injection of LPS. (× 3600)

Effects of L-Arginine, L-NMMA, Vasopressin, and Bradykinin after Near Maximal Vasodilation

L-arginine (100 μmol, n = 3), L-NMMA (10 μmol, n = 4), vasopressin, and bradykinin (10 nmol, n = 4) were injected intracisternally into control dogs whose basilar arteries showed near maximal vasodilation after LPS injection. L-NMMA suppressed significantly the vasodilation caused by LPS for following 60 min, decreasing basilar artery diameter to 70.3 ± 5.7% of its near maximal diameter (88.0 ± 6.8% of control diameter) (Fig. 2). L-arginine had no vasoactive effect on the basilar artery after near maximal vasodilation. Vasopressin had a tendency to constrict the basilar artery after treatment with LPS, but this was not statistically significant. Only bradykinin produced further vasodilation 30 min after injection, increasing the diameter to 117.7 ± 2.9% of the near maximum observed after LPS treatment (138.8 ± 7.1% of control diameter).

Concentrations of Amino Acids in CSF after Intracisternal Injection of LPS

Although concentrations of most amino-acids in CSF increased after intracisternal injection of LPS, that of L-arginine significantly decreased, from 34.8 ± 2.1 nmol/ml to 26.7 ± 2.1 nmol/ml (n = 10), whereas that of L-citrulline increased from 2.8 ± 0.7 nmol to 7.9 ± 1.5 nmol/ml (n = 10) 6 hours after LPS injection.

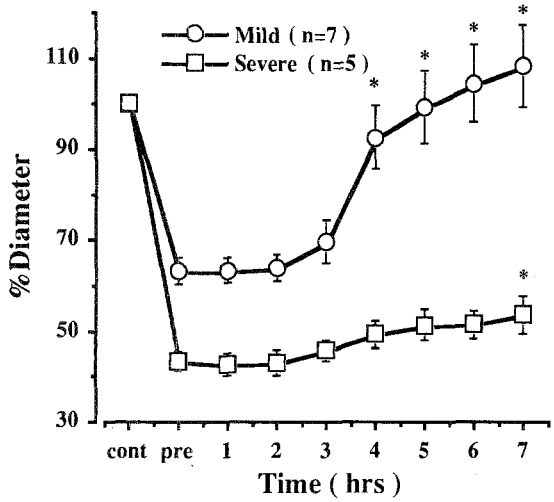

Fig. 4. Percentage change in diameter of the basilar artery after intracisternal injection of LPS in SAH model dogs. LPS (0.5 mg) was injected into the cisterna magna after the occurrence of mild or severe vasospasm was angiographically confirmed on Day 8. *Mild*: mild vasospasm group in which the diameters of the basilar arteries on Day 8 were more than 50% of those before injection of blood. *Severe*: severe vasospasm group in which the diameters of the basilar arteries on Day 8 were less than 50% of those before injection of blood. Values are mean ± S.E. * $P < 0.05$

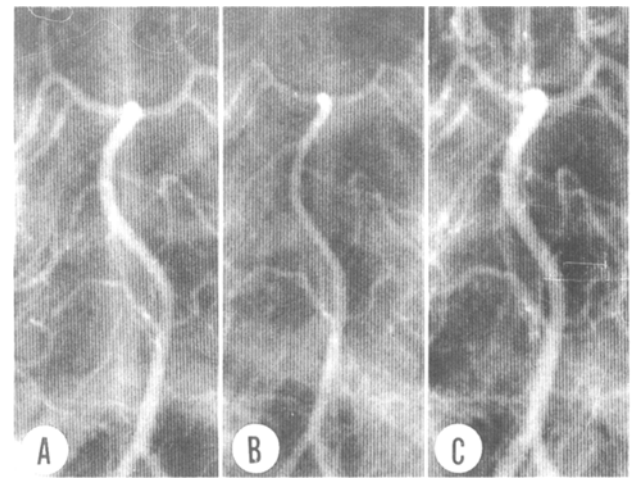

Fig. 5. Representative vertebral angiograms showing the vasodilatory effect of LPS on the basilar artery in SAH model dogs with mild vasospasm. (A) Day 1, before intracisternal injection of autologous blood. (B) Day 8, before intracisternal administration of LPS. (C) Day 8, 6 hours after intracisternal administration of LPS

Concentrations of Cytokines in CSF after Intracisternal Injection of LPS

Among the cytokines measured, only the concentration of TNF-α increased significantly in CSF from 1.3 ± 0.9 U/ml to 52.1 ± 14.7 U/ml (n = 5) 4 hours after LPS injection, before returning to the baseline value. IL-1β (n = 5), IL-6 (n = 5) and IFN-γ (n = 5) concentrations showed no obvious change throughout the 6 hours after LPS injection.

Histological Changes after LPS Administration

Compared to basilar artery structure in control dogs, arteries from LPS-treated animals showed vigorous proliferation of intimal cells, oedematous swelling of internal elastic lamina, and hyperplasia of medial cells. Protrusion of cytoplasmic processes of endothelial cells, vesiculation of medial cells, aggregations of lymphocytes and ribosomes in the medial cell matrix were prominent ultrastructural features in LPS-treated samples (Fig. 3).

Effect of LPS on the Diameter of the Spastic Basilar Artery

We examined the effects of LPS on the spastic basilar artery using both single- and double- haemorrhage model dogs. We divided the animals into two groups according to the severity of vasospasm observed: a mild vasospasm group (n = 7) whose basilar artery diameters on Day 8 were more than 50% of those on Day 1, and a severe vasospasm group (n = 5) whose basilar artery diameters on Day 8 were less than 50% of those on Day 1. In the mild vasospasm group, the mean diameter of the basilar artery on Day 8 was 63.1 ± 2.9% of that on Day 1, and it increased to 108.4 ± 9.1% of Day 1 diameter 7 hours after LPS injection. In the severe vasospasm group, the mean diameter of the basilar artery on Day 8 was 43.4 ± 2.3% of that on Day 1, and it increased to 53.8 ± 4.2% of Day 1 diameter 7 hours after LPS injection (Fig. 4). Typical angiograms are shown in Fig. 5.

In the mild vasospastic group 6 hours after administration of LPS, the vasodilator effect of LPS on the basilar artery was further increased by SOD (n = 3). Significant vasodilation was observed from 20 to 45 min after administration of SOD. Maximum arterial diameter at 20 min was 121.7 ± 1.9% of that before injection of SOD (Fig. 6).

Effect of L-Arginine on the Isolated Cerebral Arteries after SAH in vitro

To assay whether inducible nitric oxide synthase was synthesized in the smooth muscle cells after SAH, L-arginine (100 μM) was applied to endothelium-denuded cerebral arterial rings removed from

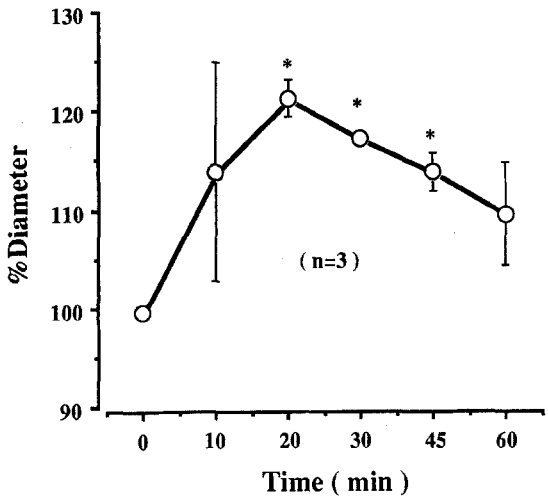

Fig. 6. Percentage change in diameter of the basilar artery in response to intracisternal administration of 6000 U superoxide dismutase (SOD) after LPS injection in experimental subarachnoid haemorrhage dogs showing mild vasospasm on Day 8 by angiography. SOD was added into the cisterna magna 6 hours after the intrathecal injection of LPS. Values are mean ± S.E. * $P < 0.05$

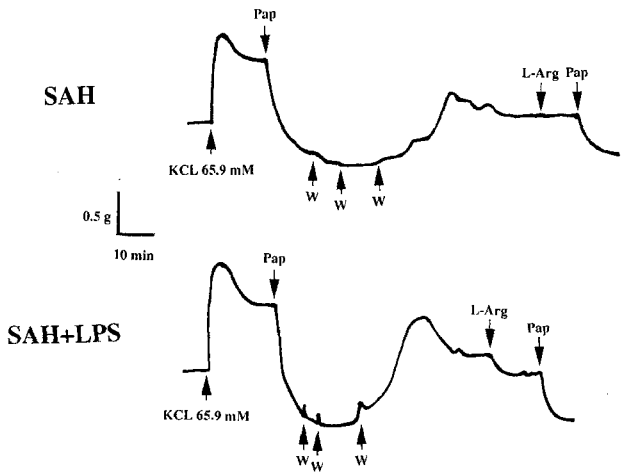

Fig. 7. Typical chart showing the vasorelaxing effect of L-arginine on the isolated basilar arteries from experimental subarachnoid haemorrhage dogs without (upper) and with (lower) treatment of LPS. Application of L-arginine produced a vasorelaxation in the endothelium-denuded basilar artery after treatment with LPS. *Pap* 100 μM papaverine HCl; *L-Arg* 100 μM L-arginine; *W* wash

SAH model dogs on Day 8. Significant vasorelaxation was detected in the basilar arterial rings form previously treated dogs with LPS, but not in those from SAH model dogs without LPS treatment (Fig. 7).

Discussion

Inducible Nitric Oxide Synthase

The resting tonus of cerebral arteries is strongly regulated by nitric oxide synthesized by constitutive nitric oxide synthase located in both vascular endothelium [9, 26, 43] and in perivascular vasomotor nerves [22, 32, 39, 40]. It seems that an approximate 20% constriction of the diameter of canine basilar arteries is maintained by continuous release of nitric oxide from the endothelium, probably in response to local hormones, changes in blood flow velocity, or endothelial sheer stress [12, 25]. There are two forms of nitric oxide synthase. One is constitutively expressed in vascular endothelial cells and in the brain [2]. The other is inducibly expressed by endotoxin- or cytokine-treated macrophages, endothelial cells and vascular smooth muscle cells [10]. Under pathological conditions including infections, injuries, or malignancies [15, 24, 38] inducible nitric oxide synthase is induced within various cell types [6, 16, 28, 34]. LPS, which is responsible for endotoxic shock, causes marked vasodilation, mediated by large amounts of nitric oxide produced from L-arginine, by inducing nitric oxide synthase in smooth muscle cells [7, 29, 37, 42].

LPS Effects on Intact Basilar Artery

Despite their low dosage (0.5 mg), our injections of LPS into the cisterna magna produced a near maximal vasodilation of the basilar artery. Vasodilation by LPS reached significance at 4 hours after injection and reached a maximal asymptote after 6 hours. This time lag corresponded well to the period required for synthesis of new, inducible, nitric oxide synthase [1, 7, 20, 21, 29, 31, 44]. The dose of LPS used in the present study appeared to be sufficient to induce the L-arginine/nitric oxide pathway only in the smooth muscle cells of cerebral arteries located in the subarachnoid space. The stable blood pressure and heart rate observed throughout the experiments indicated that the vasoactive effect of LPS was relatively restricted to the subarachnoid space, without influence on peripheral vessels or on the central pressure control centre [32, 40]. In our experiments, mild basilar dilation persisted until 72 hours after LPS injection in several dogs. This long persistence of vasodilation suggests that inducible-nitric oxide synthase activity is higher than constitutive nitric oxide synthase. The volume of nitric oxide in vessels exposed to LPS appeared to be only regulated by the extracellular concentration of L-arginine, a rate limiting factor in nitric oxide synthase activity [31].

Pharmacologic Features of LPS-Induced Vasodilation

The submaximal vasodilation induced by LPS was examined pharmacologically by intracisternal injection of nitric oxide-related substances or substances that stimulate the release of endothelium-derived relaxing factor. L-NMMA completely reversed LPS-induced dilation of the basilar artery, while high concentrations of L-arginine could not further dilate arteries after LPS treatment. The submaximal vasorelaxation induced by LPS has been supposed to result from nitric oxide synthesized from the endogenous CSF L-arginine [18, 37]. Vasopressin had the tendency to constrict the arteries after the submaximal vasodilation induced by LPS, while bradykinin produced a further, significant vasodilation. Vasopressin is known to have a dual vasoactive function on cerebral arteries, causing a vasodilation through action on endothelial receptors related to the constitutive nitric oxide pathway, as well as a vasocontraction through action on smooth muscle receptors related to Ca^{2+} influx [25, 35, 36]. It appears that the vasocontracting activity of vasopressin was dominant in arteries pretreated with LPS. Our data indicate that bradykinin acts through an NOS-independent endothelial pathway to dilate arteries, consistent with recent reports that the endothelium-derived relaxing factor released by bradykinin is different from nitric oxide [4, 5, 45].

Morphological Changes Caused by LPS

Intracisternal injection of LPS elicited inflammatory and destructive morphological changes in cerebral arteries, demonstrated by electron microscopy. Common features of endothelial derangement we observed included swelling and vigorous proliferation, hyperplasia of smooth muscle cells, and aggregations of ribosome and lymphocytes in smooth muscle cells [27]. The appearance of aggregations of ribosome in the smooth muscle cells may reflect increased protein synthesis activity, possibly mediating the expression of inducible nitric oxide synthase [23].

Biochemical Changes in CSF

In contrast to the elevated concentrations of most amino acids, including L-citrulline, observed in CSF after LPS injection, the concentration of L-arginine significantly decreased. This finding suggests that a large quantity of L-arginine was consumed as a substrate for nitric oxide production by nitric oxide synthase [33]. In addition to LPS, cytokines including TNF-α, IL-β, and IFN-γ have been proven to induce the expression of inducible nitric oxide synthase [11, 13, 14, 30]. In fact, concentrations of these cytokines were elevated after general administration of high-dose LPS [8]. Moreover, combinations of several cytokines, including TNF-α and IFN-γ, cause synergistic induction of nitric oxide synthase [3]. TNF-α, conversely, appears to downregulate an endothelial nitric oxide synthase mRNA by shortening its half-life [46]. In our experiments, only TNF-α transiently increased after exposure to LPS, while the other cytokines did not change. This observation suggests that TNF-α is the most important inducer of inducible nitric oxide synthase in the smooth muscle cells after a low-dose LPS administration, and it would also be expected to repress constitutive endothelial nitric oxide synthase activity. These results support preventive pretreatment of endotoxic shock with anti-TNF-α antibody [41].

LPS Effect on Spastic Basilar Arteries

As the concentration of cytokine, IL-6, in CSF has been reported to increase after SAH [17], it seemed possible that inducible nitric oxide synthase might be expressed after SAH. Our in vitro results, where high doses of L-arginine did not produce relaxation of endothelium-denuded basilar arteries from SAH model dogs, suggest that inducible nitric oxide does not appear in vascular smooth muscle after SAH. In contrast, L-arginine caused an obvious relaxation in basilar arteries in control and SAH model dogs exposed to LPS.

LPS had two obviously different effects depending on the severity of vasospasm, causing marked reversal of mild vasospasm to control arterial diameter but little vasodilation in severe vasospasm.We hypothesize that during vasospasm, LPS was able to induce inducible nitric oxide synthase in smooth muscle cells, causing vasodilation by generating more nitric oxide and activating a cyclic GMP-dependent mechanism. This hypothesis is supported by the further vasodilation caused by SOD, which increases the half-life of nitric oxide by inactivating superoxide anions [12]. The absence of a LPS effect on basilar arteries in vasospasm has no clear mechanism, but may result from impairment of some elements of the inducible nitric oxide-related vasorelaxing system, such as new protein synthesis, or from a decreased response of smooth muscle cells to nitric oxide due to the func-

tional correlates of the morphological alterations we observed in vascular tissue.

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Comment

The present approach of intrathecal injection of endotoxin (lipopolysaccharide) to influence constriction of brain arteries after subarachnoid haemorrhage is certainly intriguing and might have clinical perspectives. The underlying rationale was to enhance inducible nitric oxide synthase, thereby production of the vasodilatory mediator NO. Under control conditions without cerebral haemorrhage, injection of LPS (0.5 mg) led to persistent vasodilation of the basilar artery in dogs as studied by angiography. Vasodilation, however, developed gradually over hours reaching a plateau at 6 h post injection. Involvement of NO was ascertained by administration of an NO-synthase inhibitor. Further, co-administration of vasopressin was found to somewhat inhibit dilation from LPS, whereas bradykinin to enhance the dilatory response. Release of NO by LPS was further supported by observations that the NO-precursor L-arginine was decreased in CSF after LPS. As expected, LPS led to release of TNF-α in CSF, whereas other cytokines, such as IL-1β, IL-6, or interferon-γ were not affected. The authors conclude, therefore, that LPS causes release of NO by induction of NO-synthase via TNF-α. Clinically relevant experiments were made, whether LPS was antagonizing vasoconstriction in animals with SAH. Indeed, a subgroup with mild vasospasm of the A. basilaris responded with marked dilation, whereas in severe vasospasm the reaction was less impressive. The effect of LPS could be enhanced by SOD through prolongation of the NO-half life.

Taken together, the present experiments appear to open up a novel pathophysiological scenario on blood flow regulation by cytokine-controlled NO-release. Besides, antagonization of vasospasm of SAH by LPS, albeit effective only in its milder forms might have clinical implications. The current study is based on extensive experimentation in dogs including angiography, measurements of amino acids and of cytokines in CSF, histology, and electronmicroscopy, and last but not least in vitro assessments of the vasomotor response of isolated cerebral blood vessels, which were exposed in vivo to SAH.

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