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Role of nitric oxide and prostaglandins in lipopolysaccharide-induced increase in vascular permeability in mouse skin

Emiko Fujii *, Kaoru Irie, Akira Ogawa, Ken-ichi Ohba, Takamura Muraki

Department of Pharmacology, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162, Japan

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Abstract

To examine the possible role of increased vascular permeability in the circulatory shock induced by endotoxin (lipopolysaccharide), we examined whether lipopolysaccharide elicits plasma extravasation in the skin of ddY strain mice. We also studied whether nitric oxide (NO) and prostaglandins may mediate the lipopolysaccharide-induced increase in vascular permeability. Subcutaneous injection of lipopolysaccharide (100–400 μg/site) induced a dose-related and delayed increase in vascular permeability at the injection site as determined by the leakage of pontamine sky blue. Concurrent administration of aminoguanidine (a putative inducible NO synthase inhibitor) (10 mg/kg, i.v.) inhibited the lipopolysaccharide (400 μg/site)-induced dye leakage by 71%. N^G-Nitro-L-arginine methyl ester (an inhibitor for both constitutive and inducible NO synthase) (10 and 20 mg/kg, i.v.) inhibited the lipopolysaccharide-induced dye leakage by 36% and 54%, respectively, whereas the inactive enantiomer, N^G-nitro-D-arginine methyl ester (10 mg/kg, i.v.), had no effect. Pretreatment with an intraperitoneal injection of dexamethasone (500 μg/kg) or indomethacin (a cyclooxygenase-1 and -2 inhibitor) (5 mg/kg) almost completely inhibited the response induced by lipopolysaccharide, by 96% and 84%, respectively. [*N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulphonamide (a cyclooxygenase-2-specific inhibitor) (0.01–1 mg/kg, i.p.) also induced a dose-related inhibition of dye leakage elicited by lipopolysaccharide: 38% and 80% suppression at the doses of 0.1 and 1 mg/kg, respectively. Cycloheximide (a protein biosynthesis inhibitor) (35 mg/kg, s.c.) suppressed the effect of lipopolysaccharide by 74%. These results suggest that the increase in vascular permeability induced by lipopolysaccharide is mediated by both NO and prostaglandins and that synthesis of inducible NO synthase and cyclooxygenase-2 may be involved in this effect of lipopolysaccharide.

Keywords: Vascular permeability; Lipopolysaccharide; Nitric oxide (NO); Prostaglandin; Skin; (Mouse)

1. Introduction

Endotoxin or lipopolysaccharide is a major component of the cell wall of gram-negative bacteria which increases vascular permeability (for review, Cybulsky et al., 1988). Administration of lipopolysaccharide in vivo induces circulatory shock, characterized by hypotension, vascular injury and disseminated intravascular coagulation, which leads to a fatal dysfunction of various organs such as lungs, liver, kidneys and gastrointestinal tract (Hewett and Roth, 1993). Lesions in the rabbit skin following intradermal injection of *Escherichia coli* are characterized by hyperemia, increased vascular permeability, hemorrhage and infiltration of large numbers of polymorphonuclear leukocytes (Kopaniak et al., 1980; Issekutz and Bhimji, 1982); however, the mediators implicated in this process are not completely understood.

Nitric oxide (NO) is synthesized from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine by the enzyme, NO synthase (Palmer et al., 1988). At least two types of NO synthase have been identified (Moncada et al., 1991); one is constitutive, Ca^{2+}/calmodulin-dependent and releases NO for short periods in response to receptor stimulation. Another enzyme, which has been found in activated macrophages, neutrophils and endothelium (Marletta et al., 1988; Curran et al., 1989), is inducible, Ca^{2+}-independent and synthesizes NO for long periods. Endotoxin induces a Ca^{2+}-independent NO synthase in vascular endothelium (Radomski et al., 1990). Postcapillary venules are well known as the site of action of a large number of inflammatory and immune mediators, most of which have been described to induce NO produc-

* Corresponding author. Tel.: 03-3353-8111 ext. 22513; fax: 03-5269-7417.

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tion by vascular endothelial cells (Kilbourn and Belloni, 1990). Previously, Ialenti et al. (1992) showed that endogenous NO, released at the site of acute inflammation, may play a role in the carrageenin-induced increase in vascular permeability in rat skin and in dextran- and carrageenin-induced paw oedema.

On the other hand, cyclooxygenase products play an important role in the increase in vascular permeability induced by platelet-activating factor (PAF) in mouse skin (Fujii et al., 1995). Enhanced arachidonic acid metabolism generally accompanies inflammation. Prostaglandins of the E-type mediate arteriolar vasodilation and potentiate the exudation elicited by bradykinin and histamine, but have no potency to induce plasma leakage by themselves (Williams and Peck, 1977). Cyclooxygenase is the first enzyme in the pathway in which arachidonic acid is converted to prostaglandins (Vane and Botting, 1990). This enzyme, like NO synthase, exists in at least two isoforms. The constitutive isoform of cyclooxygenase (cyclooxygenase-1) is present in many types of cells, while the inducible isoform of cyclooxygenase (cyclooxygenase-2) is expressed after stimulation of cells with a variety of agents such as growth factors, interleukin-1β and endotoxin, and expression of the latter is attenuated by antiinflammatory steroids (Masferrer et al., 1992).

Pontamine sky blue as well as Evans blue, given intravenously (i.v.), bind to plasma proteins, particularly to albumin, and are used as markers of plasma extravasation in the study of vascular permeability and oedema formation (Udaka et al., 1970; Moreno et al., 1992). Recently, using the pontamine sky blue leakage technique, we found that 5-hydroxytryptamine (5-HT) and PAF increase vascular permeability in mouse skin and that endogenous NO is involved in the effect of 5-HT (Fujii et al., 1994) but not in the effect of PAF (Fujii et al., 1995).

Reportedly a number of proteins important for modulating platelet and vascular reactivity, including NO synthase and cyclooxygenase, are synthesized in endothelial cells in addition to macrophages by lipopolysaccharide (Casals-Stenzel, 1987; Salvemini et al., 1990; Moncada et al., 1991). Therefore, in the present study, we examined the possible role of NO and prostaglandins in the local effect of lipopolysaccharide on vascular permeability. To examine the potential induction of such proteins by lipopolysaccharide, we investigated the effect of cycloheximide, a potent inhibitor of protein synthesis, on lipopolysaccharide-induced plasma leakage.

2. Materials and methods

Male ddY strain mice (Sankyo Laboratory Service, Tokyo, Japan), weighing about 35 g were used. They were housed in an air-conditioned room (temperature 22 ± 2°C, humidity 55 ± 5%) with a controlled light-dark cycle (light on 06:00-20:00 h) and freely available food and water.

2.1. Assessment of vascular permeability induced by lipopolysaccharide

Vascular permeability was quantified by the extravasation of pontamine sky blue (Udaka et al., 1970). Five minutes after i.v. injection of pontamine sky blue (50 mg/kg), lipopolysaccharide (100–400 μg/site) or saline (0.1 ml/site) was administered subcutaneously (s.c.) into the back. One site of lipopolysaccharide injection was studied per animal unless otherwise stated. Two hours later, the mice were killed by cervical dislocation and the stained area of the back skin was cut out. The dye accumulated in the skin was extracted with a acetone-0.5% Na_2SO_4 mixture (14:6 v/v) and the concentration was determined colorimetrically at 590 nm.

2.2. Time course of lipopolysaccharide-induced vascular permeability

Five minutes after i.v. injection of pontamine sky blue, lipopolysaccharide and saline were injected into the right or the left side of the back (two sites per animal), and the dye accumulated in the skin was then determined at 5, 60, 120 and 180 min.

2.3. Effects of inhibitors of NO synthase, dexamethasone, indomethacin, [N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulphonamide and cycloheximide on stimulation of vascular permeability by lipopolysaccharide

Saline, aminoguanidine (10 mg/kg), N^G-nitro-L-arginine methyl ester (10 and 20 mg/kg) or N^G-nitro-D-arginine methyl ester (10 mg/kg) were administered i.v. immediately before pontamine sky blue followed by s.c. lipopolysaccharide or PAF 5 min later. Dexamethasone (500 μg/kg) was administered intraperitoneally (i.p.) 3 h before the i.v. injection of pontamine sky blue. Indomethacin (5 mg/kg) or [*N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulphonamide (0.01–1 mg/kg) was administered i.p. 35 min before, cycloheximide 65 min before the s.c. injection of lipopolysaccharide (400 μg/site, s.c.). The doses of these agents were chosen on the basis of previous studies (Rees et al., 1990a; Buchanan and Phillis, 1993; Fujii et al., 1994; Shukovski and Tsafriri, 1994; Utsunomiya et al., 1994; Gierse et al., 1995).

2.4. Drugs

The following drugs were used: lipopolysaccharide (from *Salmonella typhimurium*, code number L6511), aminoguanidine hemisulphate, N^G-nitro-L-arginine methyl ester HCl, indomethacin and cycloheximide (Sigma Chemical, MO, USA); 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (C16-PAF) and N^G-nitro-D-arginine methyl ester HCl (Nova Biochem, Switzerland); dexamethasone (Decadron, Banyu Pharm, Tokyo, Japan); [*N*-

(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide (kindly provided by Taisho Pharmaceutical Co., Saitama, Japan); pontamine sky blue 6B (Tokyo Kasei Kogyo, Tokyo, Japan). Lipopolysaccharide was dissolved in phosphate-buffered saline solution (pH 7). Indomethacin and [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide were dissolved in a small volume of absolute ethanol, then 50% propylene glycol was added to make 1 mg/ml stock solution. Just prior to use, the stock solution was diluted to the appropriate concentration with physiological saline. Saline containing 0.01% ethanol and 5% propylene glycol was used as vehicle solution. Other drugs were dissolved in physiological saline. All doses refer to the salt forms of the drugs.

2.5. Statistical analysis

The results are expressed as means ± S.E.M. Comparisons among multiple groups were evaluated non-parametrically by the Kruskal-Wallis method followed by the Wilcoxon rank sum test. For the time course study, Student's *t*-test was used.

3. Results

3.1. Effect of lipopolysaccharide on vascular permeability

Following lipopolysaccharide (400 μg/site) administration, dye leakage began to increase 1 h later to 230% of the saline control, then reached a maximum increase of 650% after 2 h without further increase at 3 h (Fig. 1). Lipopolysaccharide (100–400 μg/site) produced dose-related increases in dye leakage with a 718% increase above the saline control at the dose of 400 μg/site of lipopolysaccharide injection when determined on a μg dye/area basis (Fig. 2A). For convenient comparison to results of previous studies, we replotted the dose-response effect of lipopolysaccharide on the basis of μg dye/g wet weight skin, and obtained almost the same linear relationship,

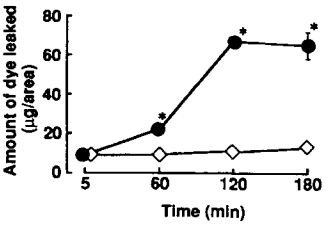

Fig. 1. Time course of the effect of lipopolysaccharide on the dye leakage in mouse skin. Five minutes after i.v. injection of pontamine sky blue (50 mg/kg), lipopolysaccharide (400 μg/site, ●) or saline (0.1 ml/site, ◇) was injected in the back skin of the mice. At the indicated times after lipopolysaccharide or saline (controls) injection, dye accumulated in the skin was determined colorimetrically. Values represent the means ± S.E.M. of five experiments. Some S.E.M. bars are inside the symbols. * $P < 0.01$ vs. saline.

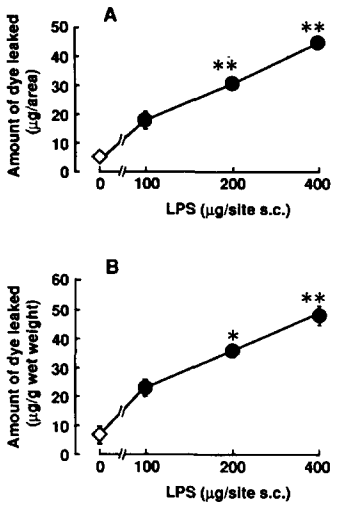

Fig. 2. Effects of increasing doses of lipopolysaccharide (LPS) on dye leakage in mouse skin. Dye leakage induced by LPS (●) and saline (◇) was assessed after 2 h. The amount of leaked dye was expressed as μg dye/area (A) and μg dye/g wet weight (B). Values represent the means ± S.E.M. of five experiments. Some S.E.M. bars are inside the symbols. * $P < 0.05$, ** $P < 0.01$ vs. saline.

with a 665% increase at a 400 μg/site dose of lipopolysaccharide (Fig. 2B). Therefore we evaluated the dye leakage induced by 400 μg/site lipopolysaccharide at 2 h and expressed dye leakage as μg/area in subsequent studies. Although we used lipopolysaccharide from *Salmonella typhimurium* in this study, essentially the same results were obtained with lipopolysaccharide from *Escherichia coli* at doses of 100–400 μg/site (data not shown).

3.2. Effects of inhibitors of NO synthase on stimulation of vascular permeability by lipopolysaccharide or PAF

To examine the role of NO, we investigated the effects of inhibitors of NO synthase on the lipopolysaccharide-induced increase in vascular permeability. Aminoguanidine (10 mg/kg), N^G-nitro-L-arginine methyl ester (10 mg/kg) and N^G-nitro-D-arginine methyl ester (10 mg/kg) themselves showed no effect on the basal dye leakage elicited by topical injection of saline. Aminoguanidine (10 mg/kg), an inducible NO synthase inhibitor, significantly inhibited the lipopolysaccharide-induced dye leakage by 71%. N^G-Nitro-L-arginine methyl ester (10 and 20 mg/kg), a non-specific NO synthase inhibitor, suppressed the response less effectively, by 36% and 54%, respectively; there was no inhibition of the effect of lipopolysaccharide by an inactive enantiomer, N^G-nitro-D-arginine methyl ester (10 mg/kg) (Fig. 3). In contrast to that with lipopolysaccharide, the PAF (0.1 μg/site)-induced increase in dye leakage was not affected by concurrent administration of the NO synthase inhibitors, aminoguanidine (10 mg/kg) or N^G-nitro-L-arginine methyl ester (10 mg/kg) (Fig. 3), confirming our previous result (Fujii et al., 1995).

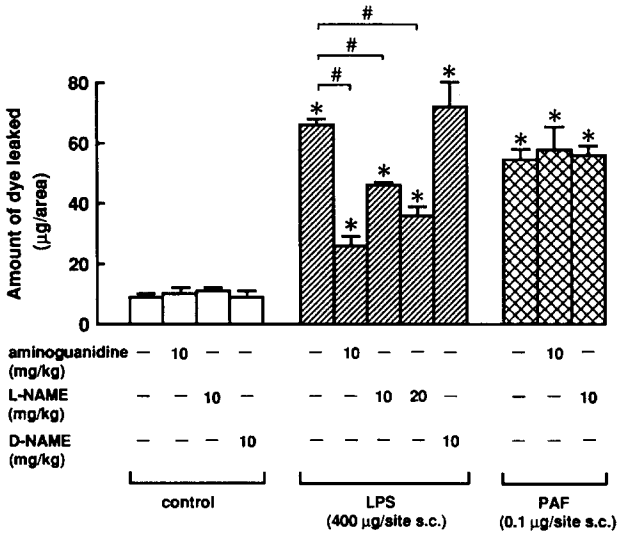

Fig. 3. Effects of aminoguanidine, N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-D-arginine methyl ester (D-NAME) on lipopolysaccharide (LPS)-induced dye leakage in mouse skin. Saline, aminoguanidine, L-NAME or D-NAME was administered i.v. immediately before pontamine sky blue followed by LPS (400 μg/site, s.c.) or PAF (0.1 μg/site, s.c.) 5 min later. The amount of leaked dye at the injection site was determined 1 h (PAF) or 2 h (LPS) later. Open columns indicate saline-treated mice, hatched columns, LPS-treated mice and cross-hatched columns, PAF-treated mice. Values represent the means ± S.E.M. of five experiments. * $P < 0.01$ vs. saline alone, # $P < 0.01$ vs. LPS alone.

3.3. Role of prostaglandins in stimulation of vascular permeability by lipopolysaccharide

To examine the role of prostaglandins we investigated the effect of dexamethasone, indomethacin and [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide on the lipopolysaccharide-induced increase in vascular permeability. Dexamethasone (500 μg/kg) but not indomethacin (5 mg/kg) inhibited the basal dye leakage elicited by topical injection of saline. Both dexamethasone (500 μg/kg) and indomethacin (5 mg/kg) almost completely inhibited the lipopolysaccharide-induced increase in dye leakage, by 96% and 84%, respectively (Fig. 4). [*N*-(2-Cyclohexyloxy-4-nitrophenyl) methanesulphonamide (0.01–1 mg/kg), a cyclooxygenase-2-selective inhibitor, dose relatedly inhibited the lipopolysaccharide-induced dye leakage in mouse

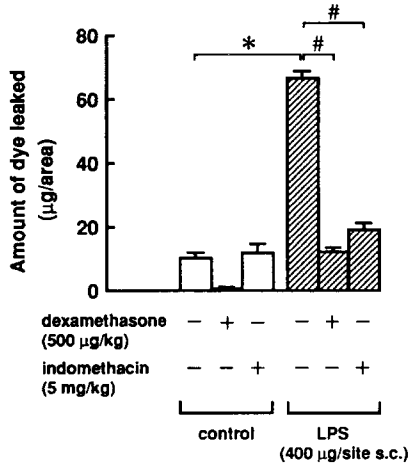

Fig. 4. Effects of dexamethasone and indomethacin on lipopolysaccharide (LPS)-induced dye leakage in mouse skin. Dexamethasone was administered i.p. 3 h before i.v. injection of pontamine sky blue. Indomethacin was administered i.p. 35 min before the s.c. injection of LPS (400 μg/site). Dye leakage induced by LPS was assessed 2 h after LPS administration. Open columns indicate mice without LPS, hatched columns show mice given with LPS. Values represent the means ± S.E.M. of five experiments. * $P < 0.001$, # $P < 0.001$.

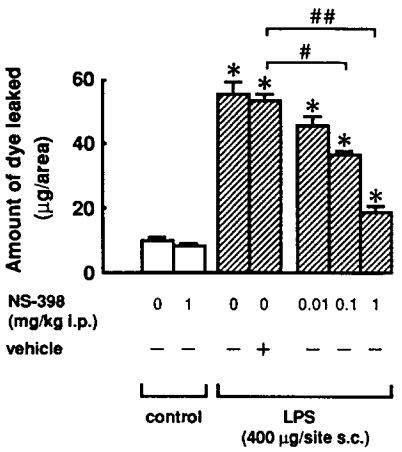

Fig. 5. Effects of increasing doses of [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide (NS-398) on lipopolysaccharide (LPS)-induced dye leakage in mouse skin. Saline, vehicle (0.01% ethanol, 5% propylene glycol in saline) or NS-398 was administered i.p. 35 min before the s.c. injection of LPS (400 μg/site) or saline (control), and dye leakage was assessed 2 h later. Open columns indicate mice without LPS, hatched columns show mice treated with LPS. Values represent the means ± S.E.M. of five experiments. * $P < 0.05$ vs. saline alone. # $P < 0.01$, ## $P < 0.001$ vs. vehicle and LPS.

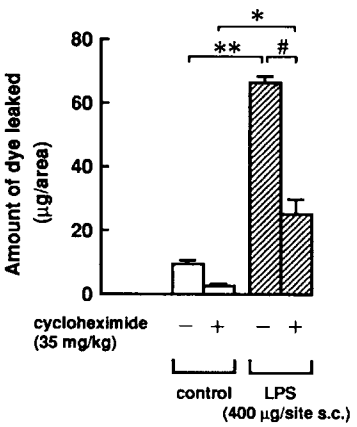

Fig. 6. Effects of cycloheximide on lipopolysaccharide (LPS)-induced dye leakage in mouse skin. Mice were treated with cycloheximide i.p. 65 min before LPS (400 μg/site, s.c.) or saline. Dye leakage induced by LPS was assessed 2 h after LPS administration. Open columns indicate mice without LPS, hatched columns show mice given with LPS. Values represent the means ± S.E.M. of five experiments. * $P < 0.05$, ** $P < 0.01$, # $P < 0.01$.

skin, reaching a maximum suppression of 80% at the dose of 1 mg/kg, whereas vehicle alone had no effect (Fig. 5). These results suggest that prostaglandins are also involved in the vascular effect of lipopolysaccharide.

3.4. Effect of cycloheximide on stimulation of vascular permeability by lipopolysaccharide

Cycloheximide (35 mg/kg) significantly suppressed the lipopolysaccharide-induced dye leakage, by 74% (Fig. 6), which indicates that induction of protein synthesis plays an important role in the effect of lipopolysaccharide.

4. Discussion

We found that s.c. injection of lipopolysaccharide increased dye leakage in mouse skin in a dose-dependent manner and this effect was delayed in onset with a peak at 2 h. This confirms the previous result with rabbit skin that intradermal injection of killed *Escherichia coli* increases the vascular permeability measured with ^{125}I-albumin accumulation and hyperemia, with a peak at 2–3 h (Kopaniak et al., 1980). Our findings that NO synthase inhibitors such as aminoguanidine and N^G-nitro-L-arginine methyl ester inhibited the lipopolysaccharide-induced increase in dye leakage, while an inactive enantiomer, N^G-nitro-D-arginine methyl ester, had no inhibitory activity, support the involvement of NO in the effect of lipopolysaccharide in mouse skin. However, due to unknown causes, the inhibitory potency of N^G-nitro-L-arginine methyl ester was weaker than that of aminoguanidine. L-Arginine analogues, N^G-monomethyl-L-arginine and N^G-nitro-L-arginine methyl ester, inhibit both the endothelial constitutive and inducible NO synthases (Rees et al., 1990b; Moncada et al., 1991), while aminoguanidine is proposed as a selective inhibitor of the inducible NO synthase (Corbett et al., 1992; Tilton et al., 1993). Therefore, effective inhibition by aminoguanidine may indicate that NO produced by inducible NO synthase plays an important role in the dye leakage induced at least partly by lipopolysaccharide. As shown for PAF, however, NO is not always involved in the vascular extravasation induced by every inflammatory mediator.

We also showed that the lipopolysaccharide-induced increase in vascular permeability was significantly inhibited by cyclooxygenase inhibitors such as indomethacin and [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide, indicating that vasodilating cyclooxygenase products play an important role in the lipopolysaccharide-induced increase in vascular permeability in mouse skin. Because [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide is a selective inhibitor of cyclooxygenase-2 (Gierse et al., 1995), the inhibition by [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide suggests that induction of cyclooxygenase-2 also plays a causal role in the dye leakage induced by lipopolysaccharide in mouse skin. Thus, we extended the work of Warren et al. (1992) on rat cutaneous vasodilation and showed that both NO and prostaglandins play a role in the lipopolysaccharide-induced increase in vascular permeability. Mice may be less sensitive to lipopolysaccharide than rats because 10 times more lipopolysaccharide was necessary to induce dye leakage in our study with mice.

Lipopolysaccharide causes the co-induction of an inducible NO synthase and cyclooxygenase-2 in murine macrophages (Mitchell et al., 1993). Protein synthesis inhibitors prevent the increase of inducible NO synthase activity elicited by either lipopolysaccharide or cytokines (Geller et al., 1993; Koide et al., 1993) and the increase in cyclooxygenase-2 protein in murine macrophages and bovine aortic endothelial cells (Akarasereenont et al., 1995). Dexamethasone is known to inhibit the expression of lipopolysaccharide-induced inducible NO synthase mRNA in several tissues of rats (Liu et al., 1993), and to inhibit cyclooxygenase-2 in murine peritoneal macrophages (Masferrer et al., 1994). In addition to the results obtained with inhibitors of inducible NO synthase and cyclooxygenase-2, the inhibition by cycloheximide and dexamethasone of the lipopolysaccharide-induced increase in vascular permeability supports the idea that newly synthesized inducible NO synthase and cyclooxygenase-2 are required for the lipopolysaccharide-induced increase in dye leakage. However, since many mechanisms such as inhibition of phospholipase A_2 have been proposed to explain the inhibitory effect of dexamethasone on inflammation (Moreno et al., 1992), the experiment with dexamethasone may not constitute conclusive evidence for the sole involvement of inducible NO synthase and cyclooxygenase-2.

The start of dye leakage elicited by lipopolysaccharide was found at 1 h and was slower than that induced by 5-HT or PAF, which increased dye leakage significantly at 5 min (Fujii et al., 1994, 1995). A possible explanation of the delayed start could be that synthesis of new proteins or enzymes such as inducible NO synthase and cyclooxygenase-2 is necessary for the acute effect of lipopolysaccharide.

Endothelial cells synthesize and release a number of proteins important for modulating platelet and vascular reactivity (Frasier-Scott et al., 1988), and a multitude of inflammatory mediators such as arachidonic acid metabolites, cytokines, PAF, NO and endothelins are released by lipopolysaccharide (Hewett and Roth, 1993). In future studies, we need to examine the role of inflammatory mediators other than NO and prostaglandins in lipopolysaccharide-induced dye leakage.

Finally, we had assumed that topical administration of lipopolysaccharide acts mainly on the vascular endothelial cells to increase vascular permeability; however, the design of the present study did not allow us to specify the site of lipopolysaccharide action. Further work is needed to examine the cellular effect of lipopolysaccharide.

In conclusion, it is suggested that the increase in vascular permeability induced by s.c. injection of lipopolysaccharide is mediated by both NO and prostaglandins and that induction of inducible NO synthase and cyclooxygenase-2 may be involved in the effect of lipopolysaccharide.

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