The Mediators Involved in Endotoxin-Induced Vascular Permeability Increase in the Rat Skin and Their Interactions

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ABSTRACT—The injection of lipopolysaccharide (LPS) from E. Coli into the dorsal skin of rats caused a dose-dependent increase in vascular permeability as measured by the extravasation over a 40-min period of intravenously injected dye. This increase caused by LPS was attenuated by pretreatment with the bradykinin (BK) receptor antagonist HOE140, the selective platelet-activating factor (PAF) antagonist TCV309, and by combined treatment with mepyramine and methysergide. Combined treatment with HOE140 and TCV309 resulted in further suppression than that achieved with a single treatment alone. By the simultaneous pretreatment with all antagonists, the response was almost totally abolished. On the other hand, indomethacin also inhibited the response induced by LPS, but not those induced by BK and PAF itself. A small dose of BK or histamine synergistically potentiated the effect of PAF when simultaneously injected. These results suggest that BK, PAF, histamine/serotonin and prostaglandins are involved in the LPS-induced increase in vascular permeability, where PAF, in addition to its direct action, potentiates the response to BK and histamine, and prostaglandins potentiate the actions of other mediators without its direct action.

Keywords: Bradykinin, Platelet-activating factor, Prostaglandin, Endotoxin, Vascular permeability

It is well-known that endotoxin (lipopolysaccharides, LPS) can induce the production or release of many vasoactive mediators (1), including platelet-activating factor (PAF) (2), prostaglandins (PG) (3), thromboxanes (4) and bradykinin (BK) (5) as well as cytokines, in particular, of interleukins and tumor necrosis factor-α (6). We reported that BK and PAF were involved in the hypotensive response of rats induced by intravenously injected LPS and we found that BK had the potentiative effect on the hypotension induced by PAF (7).

These mediators, especially those like BK and PAF, can potently increase vascular permeability (8, 9). It is difficult to measure the increase of vascular permeability when LPS is intravenously injected. On the other hand, it is easy to estimate the vascular permeability on the skin of animals (8–11). We reported that BK was involved in the increase of vascular permeability induced by LPS by comparing the response in kininogen-deficient rats with that in normal rats (10). However, it has not been clarified which of these mediators is involved in the vascular permeability increase induced by LPS.

In this study, we examined the effects of the inhibitor and several antagonists on the increase in vascular permeability caused by LPS and the interaction of the mediators. Then, from these results, the roles of these mediators involved in the LPS-induced vascular permeability increase were also clarified.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) strain specific pathogen free (SPF) rats (300–350 g; Japan SLC Co., Ltd., Hamamatsu) were used.

Agents

Pentobarbital sodium (Nembutal) was purchased from Abbott Lab. (North Chicago, IL, USA). Pontamine sky blue (Brilliant Blue 6B; Tokyo Kasei Co., Tokyo) was dissolved in 0.43% saline (50 mg/ml) and then filtered through a 0.45-micrometer membrane.

BK (Peptide Inst., Osaka) was taken from a frozen stock (10⁻⁴ M) and diluted in filtered Tyrode's just before use. PAF (Funakoshi Co., Tokyo) was dissolved in filtered Tyrode's containing 0.25% bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO, USA). Histamine hydrochloride (Wako Pure Chemicals Ind., Osaka) was also dissolved in the filtered Tyrode's. The
doses of the above agents were used as previously reported (8–11).

Endotoxic LPS (from *E. Coli* 0111:B4, lyophilized powder prepared using phenol extraction procedure; Sigma Chemical Co.) was dissolved in pyrogen-free physiological saline (Otsuka Pharmaceut. Co., Tokushima). Detoxified LPS (from *E. Coli* 0111:B4; Sigma Chemical Co.) was also used in order to test the specificity of endotoxic LPS.

HOE140 (1 mg/ml), a potent BK receptor antagonist (D-Arg-[Hyp3,Thi5,D-Tic7,Oic9]-BK, a gift from Hoechst AG, Frankfurt, Germany) (12); TCV309 (0.1 mg/ml), a novel selective PAF antagonist (3-bromo-5-[N-phenyl-N-[2-[(1, 2, 3, 4-tetrahydro-2-isoquinolyl-carbonyloxy)ethyl]carbamoyl]ethyl] carbamoyl]-1-propyl-pyridinium nitrate, a gift from Takeda Chemical Ind., Osaka) (2); mepyramine (5 mg/ml; Pfaltz Bauer, Inc., Flushing, NY, USA); and methysergide (2.5 mg/ml; a gift from Sandoz, Basel, Switzerland) were freshly prepared in pyrogen-free physiological saline for each experiment and injected intravenously 30 min before the intradermal injection of agents.

Indomethacin (Sigma Chemical Co.) was suspended in physiological saline containing 1% carboxymethylcellulose and administered intraperitoneally at a dose of 10 mg/kg 30 min before the intradermal injection of agents.

**Experiment on vascular permeability increase induced by agents**

The experiments on vascular permeability were performed by the previously reported method (9, 11). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Five minutes after intravenous injection of pontamine sky blue (50 mg/kg), 0.1 ml of various concentrations of several agents in the filtered Tyrode's solution was injected intradermally into 8–10 sites of the shaved dorsal skin of a rat. The agents were allocated to sites on the skin according to the Latin Square design, so as to avoid bias resulting from site and animal variation. The doses and kinds of the agents were different in all sites of a rat: The number of experiments equaled the number of rats used in this study. The rats were sacrificed by exsanguination 40 min after the intradermal injection of the agents. The doses of agents are expressed as moles or mg per site.

**Measurement of the exuded dye in the skin and serum of rats**

The exuded dye in the skin at each site was extracted by the method of Katayama et al. (13). In brief, the skin of each site was incubated in 1 ml of 1 N KOH at 37°C overnight, and then 2.5 ml of 0.6 N phosphoric acid was added to neutralize the base, and the exuded dye was extracted by addition of 7.5 ml of acetone (special grade, Wako Pure Chemicals Ind.). After centrifugation (1,200 × g for 20 min), the optical density of the each supernatant at 620 nm was measured by a spectrophotometer (UVIDEC-505; Japan Spectroscopic Co., Tokyo). The concentration of dye in the serum of the same rat was also determined. The amount of exuded dye at each site is expressed as serum equivalents (µl serum eq.) in the same rat for uniformity.

**Statistical analyses**

All data are expressed as means ± standard errors of the mean. The effects of various antagonists were analyzed for statistical significance by Student's *t*-test by the use of two-way analysis of variance. A *P* value of less than 0.05 was considered to be significant.

**RESULTS**

**Vascular permeability increase by endotoxic and detoxified LPS**

LPS (0.1, 0.3, 1 mg) caused a strong increase in vascular permeability in a dose-dependent manner (Fig. 1). On the other hand, detoxified LPS did not cause such a strong increase. Only the 1-mg dose of intradermally injected detoxified LPS caused a significant increase of vascular permeability (25 ± 3 µl). However, at any dose of LPS used, the increase of vascular permeability induced by endotoxic LPS was significantly (*P* < 0.05) larger than that induced by detoxified LPS. The difference of the increase of vascular permeability induced by endotoxic LPS

![Fig. 1. Dose-response of endotoxic and detoxified lipopolysaccharide (LPS) in the vascular permeability increase in the skin of rats. Endotoxic LPS (open column) or detoxified LPS (hatched column) was injected intradermally into the dorsal skin of rats. The abscissa indicates the dose of each agent in 0.1 ml of Tyrode's per site of injection. The ordinate shows the exudation of dye expressed as µl serum equivalent of the rat. Each point indicates the mean with S.E.M. from 5 rats. * indicates a significant difference at *P* < 0.05.](image-url)
from that induced by detoxified LPS was also dose-dependent. Thus, this result showed that the LPS-dose-dependent increase of vascular permeability was specific for the toxicity of LPS.

The time course of vascular permeability increase induced by BK, LPS and PAF

As shown in Table 1, BK (10 nmol), PAF (1 nmol) and histamine (500 nmol) caused the increases of vascular permeability of rats, as previously reported (8, 9, 11).

As shown in Fig. 2, the responses elicited by BK (10 nmol) and histamine (500 nmol) terminated at the end of the first 10 min after the injection, but that by PAF (1 nmol) continued for 30 min. On the other hand, the response to LPS (1 mg) seemed to be intermediate between those responses to BK and PAF. As the responses to all agents ended within 40 min after the injection and because none of the agents caused any subsequent increase in permeability between 40 min and 3 hr after the intradermal injection (15±3 µl eq. for BK, 17±2 µl eq. for PAF, 8±2 µl eq. for LPS and 10±4 µl eq. for histamine), the evaluation of the increased permeability due to an agent in this study was made at 40 min after the injection.

Inhibitory effects of several antagonists on the increased vascular permeability

The results of the effects of several antagonists on the increased permeability are indicated in Table 1.
The exudation induced by BK (10 nmol) was significantly inhibited by pretreatment with HOE140 (1 mg/kg), a BK B2-receptor antagonist, but was not changed by the pretreatment with TCV309 (0.1 mg/kg), a PAF antagonist, nor with the combination of mepyramine (5 mg/kg) and methysergide (2.5 mg/kg), a histamine antagonist and a serotonin antagonist, respectively. The response to PAF (1 nmol) was significantly lessened only by the pretreatment of TCV309, but not by pretreatment with HOE140 or concomitant treatment of mepyramine with methysergide. As for the response to histamine (500 nmol), mepyramine and methysergide decreased it, whereas pretreatment with the other antagonists did not have any effect. These results confirm the specificity of each antagonist.

Although the response to LPS was significantly inhibited by pretreatment with each individual antagonist, its inhibition was not complete. Furthermore, the combined treatment with HOE140 and TCV309 caused further suppression of the increase in permeability in response to LPS than that by each antagonist alone. When all antagonists were pretreated, the response elicited by LPS was further suppressed to a value significantly less than that obtained by the combination of both HOE140 and TCV309. The value obtained from this experiment was almost the same as that obtained by 1 mg of detoxified LPS alone.

In the vascular response to a lower dose of LPS (0.3 mg), the same pattern of inhibitory effects of these antagonists was obtained (Table 1).

**Effect of agent-interaction on the increase in vascular permeability**

Figure 3 shows the responses to mixtures of two agents compared with those to each agent alone. When a small dose of BK (1 nmol), which caused a small rise in the vascular permeability by itself, was simultaneously injected with PAF (0.1 nmol), the increase in vascular permeability elicited by PAF alone was strongly potentiated (P<0.01). Similar potentiation by histamine (5 nmol) (P<0.01) was found when histamine was administered with PAF. However, the effect of simultaneous injection of BK and histamine was only additive. Furthermore, the combination of BK, PAF and histamine caused a larger response than those obtained by the simultaneous injections of two of these agents; i.e., BK and histamine, BK and PAF, or PAF and histamine (P<0.01).

**Fig. 3.** Effect of bradykinin (BK, 1 nmol, shown with a hatched column), histamine (HIST, 5 nmol, shown with a dark column) and platelet-activating factor (PAF, 0.1 nmol, shown with a black column), along with those of their combination, on the vascular permeability in rats. In the cases of PAF plus BK, PAF plus histamine and PAF plus histamine plus BK, the effect of BK or histamine by itself (the mean) is indicated along with that of PAF by itself in order to dramatize the synergistic potentiation of the response by simultaneous injection of the two agents. A significant difference between the sum of the values by each agent and the value for the combined treatment in each animal was found (**P<0.01). And the value for the simultaneous injection of three agents were significantly (##P<0.01) different from the values for the treatment of any two agents. Each column and bar indicate the mean and S.E.M. of the 6 rats.
Effect of indomethacin on the increase in vascular permeability

As shown in Fig. 4, by pretreatment with indomethacin (10 mg/kg, i.p.), the response induced by LPS was also significantly inhibited, but those responses induced by BK, PAF and histamine were not affected. Furthermore, the response of the simultaneous injection of BK (0.1 nmol), histamine (5 nmol) and PAF (0.1 nmol) was not inhibited by pretreatment of indomethacin; 108 ± 5 µl eq. for the control and 109 ± 9 µl eq. for pretreatment of indomethacin.

**DISCUSSION**

In this study, we showed that the intradermal injection of endotoxic LPS dose-dependently increased the vascular permeability in the dorsal skin of SD strain rats. This increase was specific for the toxicity of LPS, because detoxified LPS did not cause such a strong increase of vascular permeability. The increase of vascular permeability induced by endotoxic LPS manifested itself within 10 min after the intradermal injection of LPS and appeared to have subsided by 40 min after the LPS injection. From a comparison of the time course of the rate of dye exudation seen by BK and histamine with that given by PAF (Fig. 2), it is conceivable that both BK and histamine and also PAF might be involved in the response to LPS.

Several antagonists used in this study, such as HOE140, a potent BK B₂-receptor antagonist, and TCV309, a selective PAF antagonist, were proved to be specific. That is, the results obtained with them allowed the following conclusions: neither PAF nor histamine/serotonin was involved in the response to BK itself and that the increase in vascular permeability induced by PAF was not mediated by BK and histamine/serotonin. It is reported that intravenous injection of the antagonists at these doses are sufficient to antagonize the responses of BK and PAF, respectively (7).

Pretreatment of SD rats with mepyramine/methysergide, HOE140 or TCV309, respectively, caused a significant inhibition of the permeability increase induced by LPS. Thus, BK, PAF and histamine/serotonin might be involved in the response to LPS in the skin of the animal. The combination of HOE140 and TCV309 caused significantly greater inhibition than the individual drug did. By the combined use of mepyramine/methysergide, HOE140 and TCV309, the response to LPS was almost completely diminished, because its response was the same as that induced by detoxified LPS. Thus, these results suggest that PAF, BK and histamine were the main mediators involved in the increase in vascular permeability induced by endotoxic LPS.

As shown in Fig 4, BK or histamine/serotonin could synergistically potentiate the response to PAF, whereas BK and histamine had an additive effect together. Thus, it may be suggested that in the increase of vascular permeability induced by LPS, PAF, BK and histamine/serotonin have not only direct actions but also indirect actions such as potentiation. Furthermore, since the simultaneous injection of the above three agents further potentiated the response, the potentiating action of BK would be independent of the potentiating action of histamine.

Since pretreatment with indomethacin resulted in significant inhibition of the vascular permeability increase induced by LPS, it was suggested that prostaglandins (PG), such as PG I₂ or PG E₂, might be involved in it. Furthermore, we showed that prostaglandins were not involved in the vascular permeability increase elicited by BK, histamine, or PAF itself, respectively, because these increases were not affected by the treatment with indomethacin. Thus, it was suggested that LPS might directly induce the production of prostaglandins without mediation by BK or PAF produced by LPS. However, the simultaneous treatment of the three kinds of antagonists caused the almost complete inhibition, because the level of increase was reduced to a level similar to the increase induced by detoxified LPS. Thus, it could be suggested that prostaglandins produced by LPS-stimulation did not directly act on, but potentiated the response to PAF or BK, because it is reported that prostaglandins can potentiate the increase in vascular permeability induced by PAF or BK in the skin of rats (8, 9).
This study suggested that BK, PAF, histamine/serotonin and prostaglandins are involved in the increase of vascular permeability induced by LPS, where PAF, in addition to its direct action, potentiates the responses to BK and histamine, and prostaglandins potentiate the actions of other mediators without its direct action. We found that the interaction of the mediators to cause effects such as potentiation might play an important role in the response to LPS. The results obtained from this study may suggest the interaction of the mediators involved in the increase of vascular permeability in the skin as well as the systemic response to LPS (7).

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REFERENCES

1 Klosterhalfen B, Horstmann-Jungemann K, Vogel P, Flohe S, Offner F, Kirkpatrick CJ and Heinrich PC: Time course of various inflammatory mediators during recurrent endotoxemia. Biochem Pharmacol 43, 2103–2109 (1992)
2 Terashita Z, Imura Y, Nishikawa K and Sumida S: Is platelet activating factor (PAF) a mediator of endotoxin shock? Eur J Pharmacol 109, 257–261 (1985)
3 Okuji T, Morita I, Sunada I and Murota S: Involvement of arachidonic acid metabolites in increases in vascular permeability in experimental dental pulpal inflammation in the rat. Arch Oral Biol 34, 523–528 (1989)
4 Lefer AM: Significance of lipid mediators in shock states. Circ Shock 27, 3–12 (1989)
5 Gallimore MJ, Aasen AO, Lynegaas KHN, Larsbraaten M and Amundsen E: Falls in plasma levels of prekallikrein, high molecular weight kinogen and kallikrein inhibitors during lethal endotoxin shock in dogs. Thromb Res 12, 307–318 (1978)
6 Flohe S, Heinrich PC, Scheider J, Wendel A and Flohe L: Time course of IL-6 and TNF alpha release during endotoxin-induced endotoxin tolerance in rats. Biochem Pharmacol 41, 1607–1614 (1991)
7 Ueno A, Ishida H and Oh-ishi S: Comparative study of endotoxin-induced hypotension in kininogen-deficient rats with that in normal rats. Br J Pharmacol 112, 1250–1256 (1991)
8 Oh-ishi S, Hayashi M and Yamaki K: Inflammatory effects of acetylglucerylether phosphorylcholine: vascular permeability increase and induction of pleurisy in rats. Prostaglandins Leukotrienes Med 22, 21–33 (1986)
9 Ikeda K, Tanaka K and Katori M: Potentiation of bradykinin-induced vascular permeability increase by prostaglandin E2 and arachidonic acid in rabbit skin. Prostaglandins 10, 747–758 (1975)
10 Ueno A, Tokumasu T, Naraba H and Oh-ishi S: Evidence for involvement of bradykinin in endotoxin-induced vascular permeability increase in the skin of rats: comparison between kininogen-deficient and normal rats. Eur J Pharmacol 284, 211–214 (1995)
11 Ueno A, Tanaka K, Katori M, Hayashi M and Arai Y: Species difference in increased vascular permeability by synthetic leukotriene C4 and D4. Prostaglandins 21, 637–648 (1981)
12 Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke ST, Breipohl G, Konig W, Knolle J and Scholkens BA: Hoe 140, a new potent and long acting bradykinin-antagonist. Br J Pharmacol 102, 769–773 (1991)
13 Katayama S, Shinohara H and Ohtake S: A new method for extraction of extravasated dye in the skin and the influence of fasting stress on passive cutaneous anaphylaxis in guinea pigs and rats. Microbiol Immunol 22, 89–101 (1978)