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Role of inflammatory mediators in lipid A analogue (ONO-4007)-induced vascular permeability change in mouse skin

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1 Endotoxin shock is accompanied by an increase in peripheral vascular permeability. It has been postulated that most biological activities of LPS are derived from lipid A moiety. Here we examined the effect of lipid A analogue ONO-4007 in increasing vascular permeability and the possible mediators in mouse skin by a dye leakage method.

2 Subcutaneous injection of ONO-4007 (1–2 mg site^{-1}) induced a dose-dependent increase in vascular permeability which was evident after 120 min.

3 ONO-4007-induced dye leakage was significantly attenuated by pretreatments with anti-tumour necrosis factor-α (TNF-α) and anti-interleukin-1α (IL-1α) antibodies, but not with indomethacin (5 mg kg^{-1}) or diphenhydramine (10 mg kg^{-1}). ONO-4007-induced dye leakage was significantly inhibited by a pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME) (10 mg kg^{-1}) but not with aminoguanidine (50 mg kg^{-1}). In inducible nitric oxide synthase (iNOS)-deficient mice, ONO-4007 significantly increased the dye leakage, while ONO-4007 dilated rat thoracic aortic rings pre-contracted with phenylephrine, and the L-NAME pretreatment inhibited the dilation.

4 Thus, TNF-α, IL-1α and constitutive NOSs-derived nitric oxide but not prostaglandins or histamine play a role in ONO-4007-induced increase in vascular permeability. Although ONO-4007 mimics LPS in increasing vascular permeability, mechanisms of permeability change elicited by ONO-4007 were not identical to those of LPS.

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Abbreviations: cNOS, constitutive nitric oxide synthase; COX, cyclo-oxygenase; D-NAME, N^G-nitro-D-arginine methyl ester; IL, interleukin; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NO, nitric oxide; PG, prostaglandin; PSB, pontamine sky blue; TNF, tumour necrosis factor

Introduction

Lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria exhibits a variety of biological activities. An increase in vascular permeability is one of the major manifestations observed in endotoxaemia. Previous studies have shown that vascular permeability change elicited by LPS is mediated by proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) (Edamitsu *et al*., 1995; Yi & Ulich, 1992), arachidonic acid metabolites such as prostaglandins (PGs) and leukotrienes (Fujii *et al*., 1996; Matuschak *et al*., 1990), and nitric oxide (NO) (Fujii *et al*., 1996; Laszlo *et al*., 1995; Rumbaut *et al*., 1999). LPS is constituted of R core, O side chain and lipid A. Lipid A is the hydrophobic part of LPS and supports most of the biological effects of LPS (Galanos *et al*., 1985). However, it is not known how lipid A participates in the LPS-induced vascular permeability change. Some lipid A analogues have been demonstrated to exert effects similar to LPS, while others are antagonistic to LPS (Flad *et al*., 1993; Matsuura *et al*., 1995; Takayama *et al*., 1989; Tsuchiya *et al*., 1980).

Sodium 2-deoxy-2-[3S-(9-phenylnonanoyloxy) tetradecanoyl]-amino-3-*O*-(9-phenylnonanoyl)-D-glucopyranose 4-sulphate (ONO-4007) is a monosaccharide lipid A analogue. ONO-4007 activates human monocytes/macrophages to release TNF-α only in a primed state (Matsumoto *et al*., 1998). ONO-4007 was developed as a potential anticancer drug, which exhibits strong anti-tumour activity *via* intratumoral TNF production (Yang *et al*., 1994). Since this finding suggests that ONO-4007 may also cause a systemic inflammatory response such as a vascular permeability change similar to LPS, we investigated the potency and mechanisms of ONO-4007 to induce an increase in vascular permeability in mouse skin.

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Methods

Animals and materials

Male ddY strain mice weighing approximately 35 g were obtained from Sankyo Laboratory Service (Tokyo, Japan) and male Wistar rats weighing approximately 400 g from Imamichi Institute for Animal Reproduction (Saitama, Japan). Inducible nitric oxide synthase (iNOS) deficient mice kindly provided by the Merck Research Laboratories (Rahway, NJ, U.S.A.), were raised in our laboratory and were used at 8–12 weeks old. Strain-specific and age-matched control mice bred as the F1 generation of C57BL/6J and 129 SvJ strains, obtained from Clea Japan (Tokyo, Japan), were used at 8–12 weeks old. The animals were housed in plastic cages under controlled environmental conditions (temperature 22±2°C, humidity

55±5%, light 0600–2000 h, dark 2000–0600 h). Food and water were freely available. The study protocol was approved by the institutional committee for animal study.

ONO-4007, sodium 2-deoxy-2-[3S-(9-phenylnonanoyloxy) tetradecanoyl] - amino - 3 - *O*-(9-phenylnonanoyl) -D-glucopyranose 4-sulphate, was kindly provided by ONO Pharmaceutical Co. (Osaka, Japan), and was dissolved in 50% ethanol and diluted in 5% glucose. Indomethacin was dissolved in a small volume of 100% ethanol and diluted in 0.9% NaCl. All other reagents were dissolved in 0.9% NaCl. Vehicle and all other reagents administred either locally or systemically were LPS-free. Pontamine sky blue (PSB) 6B was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), N^G-nitro-L-arginine methyl ester (L-NAME) hydrochloride (HCl), aminoguanidine hemisulphate and indomethacin from Sigma (St. Louis, MO, U.S.A.), N^G-nitro-D-arginine methyl ester (D-NAME) HCl from Nova Biochem (Läufelfingen, Switzerland), and rabbit anti-mouse TNF-α polyclonal antibody from Genzyme (Cambridge, MA, U.S.A.). Monoclonal mouse anti-human IL-1α antibody, which is a purified antibody (0.5 mg IgG_1 ml^{-1}), was kindly provided by Ohtsuka Pharmaceutical Co. (Tokushima, Japan). These antibodies were diluted by 400 fold with 0.9% NaCl and were given to mice at the volume of 10 ml kg^{-1} (Iuvone *et al*., 1999). Diphenhydramine HCl was obtained from Nacalai Tesque (Kyoto, Japan), acetylcholine chloride from Daiichi Pharmaceutical Co. (Tokyo, Japan), and *l*-phenylephrine HCl from Wako Pure Chemical Industries (Osaka, Japan).

Assessment of vascular permeability induced by ONO-4007

Vascular permeability in the mouse skin was assessed by the leakage of PSB (Fujii *et al*., 1994; 1997). Five minutes after an intravenous (i.v.) injection of PSB (50 mg kg^{-1}), ONO-4007 (1.0–2.0 mg $site^{-1}$) or vehicle (10% ethanol in 4% glucose) was subcutaneously (s.c.) administered into the back of the mouse. Two hours later, the mice were killed by cervical dislocation. The stained area of the skin was excised, weighed and minced. The accumulated dye was extracted with an acetone-0.5% Na_2SO_4 mixture (14 : 6 v v^{-1}), and the concentration determined colorimetrically at 590 nm, using a Shimadzu spectrophotometer (Kyoto, Japan).

The time course of vascular permeability changes following ONO-4007 administration was determined as follows. Five minutes after PSB i.v. injection, ONO-4007 or vehicle was s.c. injected into the back of mouse, and the dye leakage in the skin was determined at 30, 60, 120, 180 and 300 min later.

The effects of increasing doses of ONO-4007 on the vascular permeability were determined as follows. Five minutes after PSB i.v. injection, a given dose (vehicle, 1 mg $site^{-1}$, 2 mg $site^{-1}$) of ONO-4007 was s.c. injected into the back. Two hours later, the dye leakage in the skin was determined.

Treatment of mice with inhibitors and receptor antagonists of the inflammatory mediators

Indomethacin (5 mg kg^{-1}), aminoguanidine (50 mg kg^{-1}) L-NAME (10 mg kg^{-1}) or D-NAME (10 mg kg^{-1}) were intraperitoneally (i.p.) administered 30 min before PSB injection followed by ONO-4007 (2 mg $site^{-1}$) or vehicle (0.1 ml $site^{-1}$) injection. Anti-TNF-α and anti-IL-1α antibodies (diluted 400 fold) were administered s.c. at the volume of 10 ml kg^{-1} 24 h before, and diphenhydramine (10 mg kg^{-1}) was administered s.c. 15 min before PSB injection. The dosage of these agents was chosen on the basis of previous studies (Fujii *et al*., 1994; 1995; 1996; Iuvone *et al*., 1999; Nagai *et al*., 1992).

Assessment of tension of rat aorta

The thoracic descending aorta was isolated from rats and was cut into rings at approximately 3–4 mm long. The rings with the endothelium were suspended between two parallel stainless steel rods, one fixed and the other attached to an isometric transducer (model TB651T, Nihon Kohden, Tokyo Japan). The aortic rings were placed in a 5 ml water bath maintained at 37°C and filled with Krebs solution (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.0, bubbled with a mixture of 95% O_2 and 5% CO_2. The aortic rings were stretched to adjust the resting tension to 1.0 g and allowed to equilibrate for 60 min. All aortic rings were sensitized by two separate additions of 50 mM KCl. The presence of endothelium was confirmed by a vasodilation by acetylcholine (300 μM). After the aortic rings were contracted with 3 μM phenylephrine, ONO-4007 (1–100 μM) was added into the bath. In some experiments, the rings were pretreated with L-NAME (100 μM) for 20 min.

Statistical analysis

All values are expressed as mean±s.e.mean. Student's unpaired *t*-tests and one-way analysis of variance (ANOVA) were used for statistical analysis. *P*-values less than 0.05 were considered to be statistically significant.

Results

Effect of ONO-4007 on vascular permeability of mouse dorsal skin

Subcutaneous injection of ONO-4007 resulted in an increase in local vascular permeability determined by an extravasation of PSB. As shown in Figure 1, ONO-4007 at the dose of 2 mg $site^{-1}$ induced a time-dependent increase in the dye leakage, which was significant at 60 min and up to 300 min after injection. In contrast, vehicle injected mice showed no significant change in the dye leakage. ONO-4007 induced a dose-dependent increase in vascular permeability determined 2 h after injection (Figure 2).

Roles of inflammatory mediators in ONO-4007-induced dye leakage

Roles of inflammatory cytokines, namely TNF-α and IL-1α, in the ONO-4007-induced vascular permeability change were studied. As shown in Figure 3, an ONO-4007-induced increase in dye leakage was significantly attenuated by a pretreatment with anti-TNF-α antibody, or by anti-IL-1α antibody, while these antibodies did not change the vascular permeability of mouse skin injected with vehicle. We then investigated the effects of indomethacin, a nonselective cyclo-oxygenase (COX) inhibitor, and diphenhydramine, a histamine 1 (H1) receptor antagonist, on ONO-4007-induced vascular permeability change. The dye leakage induced by ONO-4007 was not significantly attenuated by a pretreatment with either indomethacin (5 mg kg^{-1} i.p.) or diphenhydramine (10 mg kg^{-1} s.c.) (Figure 4), indicating that PGs and histamine may not be major mediators related to an increase in vascular permeability elicited by ONO-4007.

To examine the role of NO, the effect of L-NAME, D-NAME and aminoguanidine on ONO-4007-induced vascular permeability change was studied. ONO-4007-induced dye leakage was significantly attenuated by the pretreatment with L-NAME but not with aminoguanidine at both early (2 h) and late (5 h) time points (Figure 5).

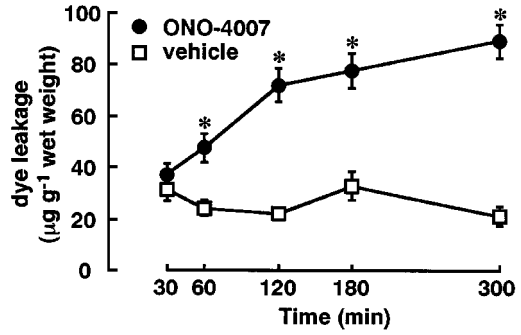

Figure 1 Time course of ONO-4007-induced vascular permeability change. Five minutes after PSB (50 mg kg^{-1}, i.v.) injection, ONO-4007 (2 mg $site^{-1}$) or vehicle (0.1 ml $site^{-1}$) was s.c. injected into the back, then the dye leakage in the skin was colorimetrically quantified at 30, 60, 120, 180 and 300 min. Values are expressed as mean ± s.e.mean of five mice. *$P<0.01$ vs vehicle.

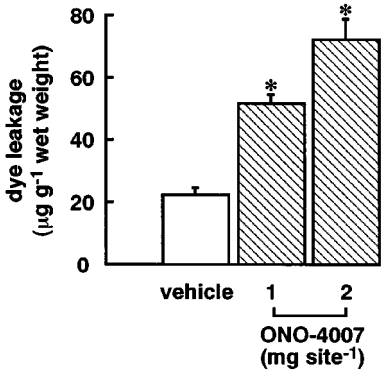

Figure 2 Dose-dependent change in the vascular permeability. Two hours after the s.c. injection of ONO-4007 or vehicle, the dye leakage in the skin was colorimetrically quantified. Values are expressed as mean ± s.e.mean of five mice. *$P<0.01$ vs vehicle.

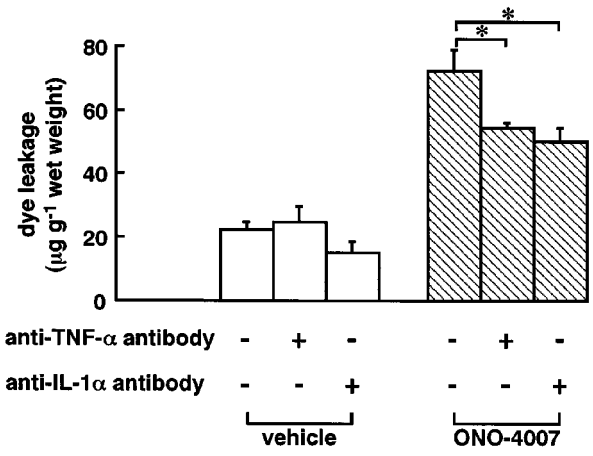

Figure 3 Effects of anti-TNF-α antibody and anti-IL-1α antibody on ONO-4007-induced dye leakage in mouse skin. In mice treated with anti-TNF-α or anti-IL-1α antibodies (1 : 400) s.c. 24 h previously, ONO-4007 (2 mg $site^{-1}$) or vehicle (0.1 ml $site^{-1}$) was s.c. injected into the back followed by determination of dye leakage colorimetrically 2 h later. Values are expressed as mean ± s.e.mean of five mice. *$P<0.05$ vs ONO-4007 alone.

Dye leakage by ONO-4007 in iNOS-deficient mice

Failure of inhibition by aminoguanidine on ONO-4007-induced vascular permeability change suggests that ONO-4007-induced dye leakage is not mediated by NO produced by iNOS. This possibility was examined in iNOS-deficient mice. The dye leakage elicited by ONO-4007 determined either 2 or 5 h after injection was increased in iNOS-deficient mice to an extent similar to that in ddY strain or wild-type mice which were used as the control of iNOS-deficient mice (Figure 6), indicating iNOS does not participate in the dye leakage elicited by ONO-4007.

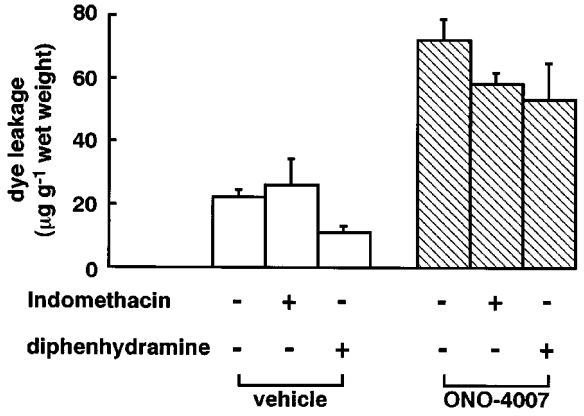

Figure 4 Effects of indomethacin and diphenhydramine on ONO-4007-induced dye leakage in mouse skin. In mice treated with indomethacin (5 mg kg^{-1} i.p.) 35 min previously or diphenhydramine (10 mg kg^{-1} s.c.) 20 min previously ONO-4007 (2 mg $site^{-1}$) or vehicle (0.1 ml $site^{-1}$) was s.c. injected into the back followed by determination of dye leakage colorimetrically 2 h later. Values are expressed as mean ± s.e.mean of five mice.

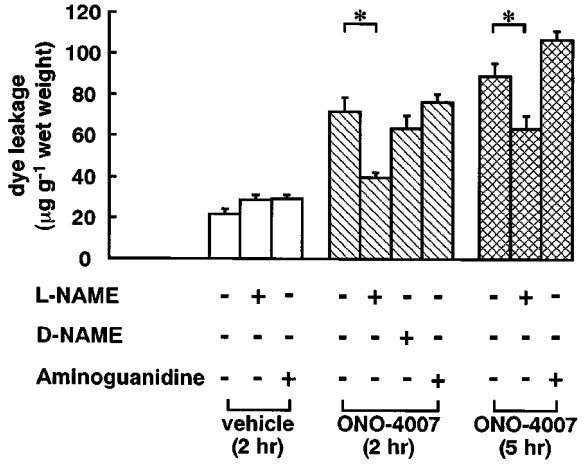

Figure 5 Effects of NOS inhibitors on ONO-4007-induced dye leakage in mouse skin. Thirty-five minutes after pretreatment with L-NAME (10 mg kg^{-1}, i.p.), D-NAME (10 mg kg^{-1}, i.p.) or aminoguanidine (50 mg kg^{-1}, i.p.), ONO-4007 (2 mg $site^{-1}$) or vehicle (0.1 ml $site^{-1}$) was s.c. injected into the back followed by determination of dye leakage colorimetrically 2 or 5 h later. Values are expressed as mean ± s.e.mean of five mice. *$P<0.01$ vs ONO-4007 alone.

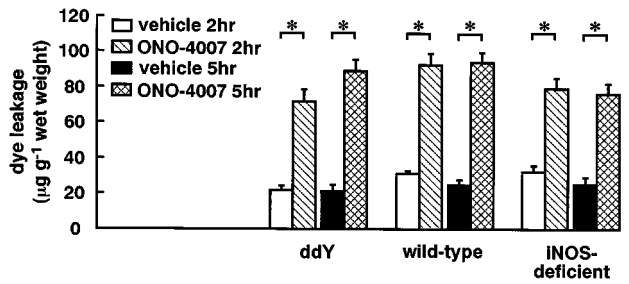

Figure 6 Effects of ONO-4007-induced dye leakage in iNOS-deficient mice. Five minutes after PSB (50 mg kg^{-1}, i.v.) injection, ONO-4007 (2 mg $site^{-1}$) or vehicle (0.1 ml $site^{-1}$) was s.c. injected into the back of ddY, wild-type or iNOS-deficient mice. Two or 5 h later, the dye leakage in the skin was colorimetrically quantified. Values are expressed as mean ± s.e.mean of five mice. $*P<0.01$ vs vehicle-treated group.

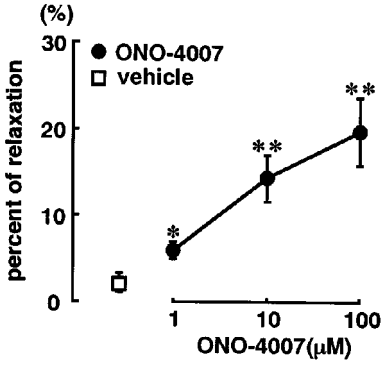

Figure 7 Effects of increasing doses of ONO-4007 on the tension of rat thoracic aortic rings pre-contracted with phenylephrine. After the ring was contracted with 3 μM phenylephrine, increasing concentrations of ONO-4007 (1, 10, 100 μM) or vehicle (0.05% ethanol) were added into the bath. The degree of relaxation was expressed as per cent decrease of the tension induced by phenylephrine alone. Values are expressed as mean ± s.e.mean of five experiments with aorta from different rats. $*P<0.05$ vs vehicle; $**P<0.01$ vs vehicle.

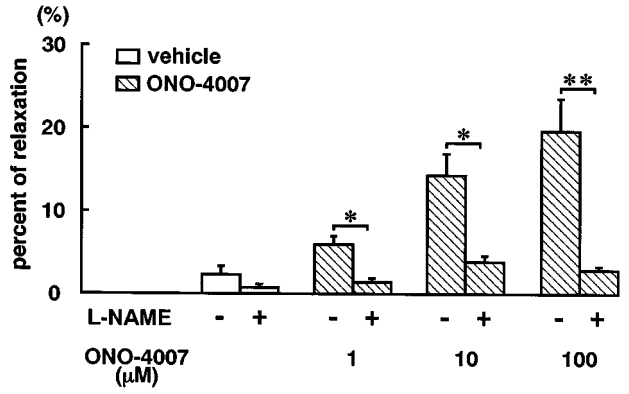

Figure 8 Effect of L-NAME on ONO-4007-induced relaxation of rat thoracic aortic rings pre-contracted with phenylephrine. The aortic rings were pretreated with L-NAME (100 μM) for 20 min prior to the pre-contraction with 3 μM phenylephrine. Then, given concentrations of ONO-4007 or vehicle (0.05% ethanol) were added into the bath. The degree of relaxation of phenylephrine-contracted aorta was expressed as per cent of relaxation. Values are expressed as mean ± s.e.mean of aorta from five different rats. $*P<0.01$ vs ONO-4007 alone; $**P<0.05$ vs ONO-4007 alone.

Vasodilatory effect of ONO-4007 on rat thoracic aortic rings

To examine the possibility that ONO-4007 may activate NO production by stimulating constitutive NOSs, we investigated whether ONO-4007 might have vasodilatory effects in endothelium-intact aortic rings. While vehicle (0.05% ethanol) showed little influence on the tension of rat thoracic aortic rings pre-contracted with phenylephrine (3 μM), ONO-4007 dilated the rings in a dose-dependent manner (1 – 100 μM) (Figure 7). In contrast, ONO-4007 had little influence on the tension of the aortic rings without endothelium (data not shown). Treatment of thoracic aortic rings with L-NAME (100 μM) prior to the pre-contraction with phenylephrine completely inhibited the relaxation induced by 1 – 100 μM ONO-4007 (Figure 8).

Discussion

In this study, we found that a lipid A analogue ONO-4007 mimics LPS in inducing an increase in the vascular permeability in mouse skin. Our previous studies have shown that s.c. injection of LPS increased dye leakage in mouse skin with the onset at 60 min after LPS injection (Fujii *et al*., 1996). While the onset of ONO-4007-induced vascular permeability change was similar to LPS, a higher dose of ONO-4007 (2 mg $site^{-1}$) was required to induce a similar degree of dye leakage than that caused by LPS (400 μg $site^{-1}$). This may suggest that ONO-4007 has a low potency in inducing shock.

Upon activation of monocytes or macrophages by LPS, serum concentrations of proinflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-6, IL-8, and interferon (INF)-γ are progressively increased (de Waal Malefyt *et al*., 1993; Nathan, 1987; Sylvester *et al*., 1993). We previously showed that an increase in vascular permeability by LPS was attenuated by antibodies against TNF-α and IL-1α, indicating the participation of TNF-α and IL-1α in the LPS dye leakage (Fujii *et al*., 1997). The results of the present study indicate that an increase in vascular permeability induced by ONO-4007 is also mediated in part by TNF-α and IL-1α. While LA-15-PP (*Escherichia coli*-type lipid A) has been shown to induce TNF-α production (Matsumoto *et al*., 1998), ONO-4007 (2.5 mg kg^{-1}) did not induce TNF-α production in spleens and sera in hepatoma-bearing rats 90 min after treatment (Kuramitsu *et al*., 1997). The reason for the discrepancy between the previous study of ONO-4007 and the effect of anti-TNF-α antibody in the present study is unclear. The dose of ONO-4007 (2 mg $site^{-1}$) may increase the cutaneous production of TNF-α or IL-1α levels locally.

In a rat model of acute endotoxaemia, vasoactive mediators such as histamine, serotonin, bradykinin, and arachidonic acid metabolites mediate vascular permeability changes (Balsa *et al*., 1997). In another study, a granulocyte/macrophage colony stimulating factor (GM-CSF) or IL-3 enhanced LPS-induced histamine formation (Takamatsu *et al*., 1996). Endotoxin increased COX activity and COX-2 protein expression in endothelial cells and macrophages (Akarasereenont *et al*., 1995). We previously showed that a nonselective COX inhibitor, indomethacin, and a H1 receptor antagonist, diphenhydramine, inhibited the LPS-induced increase in vascular permeability in mouse skin (Fujii *et al*., 1996). In contrast, both indomethacin and diphenhydramine showed little effect on the extravasation induced by ONO-4007 in the present study. Thus, while both LPS and ONO-4007 showed an ability to increase mouse skin vascular permeability, there

was a difference in the responsible vasoactive substances between LPS and ONO-4007.

NO plays a crucial role in the maintenance of vascular integrity (Moncada & Higgs, 1993). NO is generated from the terminal guanidino nitrogen atoms of amino acid L-arginine by the enzyme NOS (Moncada *et al*., 1991). Exposure to LPS stimulates expression of iNOS, an inducible form of NOSs, in blood vessels (Griffiths *et al*., 1995; Hom *et al*., 1995). Our previous studies have shown that vascular permeability change in mouse skin induced by LPS is mediated by iNOS-derived NO at least partly (Fujii *et al*., 1996). In a model of carrageenan-induced paw oedema, Salvemini *et al*. (1996) have shown that cNOS-derived NO is related to the induction of oedema at early time points, whereas iNOS-derived NO is related to the maintenance of the inflammatory response at later time points (>5 h). Hattori *et al*. (1995) reported iNOS mRNA induction by ONO-4007 in J774.2 macrophages and smooth muscle cells. In the present study, however, it was suggested that NO derived from iNOS does not mediate ONO-4007-induced vascular permeability change because: (1) aminoguanidine did not attenuate vascular permeability change, and (2) iNOS-deficient mice demonstrated the response to ONO-4007 to a similar extent in wild-type or ddY strain mice which are not deficient in iNOS expression, at both early and late phases.

The *in-vitro* studies with aorta suggested that ONO-4007 dilates the aorta by stimulating production of NO by endothelial cells. Thus, the early onset of vascular permeability change may be related to ONO-4007-induced stimulation of cNOS activities. In this regard, Kaku *et al*. (1997) described an up-regulation of endothelial NOS (eNOS) mRNA expression and eNOS promoter activity in bovine aortic endothelial cells (BAEC) treated with INF-α/β and LPS. Others found an up-regulation of eNOS mRNA expression in the liver of LPS-treated rats (Bucher *et al*., 1997) and immediate release of NO from BAEC by LPS (Salvemini *et al*., 1990). It remains to be studied whether ONO-4007 induces eNOS in the endothelium of subcutaneous microvasculature.

Our study suggested that mediators involved in the vascular permeability change induced by ONO-4007 are partly different from those in LPS treatment. Matsumoto *et al*. (1998) suggested that ONO-4007 induces TNF-α production *via* LPS binding protein (LBP)/CD14-independent pathways in monocytes, whereas LPS uses both LBP/CD14-dependent and -independent pathways. Thus, there is a possibility that cell receptors activated by ONO-4007 are different from those of LPS in the cutaneous microvasculature. Such differences in participating receptors may cause the characteristics of ONO-4007-induced vascular permeability change found in the present study.

Although less than LPS, our study suggests that ONO-4007 still has a substantial effect on mouse skin vascular permeability. It is not known whether or not the permeability effect of ONO-4007 may be desirable as an anti-tumor compound. Biological significance of such properties of lipid A requires further investigation before applying this substance for clinical use.

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