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# Evaluation of iNOS-dependent and independent mechanisms of the microvascular permeability change induced by lipopolysaccharide

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**1** Subcutaneous injection of lipopolysaccharide (LPS) increases plasma leakage in mouse skin. Pretreatment with LPS conditions mice tolerant to the LPS-induced plasma leakage. Nitric oxide (NO) has been suggested to be involved in these LPS effects. A specific role of inducible NO synthase (iNOS) was investigated in the LPS-induced plasma leakage using iNOS deficient mice.

**2** Plasma leakage in mouse skin was measured by the local accumulation of Pontamine sky blue at the site of subcutaneous injection of LPS (Sal. typhimurium). LPS (100–400 $\mu$g site$^{-1}$) produced a dose-related increase in dye leakage in both iNOS deficient and wild-type mice with about 40% less dye leakage in iNOS deficient mice.

**3** Indomethacin (5 mg kg$^{-1}$), N-[-2-cyclohexyloxy]-4-nitrophenyl methanesulphonamide (NS-398) (1 mg kg$^{-1}$), diphenhydramine (10 mg kg$^{-1}$) and anti-TNF-$\alpha$ antibody (dilution 1 : 400, 10 ml kg$^{-1}$) inhibited the LPS-induced dye leakage in both iNOS deficient and wild-type mice, whereas $N^G$-nitro-L-arginine methyl ester (L-NAME) (10 mg kg$^{-1}$) or aminoguanidine (10 mg kg$^{-1}$) inhibited that in wild-type but not in iNOS deficient mice.

**4** Pretreatment with LPS (0.15 mg kg$^{-1}$ i.p.) 4 h before decreased the LPS-induced dye leakage in wild-type but not in iNOS deficient mice. LPS pretreatment increased serum corticosterone levels in both mice, while it increased the serum nitrate/nitrite levels in wild-type but not in iNOS deficient mice.

**5** These studies indicate that an increase in vascular permeability induced by LPS is mediated by NO produced by iNOS, eicosanoids, histamine and TNF-$\alpha$. The tolerance against LPS-induced vascular permeability change may be mediated by iNOS induction but not by an increased release of endogenous corticosteroids.

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**Keywords:** Inducible nitric oxide synthase (iNOS); iNOS deficient mice; microvascular permeability; lipopolysaccharide; endotoxin tolerance

**Abbreviations:** iNOS, inducible nitric oxide synthase; LBP, LPS binding protein; NF-$\kappa$B, nuclear factor $\kappa$B; NO, nitric oxide; NS-398, N-[-2-cyclohexyloxy]-4-nitrophenyl methanesulphonamide; TLR, Toll-like receptor; TNF-$\alpha$, tumour necrosis factor-$\alpha$

## Introduction

Endotoxin or lipopolysaccharide (LPS) of the outer membrane of gram-negative bacteria mediates the gram-negative shock syndrome, which is characterized by fever, hypotension, and multiple organ failure (Ulevitch & Tobias, 1995). Once released in the circulation, LPS may bind to LPS binding protein (LBP) in the plasma followed by binding with cell surface CD14 or sCD14 in the blood (Murphy *et al*., 1998). Then Toll-like receptor (TLR)-2 or TLR-4 MD-2 complex recognizes LPS and transduces signals of LPS to NF-$\kappa$B (Yang *et al*., 1998; Shimazu *et al*., 1999). LPS induces the release of many inflammatory mediators which include eicosanoids, cytokines, platelet-activating factor (PAF), nitric oxide (NO) (Liao, 1996).

NO is generated by 3 NO synthases. Among them inducible NO synthase (iNOS) is upregulated in lungs and liver during shock and plays a role for the generation of large amounts of NO during shock or following stimulation of tissues with a variety of proinflammatory mediators (Szabó, 1995). Paradoxically, NO is antiinflammatory or proinflammatory depending on the experimental conditions (Grisham *et al*., 1999). NO plays a protective role in the protease receptor-1-mediated vascular permeability in rat hindpaw (Kawabata *et al*., 1999), while it was reported that NO acts as a proinflammatory mediator in the dermal microvessels through increasing microvascular blood flow (Ridger *et al*., 1997).

The initial responses to endotoxaemia are detectable in the microcirculation as a microvascular inflammatory response characterized by an increase in vascular permeability to macromolecules (McCuskey *et al*., 1996). When given intradermally or subcutaneously, LPS increases plasma leakage in the skin of mice and rats (Fujii *et al*., 1996a; Iuvone *et al*, 1998). It was shown that the increase in cutaneous vascular permeability elicited by LPS was mediated by many proinflammatory mediators such as cytokines, eicosanoids, PAF, histamine and NO (Fujii *et al*., 1997a,b, Iuvone *et al*., 1999). Studies with specific inhibitors showed the involvement of cyclo-oxygenase (COX)-2 and iNOS in the LPS-induced plasma leakage (Fujii *et al*., 1996a; Muraki *et al*., 1996). Intradermal injection of LPS increased the iNOS protein in the rat skin (Iuvone *et al*., 1998).

In humans and experimental animals, LPS causes an induction of acute-phase response. Some components of the acute-phase response, such as fever, anorexia and mortality are

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attenuated when LPS is repeatedly administered in short-term intervals, a phenomenon called endotoxin tolerance (Roth *et al*., 1997). Previously we showed that tolerance develops after a single injection of LPS in terms of dermal plasma leakage induced by LPS. iNOS was likely to be related to the development of tolerance because specific inhibitors for iNOS prevented the development of tolerance (Fujii *et al*., 1996b).

In an extension to our previous findings, the specific role of iNOS in LPS-induced microvascular permeability change and that of development of tolerance by LPS, mice deficient with iNOS gene were examined in the present study. The study was designed to identify iNOS-dependent and non-dependent mechanisms of one manifestation of sepsis.

## Methods

### *Animals*

Breeding pairs of iNOS deficient mice were characterized and kindly provided by Drs J. MacMicking, C. Nathan (Cornell University Medical College) and Dr J. Mudgett (Merck Research Laboratories) (MacMicking *et al*., 1995). C57BL/6 and 129Sv mice were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.). A $F_1$ hybrid between C57BL/6 and 129Sv crosses was used as wild-type controls for iNOS deficient mice. Adult mice of both sexes at 9–12 weeks of age, weighing $24.7 \pm 3.8$ g (mean $\pm$ s.e.mean, $n=132$) and $27.1 \pm 5.6$ g ($n=131$) for iNOS deficient and $F_1$ mice, respectively, were used. The animals were housed in an air-conditioned room (temperature $22 \pm 2$°C, humidity $55 \pm 5$%) with a controlled light–dark cycle (light on 6:00–20:00). The mice had free access to food and water. All experimental procedures were approved by the Institutional Animal Care Committee.

### *Materials*

Aminoguanidine hemisulphate, indomethacin HCl, LPS (Salmonella Typhimurium), $N^G$-nitro-L-arginine methylester (L-NAME) and prostaglandin (PG) $E_2$ were purchased from Sigma (St. Louis, MO, U.S.A.); diphenhydramine HCl from Nacalai Tesque (Kyoto, Japan); rabbit anti-mouse TNF-α polyclonal antibody from Genzyme (Cambridge, MA, U.S.A.). N-(2-cyclohexyloxy-4-nitrophenyl) methansulphonamide (NS-398) was kindly provided by Taisho (Saitama, Japan). Other reagents were commercially available. Indomethacin was dissolved in ethanol and diluted in 50% propylene glycol to make a stock solution (Fujii *et al*., 1996a). Drugs were dissolved in nonpyrogenic saline (0.9% NaCl).

### *Determination of plasma leakage in mouse skin*

The microvascular permeability of the skin was assessed in mice by an extravasation of Pontamine sky blue (PSB) as previously described (Fujii *et al*, 1994). Briefly, 5 min after an intravenous (i.v.) injection of PSB (50 mg $kg^{-1}$), LPS or saline (0.1 ml $site^{-1}$), was administered subcutaneously (s.c.) at the back of mice. Two hours later the mice were killed and the stained area of the skin at the site of s.c. injection was excised, weighed and minced. The skin specimen (about 1 g) was dispersed in 6 ml 0.5 % $Na_2SO_4$ and the dye was extracted by an addition of 14 ml acetone. Dye concentration was determined at an absorbance of 590 nm. L-NAME (10 mg $kg^{-1}$) and aminoguanidine hemisulphate (10 mg $kg^{-1}$) were given i.v. immediately before PSB; diphenhydramine HCl (10 mg $kg^{-1}$) was given s.c. 15 min before PSB; indomethacin (5 mg $kg^{-1}$) and NS-398 (1 mg $kg^{-1}$) were given i.p. 30 min before PSB; anti-TNF-α antibody (dilution 1:400, 10 ml $kg^{-1}$) was given 24 h prior to PSB. The dose of drugs was chosen from previous studies (Fujii *et al*., 1996a,b).

### *Determination of corticosterone and nitrate/nitrite*

Serum corticosterone was determined by radioimmunoassay with Biotrak (Amersham, Buckinghamshire, U.K.), and serum nitrate/nitrite was determined by nitrate/nitrite fluorometric assay kit (Cayman, Ann Arbor, MI, U.S.A.).

### *Statistical analysis*

Results are expressed as means $\pm$ s.e.mean of more than five mice. Results were analysed for statistic significance by 2-way or 1-way ANOVA followed by Bonferroni/Dunn's test, or Student's *t*-test.

## Results

We have shown that the dye leakage by LPS in the skin reached maximum at 2 h; therefore, we determined the dye leakage 2 h after injection of LPS in this study. Figure 1 shows that the s.c. injection of increasing doses of LPS elicited a dose-dependent increase in dye leakage in both iNOS deficient and wild-type control mice; however, the effect of LPS was less in the iNOS deficient mice. Two-way ANOVA revealed a significant effect of mouse strain ($F=109.3$, $P<0.01$) and a significant effect of dose ($F=93.7$, $P<0.01$). Pretreatment with NOS inhibitors such as aminoguanidine (10 mg $kg^{-1}$) or L-NAME (10 mg $kg^{-1}$) inhibited the LPS-induced dye leakage in the wild-type mice but not in the iNOS deficient mice (Figure 2). In contrast, COX inhibitors such as indomethacin (5 mg $kg^{-1}$) and NS-398 (1 mg $kg^{-1}$) inhibited the dye leakage induced by LPS in both iNOS deficient and wild-type control mice (Figure 3). Diphenhydramine (10 mg $kg^{-1}$), a histamine $H_1$ blocker, and anti-TNF-α antibody inhibited the LPS-induced dye leakage in both iNOS deficient and wild-type mice (Figures 4 and 5).

To examine the LPS-induced tolerance to the microvascular permeability, we injected LPS (400 μg $site^{-1}$ s.c.) 4 h after the

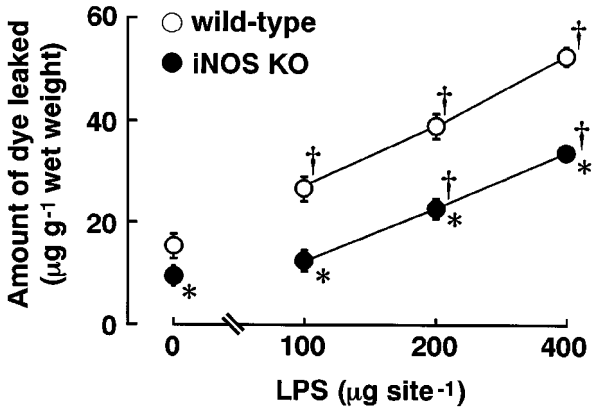


**Figure 1** Effect of increasing doses of LPS on the dye leakage in the skin of iNOS deficient and wild-type mice. Topical dye leakage in the skin was assessed 2 h after s.c. injection of LPS or saline (10 ml $kg^{-1}$). Symbols and vertical bars represent means $\pm$ s.e.mean of five experiments. $^*P<0.05$ vs wild-type mice, †$P<0.05$ vs respective saline-treated mice (0 μg LPS $site^{-1}$).

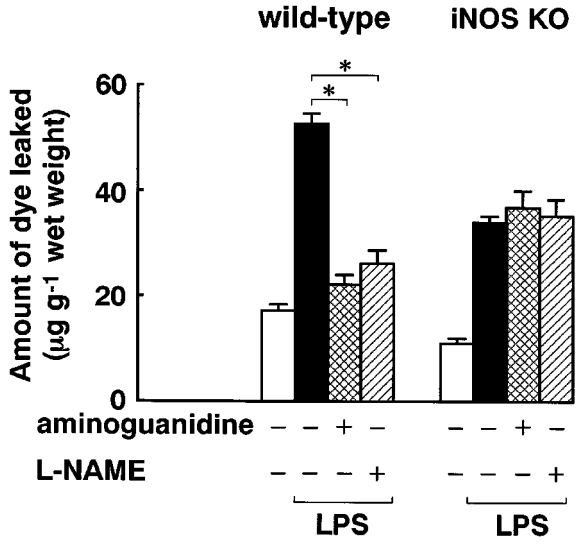


**Figure 2** Effect of iNOS inhibitors on LPS-induced dye leakage in mouse skin. Mice were treated with i.v. aminoguanidine (10 mg $kg^{-1}$) or L-NAME (10 mg $kg^{-1}$) 5 min prior to LPS (400 $\mu$g $site^{-1}$ s.c.). Control mice received saline instead of LPS. The amount of dye leakage in the skin was assessed 2 h later. Columns and bars represent means ± s.e.mean of five experiments. *$P<0.05$.

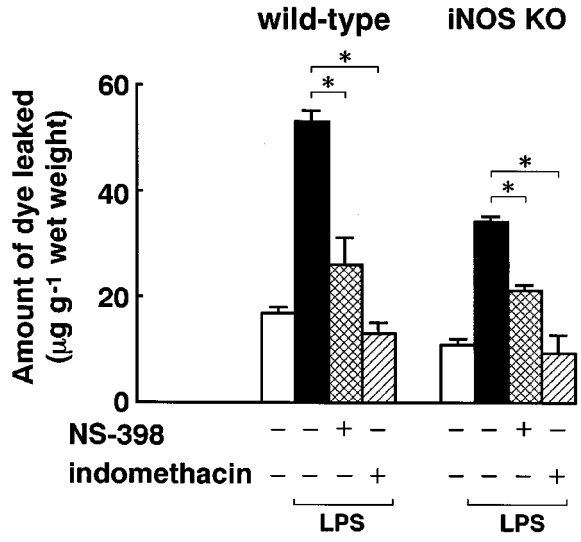


**Figure 3** Effect of COX inhibitors on LPS-induced dye leakage in mouse skin. Mice were treated with i.p. NS-398 (1 mg $kg^{-1}$) or indomethacin (5 mg $kg^{-1}$) 35 min prior to LPS (400 $\mu$g $site^{-1}$ s.c.). Control mice received saline instead of LPS. The dye leakage was assessed 2 h later. Columns and bars represent means ± s.e.mean of five experiments. *$P<0.05$.

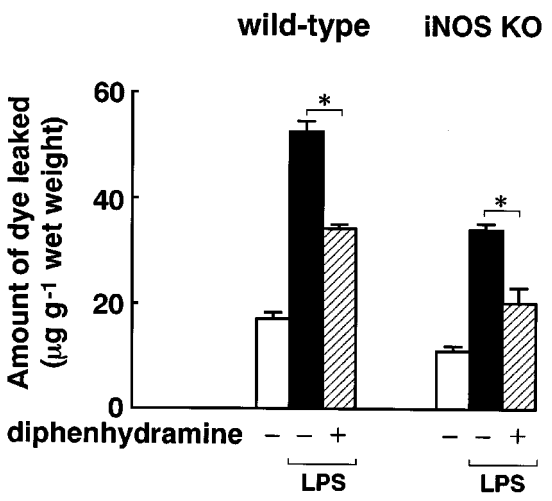


**Figure 4** Effect of $H_1$ blocker on LPS-induced dye leakage in mouse skin. Mice were pretreated with diphenhydramine (10 mg $kg^{-1}$ s.c.) 20 min prior to LPS (400 $\mu$g $site^{-1}$ s.c.). Control mice received saline instead of LPS. The dye leakage was assessed 2 h later. Columns and bars represent means ± s.e.mean of five experiments. *$P<0.05$.

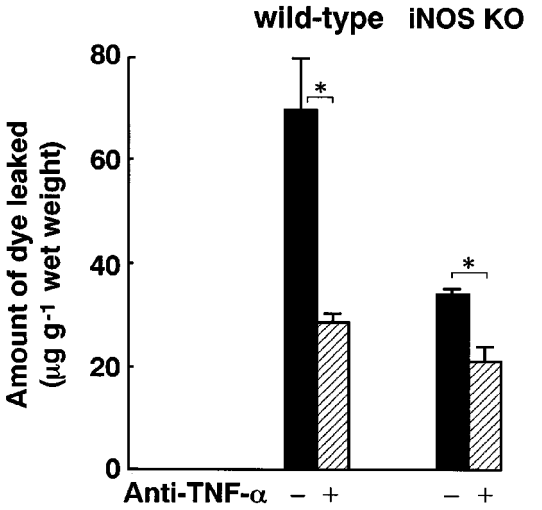


**Figure 5** Effect of anti-TNF-$\alpha$ antibody on LPS-induced dye leakage in mouse skin. Mice were treated with anti-TNF-$\alpha$ antibody (dilution 1 : 400, 10 ml $kg^{-1}$ s.c.) 24 h prior to LPS (400 $\mu$g $site^{-1}$ s.c.). The dye leakage was assessed 2 h later. Columns and bars represent means ± s.e.mean of five experiments. *$P<0.05$.

systemic pretreatment with LPS (0.15 mg $kg^{-1}$ i.p.) or saline (Figure 6). The dye leakage elicited by local LPS injection was significantly decreased in the wild-type mice pretreated with LPS as compared with saline-pretreated controls. On the other hand, LPS pretreatment showed no inhibition of LPS-induced dye leakage in the iNOS deficient mice. Our previous study showed that development of tolerance was inhibited in the adrenalectomized mice (Fujii *et al*., 1996b). To assess the possible role of NO and the pituitary adrenal system in the LPS-induced tolerance, we determined the nitrate/nitrite and corticosterone levels in serum of mice 4 h after systemic treatment with LPS or saline (Table 1). Systemic LPS treatment increased the serum corticosterone levels in both iNOS deficient and wild-type mice to a comparable degree. On the contrary, serum nitrate/nitrite levels as an index of NO production, were elevated in the wild-type but not in the iNOS deficient mice.

## Discussion

We showed that LPS elicited dye leakage dose-dependently in both iNOS deficient and wild-type control mice; however, the amount of dye leakage in iNOS deficient mice was about 60 % of the wild-type controls. In the wild-type controls, the LPS-induced dye leakage was inhibited by the NOS inhibitors, confirming our previous results with ddY strain mice (Fujii *et al*., 1996a). Contrastingly, LPS-induced dye leakage in the iNOS deficient mice was not inhibited by the NOS inhibitors. It was shown that LPS increased the iNOS proteins in the skin of LPS-treated rats (Iuvone *et al*, 1998). Collectively, these

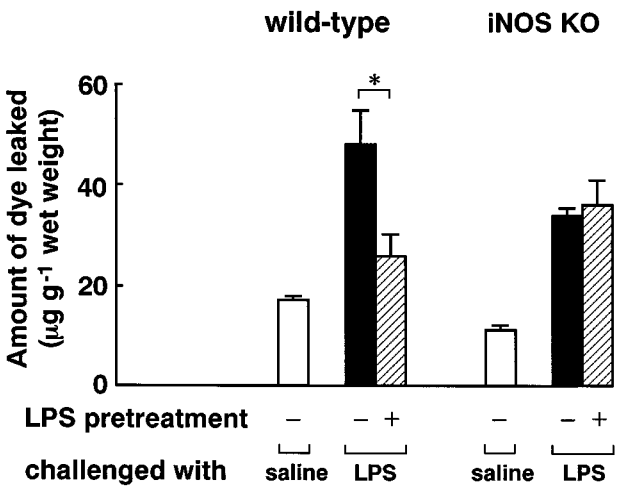


**Figure 6** Effect of systemic pretreatment with LPS on the LPS-induced dye leakage in mouse skin. LPS (0.15 mg $kg^{-1}$) or saline (10 ml $kg^{-1}$) was i.p. administered to mice 4 h previously. The mice were given LPS (400 $\mu$g $site^{-1}$ s.c.) or saline (10 ml $kg^{-1}$ s.c.) and the dye accumulation in the skin was determined 2 h later. Columns and bars represent means ± s.e.mean of five experiments. $^{*}P<0.05$.

**Table 1** Effect of LPS on serum nitrate/nitrite and corticosterone levels in iNOS deficient and wild-type mice

| | | *nitrate/nitrite* (μM) | *corticosterone* (ng $ml^{-1}$) |
|---|---|---|---|
| Wild-type | saline | 40.6 ± 7.1 (4) | 116 ± 52 (4) |
| | LPS | 85.9 ± 19.2 (4)[a] | 676 ± 59 (4)[a] |
| iNOS deficient | saline | 20.4 ± 2.4 (7)[c] | 142 ± 25 (7) |
| | LPS | 20.8 ± 2.4 (6)[c] | 530 ± 40 (6)[b] |

Serum was obtained 4 h after treatment with saline (10 ml $kg^{-1}$ i.p.) or LPS (0.15 mg $kg^{-1}$ i.p.) in wild-type and iNOS deficient mice. Results are expressed as means ± s.e. mean (number of mice) and were evaluated statistically by ANOVA followed by Bonferroni/Dunn's test. [a]$P<0.01$ vs saline-treated wild-type mice, [b]$P<0.01$ vs saline-treated iNOS deficient mice, [c]$P<0.05$ vs respective wild-type mice.

results indicate that the LPS-induced increase in microvascular permeability in the wild-type mice is largely dependent on NO produced by iNOS. In iNOS deficient mice, LPS induces vascular permeability change by mechanisms not related to iNOS. The role of other subtypes of NOS remains to be studied.

Indomethacin and NS-398, a COX-2 specific inhibitor, inhibited the dye leakage by LPS in both iNOS deficient and the wild-type controls. Thus eicosanoids produced most probably by COX-2 play a role in the dye leakage in the iNOS deficient and wild-type mice confirming our previous study with ddY strain mice (Fujii *et al*., 1996a). Studies with diphenhydramine and anti-TNF-$\alpha$ antibody revealed the role of histamine and TNF-$\alpha$ in the LPS-induced dye leakage in the iNOS deficient as well as wild-type control mice, as previously shown in mice and rats (Fujii *et al*., 1997a,b; Iuvone *et al*., 1999). The results indicated that the pathogenesis of LPS-induced dye leakage contains both iNOS-dependent and -independent components, the latter being mediated by eicosanoids, histamine and TNF-$\alpha$, but not by NO.

Although tolerance was induced usually by a repeated administration of LPS, the tolerance to LPS-induced cutaneous dye leakage appeared rapidly after a single injection of LPS (Fujii *et al*., 1996b). Due to an unknown reason, the degree of tolerance developed in $F_1$ of C57BL/6 × 129Sv crosses was less than that of the ddY strain. In contrast to the wild-type controls, systemic administration of LPS did not induce tolerance in the iNOS deficient mice at all. A previous study with a glucocorticoid receptor antagonist suggested that the elevated plasma steroid plays a central role in the development of LPS-induced tolerance by way of attenuation of induction of iNOS by LPS (Szabó *et al*., 1994). Previously, we suggested endogenous glucocorticoids as the cause of LPS-induced tolerance, because the tolerance was not induced in the adrenalectomized animals (Fujii *et al*., 1996b). However, we found that LPS pretreatment activated the pituitary-adrenocortical system without induction of tolerance in the iNOS deficient mice. Therefore, the increase in serum glucocorticoid may not be responsible for the LPS-induced tolerance. Systemic LPS increased nitrate/nitrite accumulation in serum in the wild-type mice which developed the tolerance, whereas no change in nitrate/nitrite was observed in iNOS deficient mice. Similarly, LPS treatment caused no impairment of contraction responses in carotid arteries from the iNOS deficient mice (Gunnett *et al*., 1998). These results imply that the generation of large amount of NO by iNOS contributes to LPS-induced tolerance to microvascular permeability as well as vasoconstrictory responses.

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