# Site- and Cell-Type- Specific Induction of Intestinal Inducible Nitric Oxide Synthase in a Rat Model of Endotoxemia

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**Abstract:** The intestine is one of the major organs that are involved in sepsis. The inducible isoform of nitric oxide synthase (iNOS) is known to play a critical role in the pathogenesis of septic tissue injury by generating excess amount of nitric oxide (NO) in response to cytokines and endotoxin. In this study, we examined changes in gene expression of iNOS in various regions of the intestine as well as the distribution of iNOS protein in the intestinal cells in a rat model of endotoxemia produced by intraperitoneal injection of lipopolysaccharide (LPS; 10 mg/kg). While iNOS mRNA was undetectable in the intestine of untreated control animals, it underwent marked induction following LPS treatment. Induction of iNOS mRNA in the ileum was marked and biphasic, while it was also marked but monophasic in the jejunum. The induction of iNOS mRNA was maximal in the ileum. The administration of interleukin-6 (IL-6) upregulated intestinal iNOS gene expression specifically in the ileum. Consistent with enhanced iNOS gene expression, iNOS protein was markedly expressed in the ileum after LPS treatment, exclusively in the mucosal epithelium both at crypt and villus cells, although more prominently in the former. These findings suggested that intestinal iNOS expression was upregulated both at transcriptional and protein levels not only in a site-specific, but also in a cell type-specific manner in a rat model of endotoxemia, possibly through increasing serum IL-6 levels. Differential regulation of iNOS expression along the longitudinal and crypt-villus axes of the gut might be a determinant of the pattern of sepsis-induced intestinal damage.

**Key Words:** Inducible nitric oxide synthase, lipopolysaccharide, intestinal injury, interleukin-6, sepsis, nitric oxide, inflammation.

## INTRODUCTION

The gastrointestinal tract plays an important role in the pathogenesis of sepsis, both as a site of end-organ injury and as a contributor to immune activation bacterial translocation [1, 2]. Symptoms of Gram-negative bacterial sepsis can be reproduced experimentally by treating animals with lipo-polysaccharide (LPS) [3-5]. LPS, a component of the cell wall of Gram-negative bacteria is responsible for inducing highly complex cascading events resulting in multiple organ damage [6].

The inducible isoform of nitric oxide synthase (iNOS) is an enzyme whose expression in endothelium, epithelium, and inflammatory cells requires protein synthesis, is induced by cytokines and LPS, and produces large amounts of nitric oxide (NO) for extended periods of time [7]. It has been proposed that the high output production of NO from iNOS causes tissue injury, perhaps through the generation of potent radicals such as peroxynitrite [8]. Studies have showed that expression of intestinal iNOS is upregulated by LPS treatment in rats [9-13]. However, the profile of intestinal iNOS induction has not been fully characterized in endotoxemic rats. Here we examined iNOS mRNA expression at several time points over 72 h in various regions of the intestine, and iNOS protein expression in the intestinal cells in a rat model of acute endotoxemia generated using bacterial LPS.

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## RESULTS

### Effect of LPS Treatment on iNOS Expression

We examined the effect of intraperitoneal injection of LPS on iNOS mRNA levels in various regions of the intestine. iNOS mRNA was hardly detectable in all four regions of the intestine, i.e., duodenum, jejunum, ileum, and colon in control animals (Fig. (**1**), **A** and **B**)). In contrast to control animals, iNOS mRNA levels were markedly increased following LPS treatment in the ileum and the jejunum (Fig. (**1**), **A** and **B**). iNOS mRNA level in the ilem also showed a biphasic induction pattern, which had been confirmed in three independent experiments. Namely, it markedly increased and reached the first maximum at 3 h, rapidly declined to the basal level at 6 h, then increased again to the second smaller maximum at 9 h, followed by a rapid decline to the basal level by 12 h (Fig. (**1**), **A** and **B**). In the jejunum, however, iNOS mRNA level showed a monophasic induction, which was characterized by a marked induction at 3 h after LPS treatment, then by a rapid decline to the basal level by 6 h (Fig. **(1), A and B**). In contrast to a

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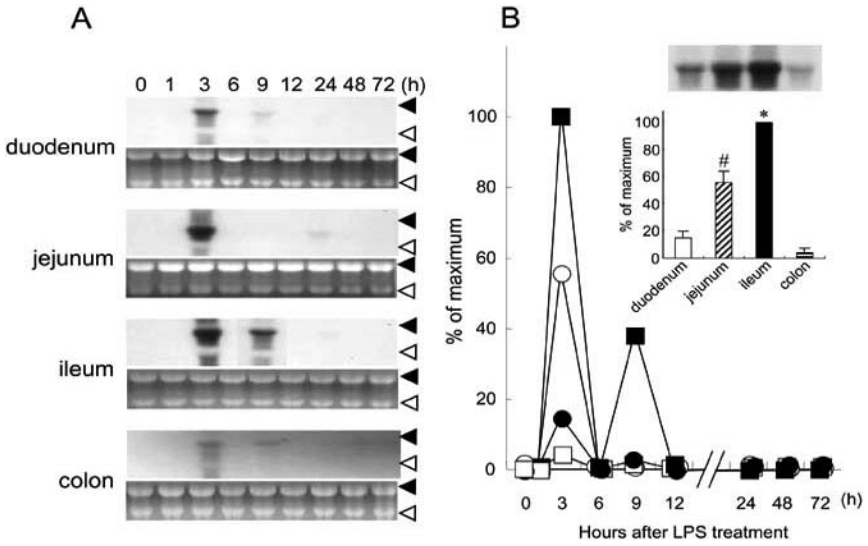


**Fig. (1). Effect of LPS treatment on iNOS gene expression in various regions of the intestine.** Intestines were excised at indicated time points following intraperitoneal injection of LPS (10 mg/kg), and dissected into four segments, i.e., duodenum (●), jejunum (○), ileum (■), and colon (□). (A) Shown are the autoradiographic signals of RNA blot hybridized with [α-$^{32}$P] dCTP labeled iNOS cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Three independent experiments showed similar results, and a typical example is shown in the figure. *Filled arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA,* (B) Levels of iNOS mRNA are expressed as relative values to the maximum level of the ileum. *Insets;* Comparative levels of the maximal expression of iNOS mRNA in various regions of the intestine after LPS treatment. Data are presented as means ± SEM (n = 3 for each region). **p* <0.05, v.s. duodenum, jejunum or colon; $^{\#}p$ <0.05, v.s. duodenum or colon.

marked increase in iNOS mRNA level in the ileum and the jejunum, its level was only slightly increased at 3 h after LPS treatment in the duodenum, and hardly increased in the colon after LPS treatment (Fig. (**1**), **A** and **B**). When the maximally induced levels of iNOS mRNA after LPS treatment were compared, the levels in the ileum and in the jejunum were more than 6-times and almost 4-times larger than that of the duodenum, respectively (Fig. (**1**), inset). Although Northern blot analysis demonstrated that iNOS mRNA was expressed in the intestine of LPS-treated animals, it was necessary to confirm the expression of the iNOS protein in the intestinal tissue. Furthermore, it was also necessary to determine what types of cell(s) in the intestine expressed iNOS. Therefore, we carried out an immunohistochemical study of iNOS in the ileum, where the most prominent expression of iNOS gene was observed, at 8 h after LPS treatment. Consistent with the increase in iNOS gene expression, positive staining for iNOS was observed in the ileum, which was confined to the mucosal epithelial cells (Fig. (**2**), **B**). Particularly strong signals were visible at crypt regions compared with villus regions (Fig. (**2**), **C**). In contrast, iNOS protein was hardly detectable the control ileum samples (Fig. (**2**), **A**). When a section of the ileum from LPS-treated animals was treated with non-immune rabbit serum, there was no positive signal, as expected (data not shown).

### Effect of IL-6 Administration on iNOS Gene Expression

We have previously demonstrated that intraperitoneal administration of LPS, 10 mg/kg, the same dose used in this study, increased serum IL-6 concentration. To examine whether IL-6 may affect iNOS induction, we administered IL-6 (10 μg/kg) to rats intravenously and examined changes in the level of iNOS mRNA in the intestine. In the ileum, there was a significant but transient increase in iNOS mRNA level following IL-6 administration (Fig. (**3**), **A** and **B**). iNOS mRNA level in the ileum markedly increased and reached a maximum at 4 h after IL-6 administration

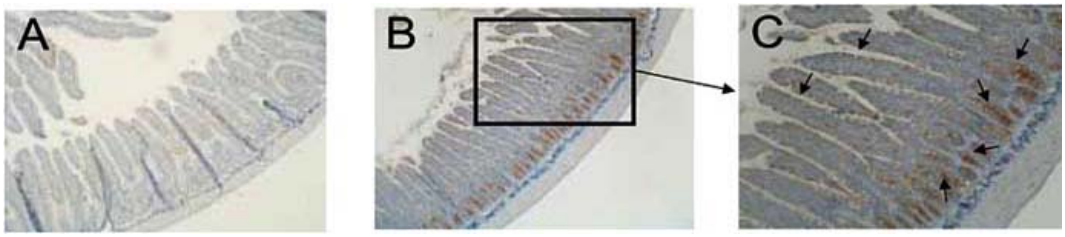


**Fig. (2). Immunohistochemistry of iNOS in the ileum of LPS-treated rats.** Ileum sections 8 h after LPS injection or vehicle were used for immunohistochemical analysis of iNOS, using rabbit polyclonal anti-rat iNOS antibody as a primary antibody. Each photograph is the representative of at least three independent experiments. Arrows indicate positive staining for iNOS protein. A, control animals; B, LPS-treated animals; C, LPS-treated animals by high-power magnification of enclosed area.(Original magnification; A and B x100; C, x200).

followed by the rapid decline to ~15% of the maximum at 6 h, and then returned to the basal level by 9 h (Fig. (**3**), **A** and **B**). In contrast to the significant induction of iNOS mRNA in the ileum, iNOS mRNA levels were not affected by IL-6 treatment in the duodenum, the jejunum and the colon (Fig. **(3), A and B**).

## DISCUSSION

Our study demonstrated that iNOS mRNA and protein were markedly induced in the mucosal epithelial cells of the intestine in a rat model of endotoxemia that was produced by intraperitoneal injection of LPS. We also showed that iNOS expression is remarkably distinct depending on the site in the intestine, the ileum being the principal site of expression and induction in response to LPS treatment. Our study is also the first to report a significant induction of iNOS mRNA specifically in the ileum by IL-6 administration, suggesting that there might be some contribution of increased serum IL-6 concentration to iNOS induction by LPS treatment.

In the ileum, iNOS mRNA level increased at 3 h and 9 h after LPS treatment, showing biphasic pattern, while its mRNA level in the jejunum increased only at 3 h (Fig. (**1**)). As noted earlier, its level in the duodenum and the colon was hardly detectable and did not increase in response to LPS treatment (Fig. (**1**)). Thus, iNOS gene expression was markedly site-specific. Morin *et al*., using the same model as ours, previously reported that, after LPS treatment, there was a marked expression of iNOS mRNA in the ileum, whereas much lower concentration of iNOS mRNA were detected in the jejunum and colon, and no iNOS mRNA were detected in the duodenum, however, examining iNOS expression only at 2 h after the treatment in the four regions of the intestine [12]. In light of our findings on the time courses of induction of iNOS mRNA, it is possible that an increase at 3 h may have been missed, and if so their results are not inconsistent with our findings in this study.

As shown in Fig. (**3**), iNOS mRNA levels in the ileum, but not in the duodenum, the jejunum and the colon, were significantly increased also following IL-6 administration. We previously reported that, using the same rat model, serum IL-6 level was significantly increased after LPS treatment [4]. It has also been reported that IL-6 upregulates iNOS expression mediated through JAK/STAT3 pathway in rat cultured myocytes [14]. The present study showed that LPS treatment induced intestinal iNOS gene expression most prominently in the ileum compared with other regions. Taken together, increased serum IL-6 levels following LPS treatment might be involved in the induction of intestinal iNOS mRNA, especially for the second peak of the induction in the ileum after LPS treatment. In support of our hypothesis, very recently, it has been reported that expression of iNOS mRNA is decreased in IL-6 knockout mice compared with that of wild-type mice in acute colitis induced by dextran sodium sulfate [15], suggesting the involvement of IL-6 in the induction of iNOS in intestinal tissue inflammation.

The mechanism behind the site-specific induction of iNOS mRNA after LPS treatment is unclear and has not been determined yet. However, it has been reported that blood flow to the ileum is selectively decreased after intraperitoneal administration of LPS to rats, whereas blood flow to the rest of the intestine was normal [16]. Furthermore, following LPS treatment, mucosal permeability was also

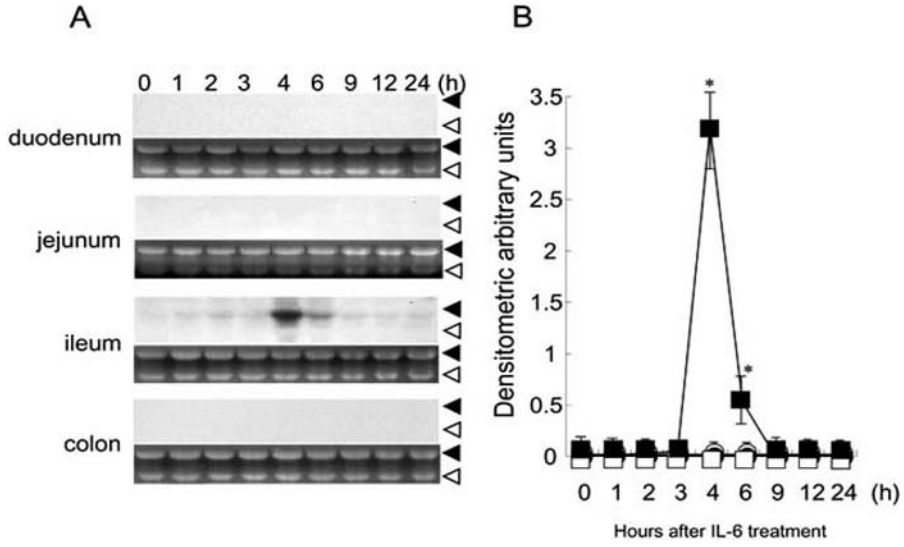


**Fig. (3). Effect of IL-6 administration on iNOS gene expression in various regions of the intestine.** Intestines were excised at indicated time points after intravenous injection of IL-6 (10 μg/kg), and dissected into four segments, i.e., duodenum (●), jejunum (○), ileum (■), and colon (□). (A) Shown are the autoradiographic signals of RNA blot hybridized with [α-$^{32}$P] dCTP labeled iNOS cDNA. Ethidium bromide staining of the same RNA is shown as a loading control. Three independent experiments showed similar results, and a typical example is shown in the figure. *Filled arrowhead, 28S ribosomal RNA; open arrowhead, 18S ribosomal RNA,* (B) Levels of iNOS mRNA are expressed as densitometric arbitrary units. Data are presented as means ± SEM (n = 3 for each region). * $p$ <0.05, v.s. duodenum, jejunum or colon for each time point.

increased at the ileum but not at sites of normal blood flow [16]. In contrast, allopurinol, a competitive inhibitor of xanthine oxidase, ameliorated the LPS-induced decrease in ileal blood flow as well as the increase in ileal permeability [16], suggesting that the ileum is more vulnerable to ischemia-reperfusion injury, which leads to the enhanced tissue inflammation. It is well known that LPS-induced inflammatory response is characterized by the cascading expression of various inflammatory mediators originating from the rapid and transient expression of tumor necrosis factor-α which leads to the expression of IL-6 as well as iNOS [17-19]. Collectively, these factors might contribute the prominent but transient induction of iNOS as well as its biphasic induction in the ileum after LPS treatment.

Consistent with the marked induction of iNOS mRNA in the ileum, iNOS protein expression was also markedly and specifically increased in the mucosal epithelial cells in the ileum (Fig. (**2**)). Moreover, when compared the intensity of iNOS expression in the villous cells to those of crypt cells, the latter is much stronger than the former (Fig. (**2**)). Cook *et al*., found immunohistochemical evidence of iNOS protein expression throughout the crypt and villus epithelium of the small intestine and that the greatest intensity of immunohistochemical staining was localized to the crypts [10]. Our results in this study confirmed these previously reported findings.

Nitric oxide can react with superoxide anion to form peroxynitrite [8]. The resultant free radical can be rapidly protonated to yield even more toxic substances such as hydroxyl radical and nitric dioxide [8]. The generation of either of these free radical species can promote lipid peroxidation and subsequent tissue injury if they are formed in excessive amounts [20-22]. Previously published study indicates that the distal small intestine is more susceptible than the proximal small bowel to NO-dependent stress [9]. We have also demonstrated that tissue inflammation and injury are more prominent in the ileum compared with those of the jejunum in LPS-treated rats, as judged both by histologic analysis and by tumor necrosis factor-α gene expression, while expression of heme oxygenase-1, which is known to play a protective role against various oxidative tissue injuries [23, 24], in the ileum is much less than that of the jejunum [4]. Moreover, very recently, Han *et al*., have reported that LPS-induced epithelial tight junction dysfunction of the ileum is iNOS dependent in mice, using pharmacologic inhibition of iNOS activity or genetic ablation of the iNOS gene [25]. Thus, enhanced iNOS expression in the epithelial cells of the ileum may play an offensive role in intestinal mucosal injury in endotoxemia.

In conclusion, LPS treatment of the rat markedly induced iNOS expression in the mucosal epithelial cells of the intestine. Gene expression of iNOS was the most significant, but biphasic in the ileum, followed by the monophasic induction in the jejunum. IL-6 administration upregulated intestinal iNOS gene expression exclusively in the ileum. These findings suggest that intestinal iNOS expression was upregulated not only in a site-specific, but also in a cell type-specific manner in a rat model of endotoxemia, possibly through increasing serum IL-6 levels.

## ACKNOWLEDGEMENT

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## METHODS

### Animals

Animal experiments were approved by the Animal Care Committee of Okayama University Medical School; care and handling of the animals were in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats, weighing 220-250 g, were purchased from Charles River (Yokohama, Japan). They were housed in a temperature-controlled (25°C) room with alternating 12-hr/12-hr light/dark cycles, and were allowed free access to water and chow diet until the start of experiments. Endotoxemia was induced by LPS treatment of rats as described previously [3-5]. Animals were injected intraperitoneally with LPS (*Escherichia coli,* 0128:B8, Sigma Chemical, St. Louis, MO; 10 mg/kg) dissolved in 1 mL of sterile physiologic saline. Control rats received the same volume of sterile physiologic saline. After injection, animals were returned to cages and allowed free access to food and water. In another set of experiments, rats were administered intravenously with 10 μg/kg of rat IL-6 (PeproTechEC Ltd., London, UK) which was dissolved in sterile physiological saline [4]. The endotoxin concentration of the IL-6 is less than 0.1 ng per μg. Under light anesthesia with ethyl ether, animals were sacrificed by decapitation at each defined time point (0 to 72 h), the entire intestine was excised and then dissected into duodenum, jejunum, ileum and colon, as described previously [4, 12]. After quick and gentle rinsing in physiologic saline, tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until used for RNA preparation.

### RNA Isolation and Northern Blot Analysis

Total RNA was isolated from the rat tissues using Tri-Reagent™ (Sigma Chemical) according to the manufacturer's protocol. Northern blotting was performed as described previously [4]. Twenty micrograms of total RNA were subjected to electrophoresis in a 1.2% agarose gel containing 6.5% formaldehyde. After blotting on a sheet of Bio-Rad Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA), RNA samples were hybridized with [α-$^{32}$P]dCTP-labeled rat iNOS cDNA probe [26] followed by washing under stringent conditions. The membrane was exposed to a sheet of Fuji Medical radiograph film with an intensifying screen at -70°C, and autoradiographs were quantified by using an image scanner (GelPrint 2000i, Genomic Solutions, Ann Arbor, MI) and a computerized image analysis software (Basic Quantifier version 3.0, Genomic Solutions) [4].

### Immunohistochemistry

Intestinal tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4- to 6-μm thickness. After deparaffinization and dehydration, sections were subjected to immunohistochemical analysis. Endogenous peroxidase activity was first blocked by 3%

hydrogen peroxide, followed by incubation with rabbit polyclonal anti-rat iNOS (Cayman CHEMICAL, Ann Arbor, MI) at 37°C for 3 h. The antigen-antibody reaction was detected using an anti-rabbit secondary antibody and an avidin-biotin immunoperoxidase staining kit (DAKO, Carpenter, CA). The positive reaction was visualized as brown stain following treatment with 3, 3'-diaminobenzidine. Normal rabbit serum was used as control for non-specific staining. Sections were counterstained with hematoxylin.

### Statistical Analysis

Statistical evaluation was performed with analysis of variance followed by Scheffé's *F*-test using Statview software (Abacus Concepts, Berkeley, CA). Data are presented as means ± SEM. Differences were considered as significant at $p < 0.05$.

## ABBREVIATIONS

IL-6 = Interleukin-6

iNOS = Inducible isoform of nitric oxide synthase

LPS = Lipopolysaccharide

NO = Nitric oxide

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