

# Effect of lipopolysaccharide on diarrhea and gastrointestinal transit in mice: Roles of nitric oxide and prostaglandin E<sub>2</sub>

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## Abstract

**AIM:** To investigate the effect of lipopolysaccharide (LPS) on the diarrheogenic activity, gastrointestinal transit (GIT), and intestinal fluid content and the possible role of nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in gastrointestinal functions of endotoxin-treated mice.

**METHODS:** Diarrheogenic activity, GIT, and intestinal fluid content as well as nitric oxide and PGE<sub>2</sub> products were measured after intraperitoneal administration of LPS in mice.

**RESULTS:** LPS dose-dependently accumulated abundant fluid into the small intestine, induced diarrhea, but decreased the GIT. Both nitric oxide and PGE<sub>2</sub> were found to increase in LPS-treated mice. Western blot analysis indicated that LPS significantly induced the protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 in mice intestines. Pretreatment with N<sup>G</sup>-nitro-L-arginine-methyl ester (L-NAME, a non-selective NOS inhibitor) or indomethacin (an inhibitor of prostaglandin synthesis) significantly attenuated the effects of LPS on the diarrheogenic activity and intestine content, but reversed the GIT.

**CONCLUSION:** The present study suggests that the pathogenesis of LPS treatment may mediate the stimulatory effect of LPS on nitric oxide and PGE<sub>2</sub> production and NO/prostaglandin pathway may play an important role on gastrointestinal function.

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**Key words:** Diarrhea; Gastrointestinal Transit; Lipopolysaccharide; Nitric Oxide; Prostaglandin E<sub>2</sub>

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## INTRODUCTION

Inflammatory reactions can cause various clinical manifestations frequently associated with abnormal motility of the gastrointestinal tract, such as nausea, vomiting, ileus, or diarrhea. It has been reported that bacterial lipopolysaccharide (LPS) could induce various alterations in gastrointestinal function. Intestinal motility, secretion and integrity of the mucosa are important barrier functions against the penetration of bacteria and endotoxins from the gut lumen to the blood stream. Impaired intestinal absorption is a major mechanism for diarrhea caused by endotoxin lipopolysaccharide (LPS) and is generally accompanied with damage to the intestine<sup>[1-3]</sup>. Besides gastric motility impairment, the gastrointestinal transit (GIT) increased during LPS-induced endotoxaemia in rats<sup>[2,4]</sup>. Several factors have been reported to be important to intestinal absorption, including intact epithelial transport processes and the adequate time for digestion and contact with the epithelium. Therefore, reductions of small intestinal and whole gut transit times may result in an impaired intestinal absorption of nutrients, electrolytes and fluid. During severe bacterial infection, endotoxin LPS would be released due to the breakdown of the cell wall of Gram-negative bacteria. It is well known that LPS derives both cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) gene expression in many tissues resulting in increase in the release of prostaglandins (PGs)<sup>[5]</sup> and nitric oxide (NO)<sup>[6-8]</sup>. In addition, many other<sup>[9-11]</sup> substances have also been shown to be stimulated by LPS, including cytokines<sup>[12,13]</sup>, vasoactive substances<sup>[14]</sup>, and procoagulant factors<sup>[15,16]</sup>.

There is evidence that diarrhea might be amplified by a variety of endogenous secretagogues, including PGs. PGs and leukotrienes also as pro-secretory in inflammatory mediators within the mucosa can not only reduce intestinal absorption<sup>[17-21]</sup> but also initiate a pro-secretory state in intestines. PGs have complex pharmacological and biological activities in the modulation of gastrointestinal functions, including contraction or relaxation of smooth muscles and inhibition or enhancement of neurotransmitter release, *etc.*<sup>[9-11]</sup>.

The discovery of NO as a neurotransmitter has radically altered the idea about synaptic transmission because NO is a labile free radical and is not stored in synaptic vesicles as other neurotransmitters. NO is believed to be an inhibitory nonadrenergic, noncholinergic neurotransmitter that mediates gastrointestinal motility in physiological and certain pathophysiologic states, such as in absorptive and secretory processes<sup>[22-24]</sup>. NO exhibits apparent dual roles in modulating intestinal transport pathway, and has both a pro-absorptive activity and a pro-secretory activity depending on the site of release and local NO concentration. It has been reported that NO could regulate PG synthesis in mouse macrophage cells<sup>[25,26]</sup>, rabbit kidneys<sup>[27]</sup> and rat uterus<sup>[28]</sup> and interact with PGs in stomach in the regulation of gastric mucosal integrity<sup>[29,30]</sup>. On the other hand, NO biosynthesized from L-arginine has been shown to be involved in various aspects of the pathophysiology of gastrointestinal mucosae<sup>[31]</sup>.

Several authors have demonstrated that LPS could trigger

the formation of NO/cyclic GMP pathways through activation of NOS to induce its effects<sup>[32-34]</sup>. Little information is available about the effects of NO and PGs on LPS-induced diarrhea. In the present study, we performed experiments to assess the effects of N-nitro-L-arginine-methyl ester (L-NAME) (an inhibitor of NOS) or indomethacin (an inhibitor of COX) on LPS-induced gastrointestinal responses in mice.

## MATERIALS AND METHODS

### Chemicals

Lipopolysaccharide 055:B4 (*E. coli* serotype), N-nitro-L-arginine-methyl ester (L-NAME), L-arginine, and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Acacia gum was obtained from Wako Pure Chemical Industries (Japan). Indomethacin was dissolved in ethanol (50%) and diluted in 0.9% saline, and other drugs were dissolved in normal saline for administration. All drugs were freshly prepared on the day of testing and injected intraperitoneally (IP) in a volume of 0.1 mL/10 g of body weight.

### Animals

Male ICR mice (Animal Center of National Taiwan University, Taipei, Taiwan) weighing 20-25 g were used in this study. The animals were maintained in an air-conditioned room with a 14:10 h light/dark cycle, fed with regular chows and allowed free access to tap water. Before experimentation, the animals were fasted but allowed to drink water for 18 h.

### Estimating gastrointestinal transit and small intestinal fluid content

Gastrointestinal transit (GIT) was measured as described by Puig *et al.*<sup>[35]</sup> with minor modifications. All experiments began at the same time of the day. The animals received intraperitoneally (IP) 5-30 mg/kg of LPS. Intestinal transit was measured 2 h after LPS injection. Thirty minutes before the scheduled time of measurement, the mice received 0.5 mL of activated charcoal meal (0.5 mL of 10% charcoal suspended in 10% gum acacia) by gavage to the esophagus of the fasted mice. Thirty min after charcoal administration, the animals were killed by cervical dislocation, and the small intestine was separated from the omentum, avoiding being stretched. The length of intestine from pyloric sphincter to the ileocecal junction and the distance traveled by the charcoal meal were measured. For each animal, GIT was calculated as the percent of the distance traveled by the charcoal, relative to the total length of the intestine (percent of GIT). The inhibitory effects of drugs on GIT were expressed as a percentage of inhibition of the GIT in a drug-treated animal (test GIT) when compared with the mean GIT measured in a group of vehicle-treated mice (vehicle GIT): % inhibition = [(vehicle GIT - test GIT) / (vehicle GIT)] × 100.

### Determination of small intestinal fluid content

For determination of small intestinal fluid content, the fasted mice were pretreated (IP) with various kinds of drugs for 15 min, and/or various doses of LPS. After 2 h, the small intestine was removed and the intraluminal fluid accumulation of intestinal fluid was weighed<sup>[36,37]</sup>.

### Diarrheogenic activity

The diarrheogenic activity was measured as described elsewhere<sup>[36,37]</sup>. Mice were given diet and water *ad libitum* before experiment, but not during testing. Animals were individually placed in a box, the bottom of which was covered with a sheet of filter paper for the observation of the fecal state. Two hours later, LPS (5-30 mg/kg) was administered in only normal mice

without spontaneous diarrhea. After administration of LPS, the diarrheogenic effect was evaluated 4 times at 1 h intervals. The filter paper was exchanged after each evaluation. Any formless or liquid stool with a stain on the filter paper indicated positive diarrhea cases. Each group consisted of 10 mice and the observation lasted 4 h in all experiments.

### Determination of PGE<sub>2</sub> and nitrite

The levels of plasma nitrate were measured as described previously<sup>[38]</sup>. Briefly, for the reduction of liquid nitrate to the gas NO, 10 μL was injected into a collection chamber containing 5% VCl<sub>3</sub>. NO was carried by a constant stream of helium gas into a NO analyzer (Seivers 270B NOA, Seivers Instruments Inc). The level of PGE<sub>2</sub> in the intestine tissues was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham).

### Gel electrophoresis and Western blotting

Tissues were stored at -80 °C before preparation for SDS-PAGE, at which time they were thawed at 4 °C. Tissue portions (1 g) were added to 3 mL of chilled RIPA buffer (0.15 mol/L NaCl, 50 mmol/L Tris, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, and 0.1% SDS), including protease inhibitors. This mixture was homogenized on ice and centrifuged at 4 °C, and the supernatant was saved. Protein analysis of extract aliquots was performed (DC protein assay, Bio-Rad, Hercules, CA). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2×SDS-PAGE sample buffer and boiled for 4 min. Lysates were loaded on a 10% SDS-polyacrylamide gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Life Science, Birmingham, UK) using an electroblotting minitransfer apparatus according to the manufacturer's protocol. Membranes were blocked at room temperature for 60 min in Tris-buffered saline plus 0.05% Tween 20 (TBST) and 5% dry powdered milk, washed twice with TBST and incubated for 1 h in a monoclonal antibody against iNOS, COX-2 or GAPDH (Transduction Lab, Lexington, KY, USA). After washing three times for 10 min each with TBST, the membranes were incubated for 45 min with horseradish peroxidase-conjugated secondary antibody. After washing three times again for 10 min each with TBST, the membranes were developed for visualization of protein by addition of enhanced chemiluminescence reagent according to the manufacturer's instructions (Amersham, Princeton, NJ). Densitometry was performed on selected blots using an appropriate software.

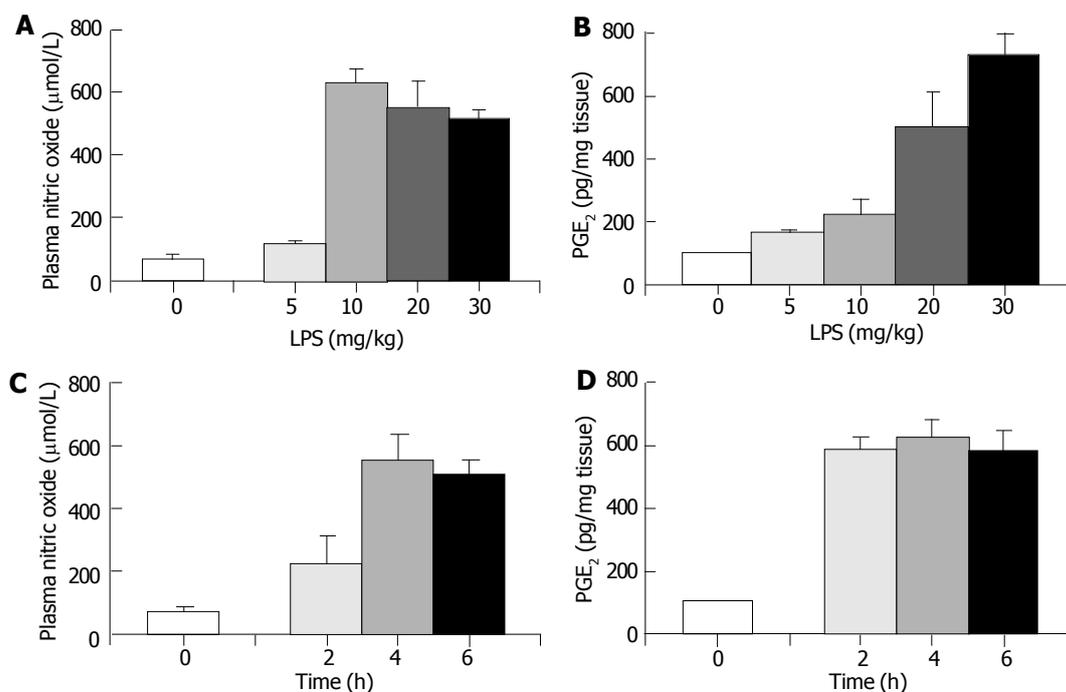
### Statistical analysis

All data were expressed as mean ± SE. Results from control, LPS, and drugs/LPS-treated animals were compared by either unpaired Student's *t*-test or ANOVA test. *P* < 0.05 was considered statistically significant.

## RESULTS

### Effect of LPS on gastrointestinal transit and small intestinal content

Intraperitoneal (IP) administration of LPS (5-30 mg/kg) produced a dose-related decrease in GIT; the inhibitory percent of GIT was from 17% to 42% (Table 1). Administration of LPS induced an increase in blood serum concentration of nitric oxide and intestine concentration of PGE<sub>2</sub> in a dose-dependent and time-dependent manner. A significant effect of LPS was observed at concentrations of 5-30 mg/kg in the production of nitrite and PGE<sub>2</sub> respectively (Figure 1A, B). The maximal level of the production of nitrite and PGE<sub>2</sub> was reached at 4 h after LPS administration (Figure 1C, D). To determine whether the



**Figure 1** Effect of lipopolysaccharide on the production of NO and PGE<sub>2</sub> in mice blood and intestine. Mice were IP administered with various doses of LPS for 4 h (A, B) or LPS (20 mg/kg) for indicated times (C, D). The blood NO and PGE<sub>2</sub> were determined as described in "Materials and Methods." Data are expressed as mean $\pm$ SE of 10 mice. For each column,  $P < 0.05$ , significantly different from individual control.

production of nitrite and PGE<sub>2</sub> resulted from the induction of iNOS and COX-2 in LPS-treated mice, Western blot analysis was performed. As shown in Figure 2, the protein expression of iNOS and COX-2 significantly increased in a time-dependent manner after administration of LPS. These results indicated that the increase of nitrite and PGE<sub>2</sub> was due to the induction of iNOS and COX-2 by LPS administration. The IP administration of the competitive inhibitors of NO synthase, L-NAME (10-20 mg/kg), following the endotoxin administration, dose-dependently attenuated delay in GIT. The effects of L-NAME (10 mg/kg) on LPS (20 mg/kg) activity could be reversed by mice treated with L-arginine (800 mg/kg) but not with D-arginine (800 mg/kg). On the other hand, pretreatment with prostaglandin synthesis inhibitor indomethacin (10-20 mg/kg) also inhibited the small intestinal responses induced by LPS (20 mg/kg) (Table 2).

Another experiment demonstrated that LPS significantly increased the intestinal content in a dose-dependent manner. The increase of intestinal content induced by LPS was also attenuated by pretreatment with L-NAME (15 mg/kg) or indomethacin (15 mg/kg) (Table 3).

#### Effect of LPS on diarrheogenic activity

Intraperitoneal administration of LPS (5-30 mg/kg) exhibited a dose- and time-dependent increase in the diarrheogenic activity, with ED<sub>50</sub> values of 5 $\pm$ 3.2 mg/kg (Table 4). There was no significant increase in the diarrheogenic activity during the first 2 h of LPS treatment. In addition, administration of normal saline did not produce diarrhea. L-NAME (10-20 mg/kg) dose-dependently attenuated the diarrheogenic activity induced by LPS (20 mg/kg). The effects of L-NAME (10 mg/kg) on LPS (20 mg/kg) activity could be reversed by mice treated with L-arginine (800 mg/kg) but not with D-arginine (800 mg/kg). In addition, the inhibitory effect of indomethacin on LPS-induced diarrheogenic activity could be reversed by addition of L-arginine (800 mg/kg) (Table 5). These results indicated that both nitric oxide and PGE<sub>2</sub> were involved in the diarrhea activity in LPS-treated mice.

**Table 1** Effect of lipopolysaccharide (LPS) on gastrointestinal transit (GIT) in mice (mean $\pm$ SE)

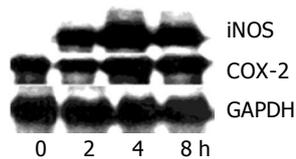
Treatment	Percent of GIT	% Inhibition of GIT
Saline	66.4 $\pm$ 6.5	0
LPS 5 mg/kg	55.1 $\pm$ 5.7	17
LPS 10 mg/kg	50.5 $\pm$ 3.0 <sup>a</sup>	24.38 <sup>a</sup>
LPS 20 mg/kg	47.5 $\pm$ 6.0 <sup>a</sup>	28.46 <sup>a</sup>
LPS 30 mg/kg	38.5 $\pm$ 6.2 <sup>a</sup>	42 <sup>a</sup>

<sup>a</sup> $P < 0.05$  vs the values of normal saline-treated mice.

**Table 2** Effects of L-NAME or indomethacin, and arginine on lipopolysaccharide (LPS)-induced gastrointestinal transit (GIT)

Treatment	Percent of GIT	% Inhibition of GIT
Saline	65.6 $\pm$ 6.1	0
LPS 20 mg/kg	35.3 $\pm$ 5.4 <sup>a</sup>	43
LPS 20 mg/kg+L-NAME 10 mg/kg	56.7 $\pm$ 6.9 <sup>c</sup>	14
LPS 20 mg/kg+L-NAME 20 mg/kg	58.9 $\pm$ 6.6 <sup>c</sup>	10
LPS 20 mg/kg+L-NAME 10 mg/kg+L-arginine 800 mg/kg	44.2 $\pm$ 6.5	33
LPS 20 mg/kg+L-NAME 10 mg/kg+D-arginine 800 mg/kg	55.6 $\pm$ 6.2 <sup>c</sup>	15
LPS 20 mg/kg+indomethacin 10 mg/kg	53.1 $\pm$ 6.3 <sup>c</sup>	22
LPS 20 mg/kg+indomethacin 20 mg/kg	57.6 $\pm$ 5.7 <sup>c</sup>	15
LPS 20 mg/kg+indomethacin 10 mg/kg+L-arginine 800 mg/kg	43.3 $\pm$ 4.2	36
LPS 20 mg/kg+L-NAME 10 mg/kg+D-arginine 800 mg/kg	55.6 $\pm$ 6.2 <sup>c</sup>	15

<sup>a</sup> $P < 0.05$  vs the values of normal saline-treated mice, <sup>c</sup> $P < 0.05$  vs the values of LPS alone-treated mice.



**Figure 2** Time course of lipopolysaccharide (LPS) on protein expression of iNOS and COX-2 in the small intestine of mice. Mice were administered with LPS (20 mg/kg) for indicated times and the total cell lysates were used to perform the Western blot as described in the "Materials and Methods."

**Table 3** Effects of L-NAME and indomethacin on lipopolysaccharide (LPS)-induced change of small intestinal content (mean±SE)

Treatment	Intestinal content (g/mouse)	% Intestinal content
Saline	0.14±0.02	100
LPS 5 mg/kg	0.18±0.02	129
LPS 10 mg/kg	0.21±0.01 <sup>a</sup>	150
LPS 20 mg/kg	0.25±0.03 <sup>a</sup>	179
LPS 30 mg/kg	0.26±0.03 <sup>a</sup>	185
LPS 20 mg/kg+L-NAME 15 mg/kg	0.16±0.03 <sup>c</sup>	114
LPS 20 mg/kg+indomethacin 15 mg/kg	0.16±0.02 <sup>c</sup>	114

<sup>a</sup>*P*<0.05 vs the values of normal saline-treated mice, <sup>c</sup>*P*<0.05 vs the values of LPS alone-treated mice.

**Table 4** Effects of lipopolysaccharide (LPS)-induced diarrheogenic activity in mice (mean±SE)

Dose (mg/kg)	Diarrheogenic activity (%)	Time course (h)	Diarrheogenic activity (%)
Saline	0	0	0
LPS, 5	49.6±3.0 <sup>a</sup>	1	10.0±10
LPS, 10	56.6±3.9 <sup>a</sup>	2	31.0±15
LPS, 20	87.7±4.0 <sup>a</sup>	4	90.0±10 <sup>a</sup>
LPS, 30	97.5±1.7 <sup>a</sup>	6	94.0±8 <sup>a</sup>

<sup>a</sup>*P*<0.05 vs the values of normal saline-treated mice.

**Table 5** Effects of L-NAME or/and arginine on lipopolysaccharide (LPS)-induced diarrheogenic activity (mean±SE)

Treatment	Diarrheogenic activity (%)
Saline	0
LPS 20 mg/kg	87.7±4.0 <sup>a</sup>
LPS 20mg/kg+L-NAME 10 mg/kg	40±4.36
LPS 20 mg/kg+L-NAME 20 mg/kg	30±4.46 <sup>c</sup>
LPS 20 mg/kg+L-NAME 10 mg/kg +L-arginine 800 mg/kg	70±2.15
LPS 20 mg/kg+L-NAME 10 mg/kg +D-arginine 800 mg/kg	50±3.23
LPS 20 mg/kg+Indomethacin 10 mg/kg	30±4.25 <sup>c</sup>
LPS 20 mg/kg+Indomethacin 20 mg/kg	20±2.41 <sup>c</sup>
LPS 20 mg/kg+Indomethacin 15 mg/kg+L-arginine 800 mg/kg	50±3.62

<sup>a</sup>*P*<0.05 vs the values in corresponding saline-treated mice, <sup>c</sup>*P*<0.05 vs the values in LPS (20 mg/kg)-treated mice.

## DISCUSSION

Lipopolysaccharide (LPS) is an endotoxin in cell walls of Gram-negative bacteria, which could cause multiple hemodynamic and metabolic changes<sup>[39]</sup>. LPS involves the activation of macrophages that are known to secrete at least 100 biologically active substances, including arachidonic acid metabolites such as prostaglandins and other eicosanoids, as well as nitric oxide and cytokines<sup>[40]</sup>. Several reports strongly suggested that the intestinal muscularis resident macrophage network should be an important mediator of endotoxin-induced gut dysmotility<sup>[9,41,42]</sup>. In addition, LPS could increase iNOS expression and stimulate NO production in muscularis resident macrophages, resulting in the inhibition of smooth muscle contraction. In this study, we found that LPS significantly increased the diarrheogenic activity, small intestinal fluid content, and decreased the small intestinal propulsion. Additional results showed that LPS increased the protein expression of iNOS and COX-2 in the intestine (Figure 2). These effects were attenuated by pretreatment with NOS inhibitor L-NAME or prostaglandin synthase inhibitor indomethacin, indicating that both nitric oxide and prostaglandin were involved in the diarrheogenic activity, small intestinal fluid content, and gastrointestinal motility. It is possible that the production of nitric oxide and prostaglandin was mediated by the intestinal resident macrophages during endotoxemia. However, we cannot rule out the possibility of the induction of nitric oxide and prostaglandin as exhibited in intestinal smooth muscle cells and other intestinal cells.

The present data also showed that the diarrhea observed after administration of LPS was due to accumulation of abundant fluid in the small intestine rather than intestinal hypermotility. Under normal conditions the rate of lumen-to-blood fluid influx is several-fold greater than actual net absorption, indicating a respectable blood-to-lumen fluid efflux. In diarrhea, the water efflux outpaces the influx? And results in a net secretory condition and an increase of small intestinal fluid content. Several authors have presented evidence that LPS could trigger the formation of NO/cyclic GMP pathways through activation of NOS and inhibition of gut motility. NO has also been demonstrated to play an important role in the control of secretion and conditions associated with pathologic changes of the gut. For example, excess NO could lead to gut secretion via elevation of intracellular cGMP and cAMP concentrations<sup>[43]</sup>. In this study, LPS led to diarrhea and cGMP and cAMP levels were mediated by NO from iNOS. In addition, LPS might change the normal influx/efflux ratio, and particularly decreased the lumen-to-blood fluid influx. Therefore, LPS treatment increased both small intestinal content and diarrheogenic activity.

Taken together, the present study suggests that NO and prostaglandins play an important role in inducing diarrhea and further support the interaction between NO and prostaglandin.

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