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Inducible nitric oxide synthase and tumor necrosis factor-α in delayed gastric emptying and gastrointestinal transit induced by lipopolysaccharide in mice

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Abstract

Gastrointestinal motility disturbances during endotoxemia are probably caused by lipopolysaccharide (LPS)-induced factors: candidates include nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-1ß, and interleukin-6. Flow cytometry was used to determine the effects of LPS and these factors on gastric emptying (evaluated indirectly by determining percent gastric retention; %GR) and gastrointestinal transit (GIT) in male BALB/c mice (23-28 g). NO (300 µg/mouse, N = 8) and TNF-α (2 µg/mouse, N = 7) increased ($P < 0.01$) GR and delayed GIT, mimicking the effect of LPS (50 µg/mouse). During early endotoxemia (1.5 h after LPS), inhibition of inducible NO synthase (iNOS) by a selective inhibitor, 1400 W (150 µg/mouse, N = 11), but not antibody neutralization of TNF-α (200 µg/mouse, N = 11), reversed the increase of GR (%GR 78.8 ± 3.3 *vs* 47.2 ± 7.5%) and the delay of GIT (geometric center 3.7 ± 0.4 *vs* 5.6 ± 0.2). During late endotoxemia (8 h after LPS), both iNOS inhibition (N = 9) and TNF-α neutralization (N = 9) reversed the increase of GR (%GR 33.7 ± 2.0 *vs* 19.1 ± 2.6% (1400 W) and 20.1 ± 2.0% (anti-TNF-α)), but only TNF-α neutralization reversed the delay of GIT (geometric center 3.9 ± 0.4 *vs* 5.9 ± 0.2). These findings suggest that iNOS, but not TNF-α, is associated with delayed gastric emptying and GIT during early endotoxemia and that during late endotoxemia, both factors are associated with delayed gastric emptying, but only TNF-α is associated with delayed GIT.

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Introduction

Endotoxemia, which occurs after lipopolysaccharide (LPS) injection, delays gastric emptying and disrupts intestinal transit (1-7). However, it is not clear whether the latter effect involves a delay or acceleration since both have been reported (1,5-7). While uncertainties remain about the nature of the effect of LPS on gastrointestinal motility, the effect is suspected to involve various cytokines that are produced in response to LPS (1-8). Intraperitoneal (*ip*) injection of LPS in mice elicits a surge in serum tumor necrosis factor-α (TNF-α) at 1-2 h, followed by an increase in serum interleukin-1ß (IL-1ß), and a subsequent increase in serum levels of IL-6 (5). In addition, serum nitric oxide (NO) increases soon after LPS injection and remains high for up to 12 h (5). These factors are likely to cause distur-

bances of gastrointestinal motility, but it is not known which of them causes the disturbance during endotoxemia.

Since there is some controversy about the effect of LPS on intestinal transit, the first aim of this study was to determine the effect of LPS on gastric emptying and gastrointestinal transit in mice using a new method, the flow cytometric method, that is exquisitely sensitive to the effects of drugs on gastrointestinal motility (9,10). The method has advantages over other standard methods such as those using radioactive chromium or phenol red as markers in that it does not use an isotope and can measure the center of gravity of the marker (a measure of the distribution of the marker within the gastrointestinal tract) as well as how far the marker progresses along the intestine, thus greatly increasing the accuracy of measurement of gastrointestinal transit. The second aim was to determine whether NO, TNF-α, IL-1ß, or IL-6 have an endotoxemia-like effect on gastric emptying and gastrointestinal transit in normal mice. Third, we sought to further investigate any such endotoxemia-like effect by determining whether inhibition of NO, TNF-α, IL-1ß, or IL-6 reversed the disturbances during endotoxemia.

Material and Methods

Mice

Male BALB/c mice (Charles River, Yokohama, Japan), aged 6-7 weeks and weighing 23-28 g, were used. The study was approved by the Institutional Committee on Animal Research. The mice were housed under standardized environmental conditions, with a 12-h light/dark cycle. The animals were fasted for 16-24 h but were allowed free access to water until 20-30 min before having a test liquid introduced into the stomach. Each animal was housed individually in a wire-mesh cage to prevent coprophagy during fasting. All experiments measuring gastrointestinal motility were started between 6:00 and 9:00 am.

Measurement of gastrointestinal function

We evaluated gastric emptying and gastrointestinal transit by infusing liquid containing fluorescent and nonfluorescent microbeads into the stomach and measuring the quantity of the fluorescent microbeads at a fixed time thereafter using a flow cytometer (9,10). A mixture of fluorescent polystyrene microbeads 6 μm in diameter (Flow Check High Intensity Alignment Grade Particles, Polysciences, Inc., Warrington, PA, USA), and nonfluorescent microbeads 2.14 μm in diameter (Sphero UV fluorescent particles, BD Biosciences, San Jose, CA, USA) was used. The manufacturers provided these microbeads in water: 1 μL contained 2×10^3 6-μm microbeads or 6×10^6 2.14-μm microbeads.

Each mouse was lightly anesthetized with halothane and 0.2 mL saline containing the 50 μL fluorescent and 2 μL nonfluorescent microbeads was infused via a metal cannula (PS 7912, ISIS Co., Ltd., Osaka, Japan) into the stomach (9,10). An excess of smaller 2.14-μm nonfluorescent microbeads was included to block the absorption of the fluorescent markers by the gastrointestinal mucosa (9,10).

Thirty minutes later, the mouse was killed by an overdose of halothane. This time interval was selected to obtain the geometric center of 6-7 and to prevent the leading edge of the test fluid from going beyond the ileocecal junction (9,10). The esophagus, just proximal to the gastric fundus, and the duodenum, just distal to the pylorus, were cross-clamped, and the stomach and small intestinal tract were removed. If there was chyme in the stomach or small intestinal tract, the data were not used. The intestinal tract was placed on a ruled template and divided into ten equal segments. The stomach and each segment of the intestine were placed in individual tubes containing 5 mL phosphate-buffered saline. Each tube was vortexed

and 600 µL of the supernatant was filtered through a strainer (Cell-Strainer; BD Biosciences).

The quantity of fluorescent microbeads in each sample was measured using a flow cytometer (FACScan; BD Biosciences). The fluorescent microbeads are labeled with a fluorescent yellow-green dye, which absorbs the light of a 488-nm argon laser and emits yellow-green fluorescence. During flow cytometry, the fluorescent microbeads were gated by their distinct forward and side light-scatter profiles. The gated particles were further analyzed for the presence of intense fluorescence. The number of particles with high intensity fluorescence was counted for 30 s at a "high flow rate" (9,10) (Figure 1). Gastric emptying of the liquids was indirectly evaluated by determining the percent of gastric retention (%GR) (11) as described below:

$$\%GR = (\text{stomach count}/\text{total count}) \times 100,$$

where total count = stomach count + Σ(count in each intestinal segment).

Gastrointestinal transit was assessed us-

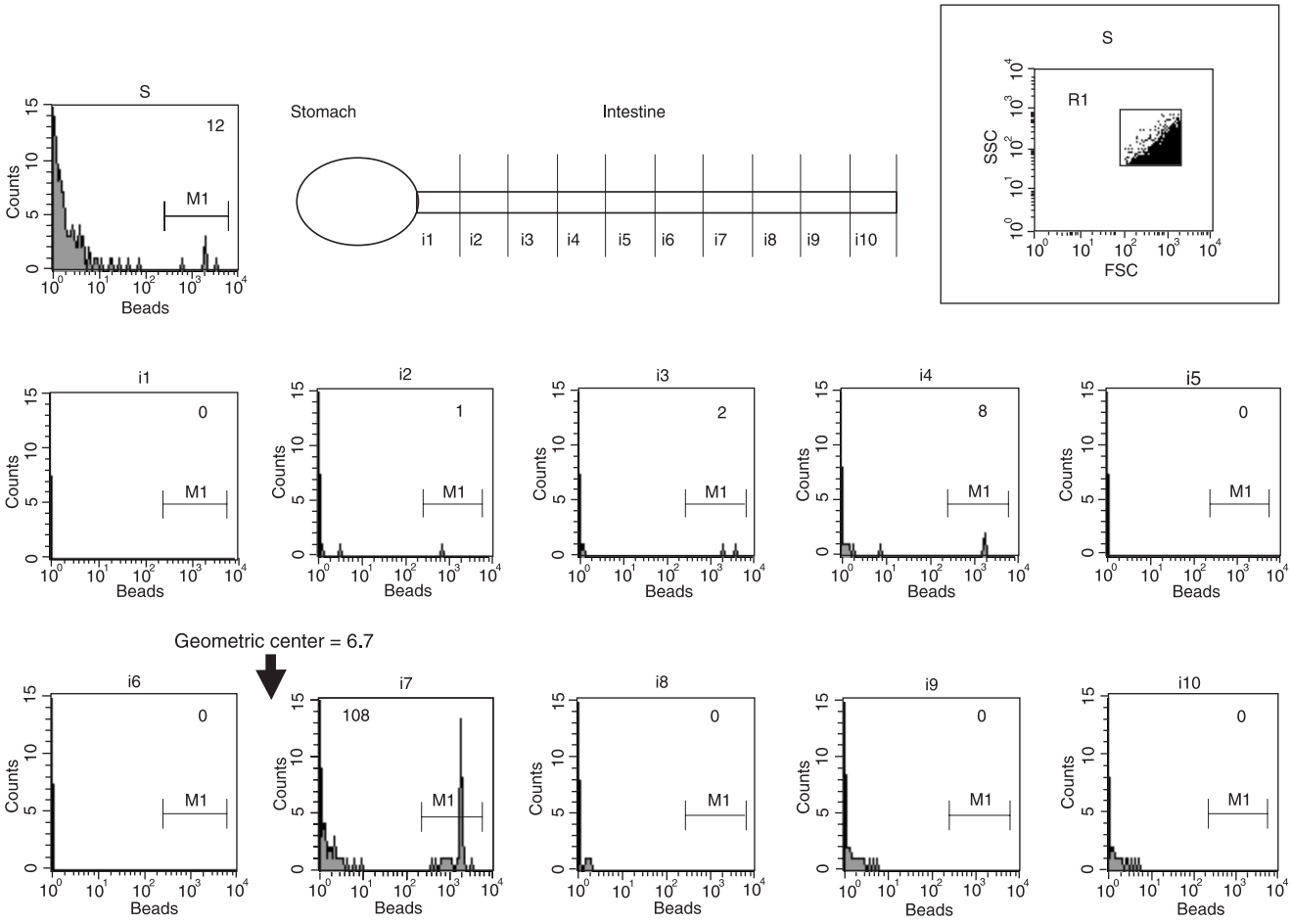

Figure 1. Measurement of gastric emptying and gastrointestinal transit by flow cytometry. This was done by infusing liquid containing fluorescent microbeads into the stomach and measuring the quantity of the beads at 30 min thereafter. The stomach and ten-equally divided segments of the small intestine were placed in individual tubes containing phosphate-buffered saline. The tubes were vigorously vortexed and the number of beads in the supernatant was counted using flow cytometry. In flow cytometry, the fluorescent microbeads were gated by their distinct forward (FSC) and side (SSC) light-scatter profiles (inset scatter plot). The gated (R1) particles were further analyzed for the presence of intense fluorescence, and the number of the particles with high intensity fluorescence (M1) was counted. Numbers in histograms indicate the amount of beads within each segment. S, stomach; i1-i10, small intestinal segments.

ing the "geometric center" (GC; the center of gravity). This was calculated by a method described previously (9,10): GC = Σ (count in intestinal segment x intestinal segment number)/Σ (count in each intestinal segment).

Effect of lipopolysaccharide

Since a previous study indicated that the cytokine profiles, especially those of TNF-α and NO, and the resulting disturbances in gastrointestinal transit were different at early (1.5 h) and late (8 h) times after bolus *ip* injection of LPS (5), we measured gastric retention and gastrointestinal transit at both times. Mice were randomly allocated to receive an *ip* injection of saline (250 μL) or LPS (10 or 50 μg in 250 μL saline; *Escherichia coli* O111:B4, Sigma, St. Louis, MO, USA). We selected the LPS doses based on our aim to study the toxin's effect at well below the lethal dose in mice. We determined the maximum sublethal dose of the lot of LPS used (lot No. 50K4088) to be 100 μg for male BALB/c mice. To further determine the efficacy of LPS, we also measured the serum TNF-α levels at 1.5 h after LPS (when the levels are considered to be highest) and tissue inducible NOS (iNOS) and neuronal NOS (nNOS) protein expressions at 8 h after LPS (when iNOS expression is considered to be high), as described below.

Enzyme-linked immunosorbent assay

Serum was separated by blood centrifugation (4ºC, 20,000 *g*, for 5 min) and stored at -80ºC until measurement. Concentrations of TNF-α were measured by enzyme-liked immunosorbent assay (Biosource International, Camarillo, CA, USA) according to manufacturer instructions. All samples were measured in triplicate. The inter- and intra-assay coefficients of variation of the kit were ≤8.7 and ≤6.5%, respectively, and the detection limit was 3 pg/mL.

Western blotting

Mid jejunum was frozen in liquid nitrogen, homogenized, and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% NP-40, 5 mM EDTA, 150 mM NaCl, 2 mM DTT, 1 mM sodium orthovanadate; Sigma), and a protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). The lysates (20 μg per lane) were then separated on a 7.5% SDS-polyacrylamide gel and transferred to a PVDF membrane (Sequi-Blot PVDF Membrane; Bio-Rad Laboratories, Hercules, CA, USA). iNOS and nNOS were detected with anti-iNOS antibody (1:2500; Pharmingen, San Diego, CA, USA) and nNOS antibody (1:1000; Zymed Laboratories Inc., San Francisco, CA, USA), respectively, and actin was detected with anti-actin antibody (1:2000; C-11; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). These proteins were then visualized using anti-IgG-HRP of appropriate species (Zymed Laboratories) and ECL plus Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK).

Effects of cytokines

Mice were randomly allocated to receive 250 μL saline *ip*, 2 μg recombinant murine TNF-α, IL-1ß, or IL-6 (endotoxin level <1 EU/μg) (PeproTech EC Ltd., London, UK) in 250 μL saline *ip*, or 300 μg of 1-hydroxy-2-oxo-3,3-bis (2-aminoethyl)-1-triazene (NOC18; an NO donor; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) (12) subcutaneously (*sc*) in 100 μL saline. Gastric retention and gastrointestinal transit were measured 30 min after *ip* saline or cytokine injection, or 1 h after *sc* NOC18 injection. The doses, routes, and time intervals were selected on the basis of previous studies (12-15), and a preliminary study indicated that these doses were likely to affect gastric emptying, thus precluding insufficient dosing. We limited the time to 30 min in order to measure the injected

cytokine (or NO) effect and minimize the effects of other cytokines induced *in vivo* by injected cytokines (or NO).

Effects of inhibitors

To determine the effect of inhibitors of iNOS and TNF-α on gastric emptying and gastrointestinal transit in early and late endotoxemia, mice were given *ip* saline, 150 µg of the selective iNOS inhibitor N-(3-(aminomethyl) benzyl) acetamidine (1400 W; Alexis Biochemicals, Lausen, Switzerland), or 200 µg anti-mouse TNF-α antibody. The anti-mouse TNF-α polyclonal antibody was purified, had a low endotoxin content (<2.91 EU/mg), and was preservative- and carrier-free (Pierce Endogen Inc., Woburn, MA, USA). Each inhibitor was administered *ip* in a volume of 250 µL saline 1 h before measurement. The doses of the inhibitors used were based on previous studies (6,16) and our preliminary experiments showing that any further increase in the dose failed to further affect the gastric emptying/intestinal transit. Since a previous study (6) and our preliminary results indicated that N^{ω}-nitro-L-arginine methyl ester (a non-selective inhibitor) and 1-(2-trifluoromethylphenyl) imidazole (a selective nNOS inhibitor) both delay gastric emptying in normal mice, we chose to use a selective iNOS inhibitor.

Statistical analysis

Data are reported as means ± SEM. One-way analysis of variance with Bonferroni correction was used to compare data between groups, with the level of significance set at $P < 0.05$.

Results

Efficacy of LPS treatment

At 1.5 h after LPS administration, serum concentrations of TNF-α increased significantly (495.8 ± 47.7 pg/mL with LPS *vs* below the detection limit with saline, N = 3 for each group). At 8 h after LPS, iNOS was expressed in the intestine, whereas nNOS expression became undetectable (Figure 2). These findings are consistent with previous reports showing that iNOS expression is induced and nNOS expression is down-regulated by LPS treatment (17,18), demonstrating the efficacy of endotoxemia induction in our study.

Effect of lipopolysaccharide

LPS increased gastric retention and delayed gastrointestinal transit during both early (1.5 h) and late (8 h) endotoxemia ($P < 0.05$; Figure 3A). The transit histogram shows that, with increasing LPS, the marker distribution at both times became broader and the distinct peak of distribution became less evident (Figure 3B).

Effects of cytokines

NO and all the cytokines tested increased gastric retention ($P < 0.05$; Figure 4). Gastrointestinal transit was delayed only by NO and TNF-α ($P < 0.01$; Figure 4). Therefore, the effect of NO and TNF-α mimicked that observed during endotoxemia.

Effects of inhibitors

At 1.5 h after LPS administration, inhibition of iNOS with 1400 W reversed the increase in gastric retention and the delay in

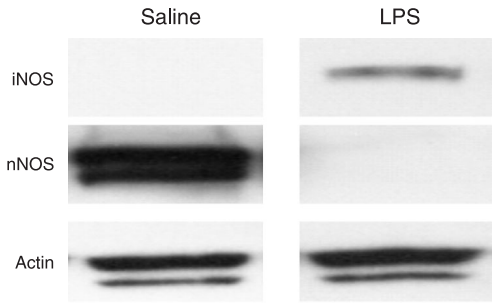

Figure 2. Expression of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) in jejunum at 8 h after *ip* lipopolysaccharide (LPS) (50 µg) or saline. Representative data of five independent experiments.

gastrointestinal transit ($P < 0.01$), while TNF-α neutralization with anti-TNF-α antibody had no significant effect (Figure 4). At 8 h after LPS administration, both iNOS inhibition and TNF-α neutralization reversed the increase in gastric retention ($P < 0.01$), while only TNF-α neutralization was able to reverse the delay in gastrointestinal transit ($P < 0.01$) (Figure 5). In otherwise untreated mice, 1400 W had no significant effect on gastric retention or gastrointestinal transit (%GR: 12.2 ± 2.1 with saline *vs* 14.8 ± 2.3% with 1400 W, GC: 6.9 ± 0.3 with saline *vs* 6.8 ± 0.4 with 1400 W, N = 5 for each group, $P > 0.05$). Anti-TNF-α antibody also had no significant effect on gastric retention or gas-

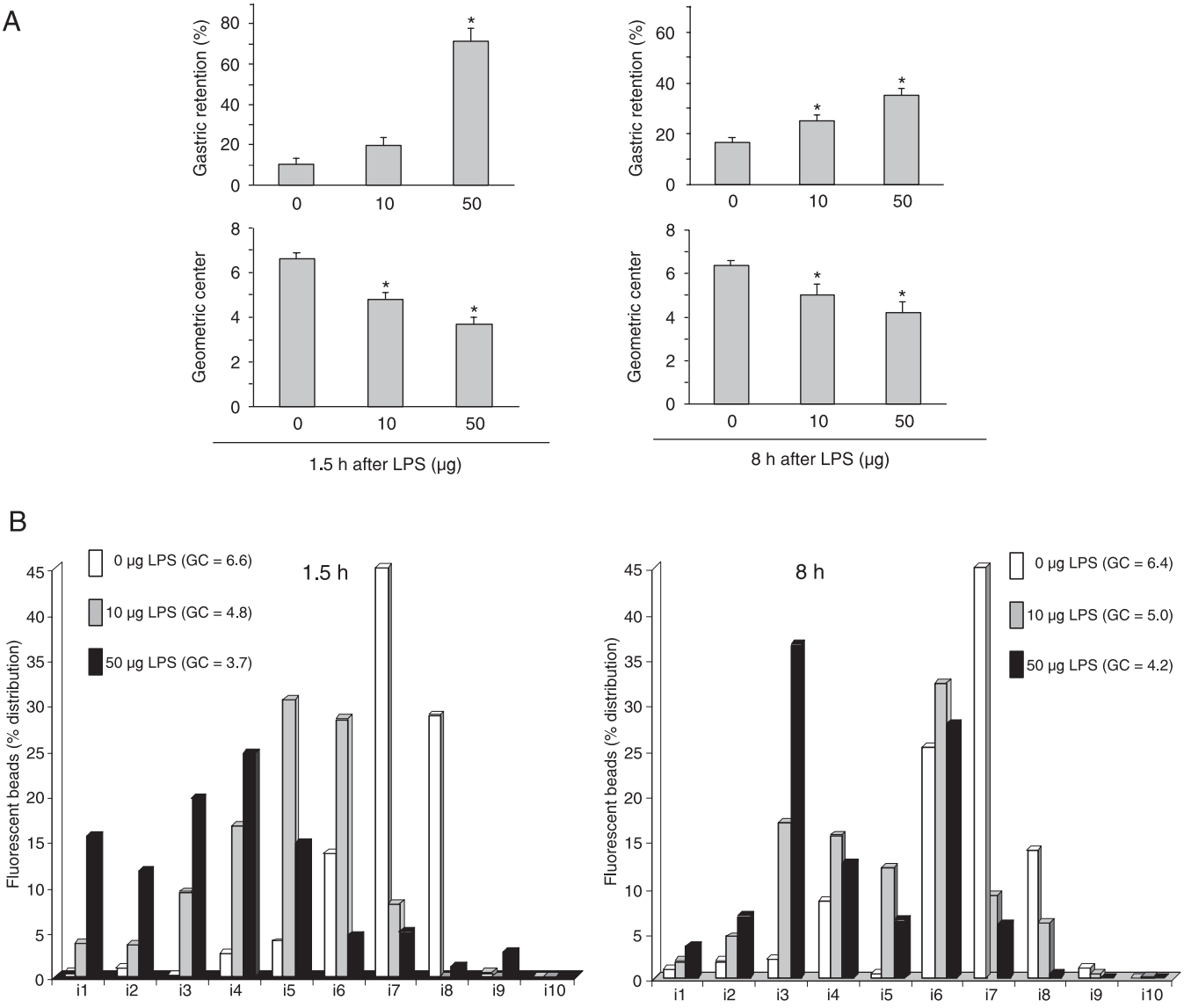

Figure 3. Effect of lipopolysaccharide (LPS) on gastric emptying and gastrointestinal transit. *A*, Gastric retention and geometric center (GC) at 1.5 h and 8 h after LPS injection. (GC indicates how far the center of gravity of the distribution of the microbeads reached beyond the pylorus into the small intestine. GC of 1 = minimum transit; GC of 10 = maximum transit). Results are reported as means ± SEM for 7-8 animals in each group. *P < 0.05 *vs* control (one-way analysis of variance and Bonferroni *t*-test). *B*, Transit histograms. The y-axis indicates the amount of fluorescent beads within each segment as a percentage of the total amount of beads in the intestinal tract. Means are shown for 7-8 animals in each group. The x-axis represents small intestinal segments i1 to i10.

trointestinal transit in otherwise untreated mice (%GR: 18.0 ± 2.5 with saline *vs* 12.8 ± 0.9% with anti-TNF-α, GC: 6.4 ± 0.1 with saline *vs* 6.7 ± 0.1 with anti-TNF-α, N = 5 for each group, P > 0.05).

Discussion

The main new findings of the present study are that the LPS-induced delay in gastric emptying in early endotoxemia (1.5 h after LPS administration) was associated with iNOS but not TNF-α, whereas in late endotoxemia (8 h after LPS administration) the delay was caused by both iNOS and TNF-α. These findings indicate that the role of TNF-α in gastric emptying differs depending on the time after LPS administration, whereas NO appears to contribute to the delay in gastric emptying at both times.

Although iNOS inhibition significantly reversed the increase in gastric retention in early endotoxemia, consistent with the results of previous studies (2,6), the reversal was incomplete. The level of iNOS mRNA in the gastrointestinal tract, which is already detectable in the absence of LPS, increases upon LPS stimulation, peaking at 3 h after *ip* LPS administration in rats (19). Thus, the activity of iNOS may not yet be fully realized 1.5 h after LPS administration. Therefore, in addition to iNOS, other mechanisms may be involved in the delay in gastric emptying during early endotoxemia. Molecules such as cyclooxygenase-2 may play a role, as also reported by others (20,21). During late endotoxemia, iNOS inhibition also reversed, almost completely, the increase in gastric retention. This finding is consistent with a previous study in rats, in which iNOS inhibition with aminoguanidine (a selective iNOS inhibitor) administered 5 h after LPS administration partially reversed the delay in gastric emptying in late endotoxemia (2).

What is most intriguing is that in late endotoxemia TNF-α neutralization also reversed the increase in gastric retention. The remarkable difference in gastric retention between the values observed at 1.5 h and those observed at 8 h may suggest that the mechanisms of dysmotility are different. The

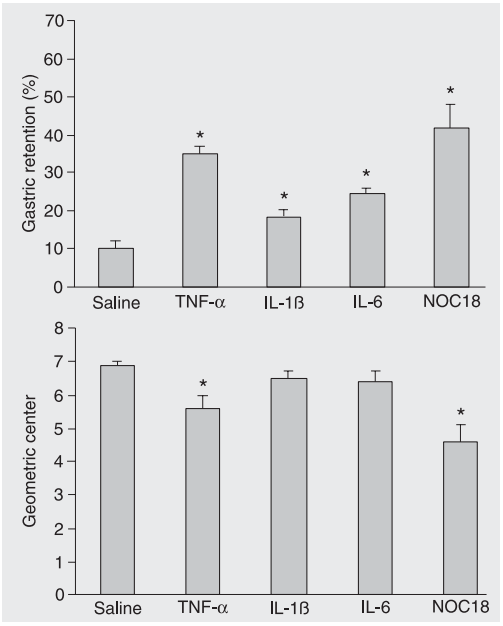

Figure 4. Effect on gastric retention and gastrointestinal transit of 2 µg *ip* tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß), and IL-6, and 300 µg *sc* 1-hydroxy-2-oxo-3,3-bis (2-aminoethyl)-1-triazene (NOC18, an NO donor). Gastric retention and geometric center were measured 30 min after saline or cytokine, or 1 h after NOC18 administration. Data are reported as means ± SEM for 7-9 animals in each group. *P < 0.05 *vs* saline (one-way analysis of variance and Bonferroni *t*-test).

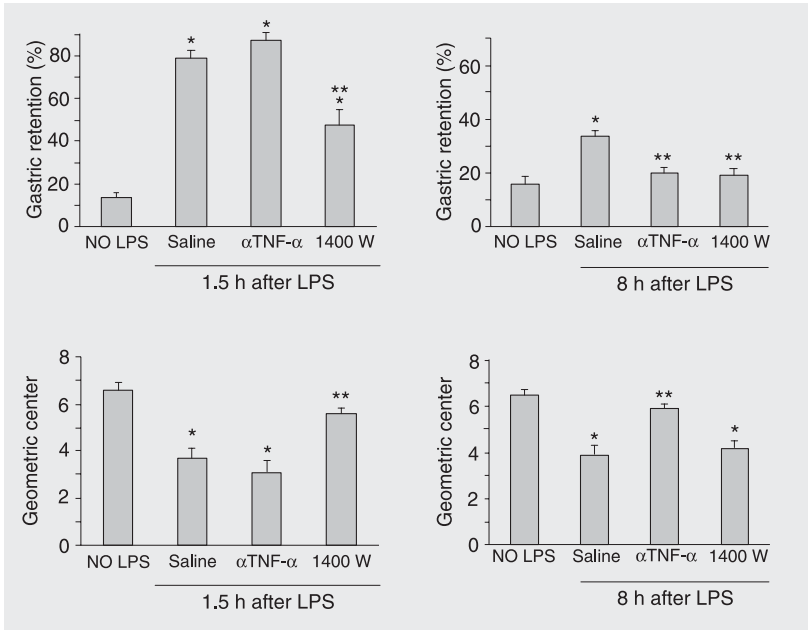

Figure 5. Effect of saline (*ip*), N-(3-(aminomethyl) benzyl) acetamidine (1400 W, a selective inducible nitric oxide (NO) synthase inhibitor, 150 µg, *ip*), and an antibody to tumor necrosis factor-α (αTNF-α, 200 µg, *ip*) during early (1.5 h after 50 µg *ip* lipopolysaccharide, LPS) and late (8 h after LPS administration) endotoxemia. Data are reported as means ± SEM for 9-11 animals in each group. *P < 0.01 *vs* NO LPS; **P < 0.01 *vs* saline (one-way analysis of variance and Bonferroni *t*-test).

reason for the successful reversal with both TNF-α neutralization and iNOS inhibition in late endotoxemia is not known but it may be that both TNF-α and iNOS contribute independently to the delay in gastric emptying. We assume that the effect of these two substances on the delay in gastric emptying is more than additive; thus the inhibition of either one can dramatically improve the delay. Another possible reason may be that TNF-α delays the gastric emptying via iNOS activation, as TNF-α has been shown to stimulate NOS activity (13).

There has been only one report describing the effect of NO and TNF-α on intestinal transit during endotoxemia (5). The study suggested that the LPS-induced disturbances in gastrointestinal transit during early endotoxemia (1.5 h after LPS administration) are not induced by TNF-α (but most likely by NO), while in late endotoxemia (8 h after LPS administration) the delay in intestinal transit is induced by TNF-α. In the same study, lactoferrin was administered orally to endotoxemic mice in order to indirectly deplete TNF-α. The lactoferrin treatment effectively reversed the LPS-induced delay in intestinal transit (5); however, since the depletion of TNF-α was indirect, the possibility of other effects of lactoferrin (such as the depletion of IL-6) could not be ruled out. In the present study, we directly neutralized TNF-α using an antibody, thus clearly demonstrating that TNF-α is a primary cytokine that delays gastrointestinal transit during late endotoxemia.

In our study, treatment with 1400 W had no effect on the LPS-induced delay of gastrointestinal transit in late endotoxemia. However, in a previous study in which 1400 W was given before LPS and gastrointestinal transit was measured 18 h later (6), the delay in gastrointestinal transit was reversed. One possible explanation for these findings is that the improved transit observed in the previous study was due to attenuated endotoxemia because the general well-being of the mice was also significantly improved (6).

Other research has shown that LPS-induced TNF-α production occurs in the early phase of sepsis and that serum as well as intestinal levels of TNF-α decrease in the later phase (5,22). It is therefore unclear why TNF-α contributed significantly to both the delay in gastric emptying and gastrointestinal transit in late endotoxemia (when TNF-α concentrations are low) but not the delay in early endotoxemia (when TNF-α concentrations are high). This issue was addressed in a previous study investigating the role of TNF-α in LPS-induced delay in gastrointestinal transit, without clear answers (5). We found that TNF-α per se delayed gastric emptying and gastrointestinal transit. Our study suggests that the TNF-α surge in the early phase of sepsis may be unrelated to the delay in gastrointestinal motility in late endotoxemia, because TNF-α neutralization was performed just before the 8-h measurement. One possible explanation is that the sensitivity of the TNF receptor to TNF-α may be altered in the cytokine/humoral milieu of endotoxemia.

The effect of LPS on intestinal transit is still controversial (1,5-7). We found that gastrointestinal transit was delayed by LPS, which is consistent with the reported "ileus" with endotoxemia (5-7), but some studies have shown accelerated transit during endotoxemia (1,5). There are several possible explanations for this discrepancy. These include differences in the doses of LPS used, the types of LPS used, and the timing of LPS administration. We suggest that differences in measurement methods could also account for the discrepancy. There are two ways of assessing gastrointestinal transit: measuring the position of the leading edge of the marker or measuring the distribution of the marker within the gastrointestinal tract (such as the geometric center). In endotoxemia, especially in the early phase, massive amounts of fluid may accumulate in the intestinal tract.

This fluid may easily be displaced distally when the gastrointestinal tract is removed in preparation for the measurement. We believe that the distal displacement of markers may severely affect the results with the leading edge method, but minimally affect the results with the geometric center method (which we employed), because the latter measures the center of gravity of the marker along the intestinal tract. As shown in the transit histogram (Figure 3B), at 1.5 h after treatment with 50 μg LPS (when the gastrointestinal tract becomes severely edematous and contains an excessive amount of fluid within the lumen) the leading edge of the beads progressed more distally, while the geometric center was positioned more proximally than in the absence of LPS treatment. The discrepancy between the position of the leading edge and the geometric center may result in an accelerated measurement when intestinal transit is assessed using the leading edge method. We did not observe such a discrepancy at 8 h, when edema of the tract and fluid accumulation in the lumen were considerably relieved. However, as we did not design the protocol to specifically study this issue and we were very careful to minimize the distal displacement of accumulated fluid containing the fluorescent marker during preparation of the intestine, further studies will be needed to test this hypothesis.

The mechanisms underlying LPS inhibition of gastrointestinal motility are complex. It appears that, iNOS and TNF-α contribute to the inhibition, and the extent of their participation differs at different times after LPS administration. iNOS contributed to the disturbances in gastrointestinal motility in early and late endotoxemia, while TNF-α primarily contributed to the disturbances in late endotoxemia. However, sepsis in clinical settings is far more complicated than in this animal model which utilizes the administration of LPS (23). Although we believe that our findings provide important insights into the mechanism of sepsis-induced gastrointestinal motility disturbances, great care should be taken when attempting to extrapolate our results to sepsis-induced gastrointestinal complications in patients.

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