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Protective Effects of L-Arginine on Rat Terminal Ileum Subjected to Ischemia/Reperfusion

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ABSTRACT

Objectives: Studies have shown that nitric oxide (NO) may play a major role in sustaining mucosal integrity; however, NO has been also implicated in the pathogenesis of ischemia/reperfusion (I/R)–related tissue injury. We investigated the effects of L-arginine and N^G-nitro L-arginine methyl ester (L-NAME) on the acetylcholine-induced contractile response of ileum and the levels of malondialdehyde (MDA) and reduced glutathione (GSH). Histopathological changes were also evaluated in ileal preparations.

Materials and Methods: Male Wistar Albino rats were subjected to mesenteric ischemia (30 min) followed by reperfusion (3 hours). Four groups were designed: sham-operated control; I/R; I/R and L-arginine pretreatment; and I/R and L-NAME pretreatment. After reperfusion, ileum specimens were collected to determine the parameters mentioned above.

Results: Following reperfusion, a significant decrease in acetylcholine-induced contractile response, an increase in lipid peroxidation, a decrease in GSH content, and mucosal damage of the ileal preparations were observed. We showed that decreased contractility, increased lipid peroxidation, and reduced GSH content have been reversed by L-arginine but not by L-NAME. Mucosal injury was significantly lowered in the L-arginine group.

Conclusions: Treatment with L-arginine exerted a protective effect in intestinal I/R injury, which was mediated in part by regulating MDA and GSH levels, consequently ameliorating impaired contractile response and mucosal injury. ***JPGN 46:29–35, 2008.*** **Key Words:** Contractile activity—Ileum—Ischemia and reperfusion—Nitric oxide. © 2008 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

Intestinal ischemia/reperfusion (I/R) is associated with various pathological conditions and surgical procedures that cause disruption of intestinal barrier function, impairment of gut motility, and increased intestinal permeability (1). Several mechanisms have been accounted for the pathogenesis of postischemic lesions in the intestine. Oxygen free radicals (OFRs) play a significant role in the development of intestinal I/R (2–5). OFRs are potent oxidizing agents that cause damage to cellular membrane by lipid peroxidation, which is a major consequence exerted through OFRs (3). Xanthine oxidase has been proposed as the primary source of OFRs because pretreatment with xanthine oxidase inhibitors such as allopurinol prevent postischemic mucosal injury. Another potential source of oxygen radicals is the inflammatory neutrophil. (6–9).

In intestinal I/R injury, motor alterations have been reviewed extensively. A delay in rat intestinal transit, derangement of piglet migration complex, and decreased sensitivity to cholinergic agonists in terms of contractile activity have been described in various experimental models (10). Muscle and nerve cells in intestinal tissue are vulnerable to ischemia. In intestinal tissue exposed to ischemia, energy depletion occurs in the cells (11). Furthermore, during the reperfusion period, generation of OFRs interfere with the function of cells by disturbing the cellular ionic homeostasis (2). Additionally, changes in the production of nitric oxide (NO) (12,13) and inflammatory mediators contribute to the alteration of intestinal motility during I/R (6–8,12,13).

A substantial number of studies suggest that NO is cytoprotective in I/R injury (14,15). Under conditions of oxidant stress, part of the NO can combine with superoxide anion, leading to the production of peroxynitrite, which results in cytotoxic effects (15). Conversely, there are also data showing that NO reduces mucosal injury

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after I/R of the intestine. Compounds that ultimately cause NO production significantly reduce mucosal injury after intestinal I/R. N^G-nitro L-arginine methyl ester (L-NAME), a potent inhibitor of NO synthase, greatly exacerbates intestinal injury and disruption of mucosal barrier function (7,14–17).

In the light of these observations, the aim of the present study was to determine the effects of NO donor, L-arginine, and the NO inhibitor L-NAME on ileal muscle contractility in addition to measure the levels of malondialdehyde (MDA) as an indicator of lipid peroxidation and reduced glutathione (GSH). Histopathological changes induced by I/R injury were also evaluated in ileum samples.

MATERIALS AND METHODS

A total of 28 Wistar adult male rats weighing 220 ± 20 g were used in the study. The animals were obtained from the Zonguldak Karaelmas University Animal Care Unit and housed under standard conditions. They had free access to commercial chow and water. On the day before the surgical procedures, the animals were fasted overnight but allowed ad libitum access to water. They were maintained in their cages at a constant room temperature using a 12 h:12 h light/dark cycle. All of the procedures described here were approved by the local animal ethics committee of the institution.

Induction of Ischemia

Rats were anesthetized with sodium thiopenthal intraperitoneally (60 mg/kg) followed by conducting laparotomy through a midline incision into the peritoneal cavity. After the small bowel was exteriorized gently to the left onto moist gauze, animals were subjected to 30 min of ischemia by ligation of the superior mesenteric artery (SMA), using 3/0 silk thread. Intestinal ischemia was confirmed by the lack of pulse in the mesenteric artery and the pale color of the jejunum and ileum. Afterward, the intestines were returned to the abdomen, which was closed with 2 small clamps. At the end of 30 min of ischemia, the thread was gently removed to allow reperfusion of the blood flow, which was confirmed by observing the pulsation of the artery and its branches on the intestine. Body temperature was maintained at 37°C by heating limb during the I/R procedure. In the experimental protocol, animals were divided into 4 groups each of 7 animals:

- Sham-operated control group: the rats underwent laparatomy without performing the occlusion of SMA
- I/R group: the SMA was occluded for 30 min followed by 3 hours of reperfusion
- L-arginine-treated group: rats subjected to I/R were treated with L-Arginine
- L-NAME-treated group: rats subjected to I/R were treated with L-NAME

L-NAME and L-arginine (Sigma Chemical, St. Louis) was dissolved in sterile saline. Each compound was administered at a dose of 10 mg/kg with a volume of 100 μL via tail vein 5 min before reperfusion. Animals in the sham-control and I/R groups were instead given sterile serum physiological solution in the same volume. Following the reperfusion period, the animals were killed by administration of anesthetic at lethal doses; thereafter, ileal samples were collected and processed for each experimental protocol. All of the other reagents and compounds, including trichloroacetic acid (TCA), thiobarbituric acid, butylated hydroxy toluene, and dithiobisnitrobenzoate were obtained from Sigma Chemical.

Ileal Longitudinal Muscle Contractility

The ileal longitudinal muscle contractile activity was evaluated in isolated ileal segments after 3 hours of reperfusion in an organ bath. Strips of longitudinal muscle at 5-mm length were removed 1 cm away from the ileocecal junction. After removing the first strip for histological evaluation, second, third, and fourth samples at the same size were collected for contractility and biochemical analyses. Strips were longitudinally suspended under 2-g load in 20 mL organ bath filled with Krebs solution (in mmol/L: NaCl 118.5, KCl 4.8, KH^2PO_4 1.2, $MgSO_4$-$7H_2O$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25, glucose 10.1). The solution was gassed with a mixture of 5% CO_2 and 95% O_2 and maintained at 37°C. After 60 min equilibration with 2 g load, acetylcholine was added to the organ bath at final various concentrations ranging from 10^{-8} to 10^{-3} mol/L. Active force development was recorded at each concentration to determine the dose-response relationship. Isometric force was monitored by external force displacement transducer (FDA-10A, Commat İletisim Co, Ankara) using MP 30 software (MP30 Biopac Systems Inc, Santa Barbara, CA). To evaluate the effects of I/R and the drugs on response to acetylcholine, the maximal contractile response (E_{max}, as gram contraction) and pD_2 values (ie, the negative logarithm of the concentration for the half-maximal response, ED_{50}) (18,19) were calculated by Graphpad Prism Software version 3.0 (San Diego, CA). Samples of full-thickness segments that were to be analyzed for levels of GSH and MDA were frozen immediately after the reperfusion period and stored at −40°C. In addition, the ileal segments from each group were fixed by formaldehyde immediately for histopathological examination.

MDA Determination

Intestinal lipid peroxide levels were measured by a method described by Casini et al (20). Briefly, by using a motor-driven pestle, tissue samples were homogenized in ice-cold TCA by adding 10 mL of 10% TCA per gram of tissue. After centrifugation, 750 μL supernatant was added to equal volume of 0.67% thiobarbituric acid and heated to 100°C for 15 min. The absorbance of the samples was then measured spectrophotometrically at 535 nm.

GSH Determination

Glutathione content of the samples was measured by a modified Ellman method (21). To the 0.5 mL of supernatant obtained by using the same homogenization procedure as described above, 2 mL 0.3 mol/L Na_2HPO_4 solution was added.

Solution of dithiobisnitrobenzoate (0.2 mL) was added into the mixture immediately followed by vortexing; the absorbance was then measured at 412 nm.

Histopathological Evaluations

Segments of ileum were fixed in 10% formalin and embedded in paraffin. Intestinal paraffin sections were stained with hematoxylin and eosin for morphological analysis. Histopathological examinations of reperfused intestinal tissue were performed based on a staging method described by Hierholzer et al (6), and the evaluation was graded from 0 to 4. In grade 0, no specific pathological changes are observed: normal structure of gut wall, including villi, crypts, lamina propria, and muscularis externa. In grade 1, mild mucosal damage is assessed: denudation of villi epithelium, otherwise normal structure. In grade 2, moderate damage occurs: loss of villus length and epithelial sloughing with evidence of congestion, hemorrhage, and inflammation in the mucosa, but no change in submucosa or muscularis externa. In grade 3, extensive damage is observed: loss of a large number of villi including denudation, sloughing, and the presence of granulomatous tissue with the damage localized to submucosa and muscularis. In grade 4, there is severe damage and necrosis: inflammation and necrosis in areas throughout the thickness of the intestinal wall.

Statistical Analysis

All of the data are presented as the mean ± SD. One-way analysis of variance (ANOVA) was used for statistical comparison of groups, with multiple post-hoc comparison performed using the Tukey-Kramer test. $P < 0.05$ were considered significant. All of the statistical procedures were performed using SPSS 11.0 statistical software (SPSS Inc, Chicago).

RESULTS

Ileal Longitudinal Muscle Contractility

The addition of acetylcholine in a cumulative fashion at concentrations from 10^{-8} to 10^{-3} mol/L into the organ bath fluid resulted in a dose-dependent contractile effect on the terminal ileum segments from all groups (Fig. 1), providing sigmoid curves with E_{max} and pD_2 values. E_{max} for the acetylcholine was significantly lower in the I/R group than in the sham-control group (1.89 ± 0.08 g vs 4.22 ± 0.11 g). In other words, the contractile responses induced by acetylcholine were significantly inhibited by induction of I/R ($P < 0.05$). The I/R-induced reduction in the contractile responsiveness to acetylcholine was significantly restored in the L-arginine-treated group but not in the L-NAME-treated group (Fig. 1). Comparison of the E_{max} values showed that ileal preparations in I/R group contracted 44% of the sham-control, whereas those in the L-arginine treated showed 90% of the sham-control. In the L-NAME treated group, E_{max} was found to be 26% of the sham-control. There was no statistically significant change in the corresponding pD_2 values in all groups (Table 1).

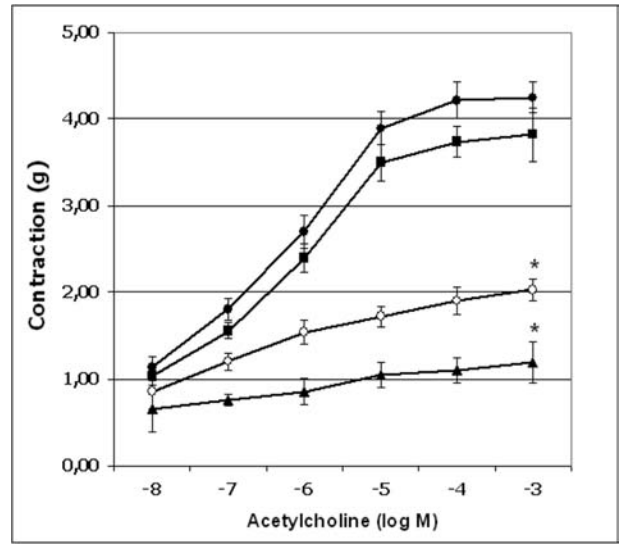

FIG. 1. Concentration-response curves of Ach in longitidunal ileum muscle isolated from rats in sham-control (●), I/R (○), L-arginine-treated (■), and L-NAME-treated (▲) groups. Data are expressed as mean ± SD. Asterisk denotes statistical difference from sham-control and L-arginine-treated groups, with $P < 0.05$.

I/R caused an ~56% decrease in the contractility as shown in Table 1. Administration of L-arginine caused a significant decrease in the inhibition of contractility observed due to I/R. In the case of L-arginine treatment, E_{max} was 90% of that from the sham-control group (3.77 ± 0.08 g vs 4.22 ± 0.11 g) and was not statistically different from the sham-control group ($P > 0.05$). In L-NAME-treated animals, however, E_{max} to acetylcholine was not statistically different from that observed in the I/R group (1.10 ± 0.05 g vs 1.89 ± 0.08 g) ($P > 0.05$).

TABLE 1. *E_{max} and pD_2 values of acetylcholine in each experimental group*

	Sham-control	I/R	L-arginine-treated group	L-NAME-treated group
E_{max}	4.22 ± 0.11	1.89 ± 0.08$^{\#,*}$	3.77 ± 0.08	1.10 ± 0.05$^{\#,*}$
pD_2	5.99 ± 0.12	6.34 ± 0.31	5.98 ± 0.10	5.85 ± 0.39

Data expressed as mean ± SD. * and $^{\#}$ $P < 0.05$, statistically different from sham-control group and L-arginine-treated group, respectively. E_{max}, maximal contractile response (absolute tension as gram contraction); pD_2: negative logarithm of the concentration for the half-maximal response, ED_{50}.

TABLE 2. *Values of MDA and GSH, measured in each experimental group (7 rats/group)*

Groups	MDA nmol/g tissue*	GSH μmol/g tissue*
Sham-control	54.17 ± 3.27^{a}	3.58 ± 0.59^{a}
I/R	95.47 ± 19.70^{b}	2.34 ± 0.58^{b}
L-arginine-treated group	50.54 ± 8.41^{c}	4.01 ± 0.41^{c}
L-NAME-treated group	96.60 ± 11.41^{d}	2.22 ± 0.59^{d}

Data expressed as mean ± SD. Using analysis with 1-way ANOVA, overall significance among groups: $P < 0.05$. Analysis with Tukey-Kramer post hoc test. MDA, malondialdehyde; GSH: reduced glutathione.

(a,b), (a,d), (b,c), (c,d) $P < 0.05$.

MDA Levels

Malondialdehyde contents of homogenates obtained from intestinal samples of sham-control animals averaged 54.17 ± 3.27 nmol/g tissue, whereas those of animals subjected to only I/R were found to be 95.47 ± 19.7 nmol/g tissue (Table 2). I/R resulted in an ~1.7-fold increase in MDA content of intestinal homogenates, which is significantly different from MDA levels measured in sham-control homogenates ($P < 0.01$). It was interesting to observe that L-arginine administration before the reperfusion significantly reduced the intestinal MDA content to the sham-control levels. With an average value of 50.54 ± 8.41 nmol/g tissue, the L-arginine-treated I/R group was statistically indistinguishable from the sham-control group in terms of MDA content. In the case of L-NAME administration into animals subjected to I/R, the MDA content did not differ from that of I/R-only group (96.60 ± 11.94 vs 95.47 ± 19.7; $P > 0.05$).

GSH Levels

The amount of GSH measured in intestinal tissues subjected to I/R decreased approximately 35% compared with that in sham-control samples (Table 2). Administration of L-arginine significantly ameliorated the decreased amount of GSH. Amounts of tissue GSH were indistinguishable in both sham-control and L-arginine-treated animals ($P > 0.05$), 3.58 ± 0.59 μmol/g tissue and 4.01 ± 0.41 μmol/g tissue, respectively. The administration of L-NAME appeared not to be beneficial in returning the GSH values to sham-control levels. In the L-NAME-treated animals, the amount of GSH was found to be 2.22 ± 0.59 μmol/g tissue, which was statistically indifferent from the value measured in the I/R group, 2.34 ± 0.58 μmol/g tissue ($P > 0.05$); however, it was statistically different from the values measured in both sham-control and L-arginine-treated groups ($P < 0.05$).

TABLE 3. *Semiquantitative histological grading of cross-sections of the rat ileum (6 sections/group)*

Groups	Mean ± SD
Sham-control	0
I/R	2.29 ± 0.48^{a}
L-arginine-treated group	1.14 ± 0.37^{b}
L-NAME-treated group	2.42 ± 0.53^{c}

Using analysis with 1-way ANOVA, overall significance among groups $P < 0.004$. Analysis with Tukey-Kramer post hoc test.

(a,b) $P = 0.001$; (b,c) $P = 0.0001$.

Histopathological Findings

Table 3 shows the scores for mucosal injury in the groups. Based on the histopathological analysis of 6 sections for each group, grading scores were calculated and analyzed statistically as demonstrated in Table 3. Using analysis of 1-way ANOVA, we observed a significant difference among groups ($P < 0.0001$). The most extensive changes in morphology were detected in the I/R group, which was statistically indistinguishable from the group pretreated with L-NAME ($P > 0.05$). Statistical evaluation of grading scores also showed that the treatment with L-arginine restored significantly the alterations in morphology ($P = 0.001$).

In sham-control group, no change was detected as depicted in Fig. 2A (grade 0). In sections from the group injured by I/R, mucosal damage and sloughing on some of the surface epithelium were obvious (grade 2.28 ± 0.48) as seen in Figure 2B. In the group treated with L-arginine, however, mild mucosal damage with only minimal focal denudations of villi epithelium was noticed and considered to be grade 1.14 ± 0.37 (Fig. 2C). In Figure 2D, which shows the section from the L-NAME-treated group, the surface epithelium was detected to be sloughed. Furthermore, congestion, hemorrhage, and inflammatory cell infiltration were evident in this group (grade 2.42 ± 0.53).

DISCUSSION

Our results showed that intestinal I/R resulted in decreased ileal contractility in response to acetylcholine, classic excitatory neurotransmitter in the small intestine, therefore influencing receptor-mediated induction. That the pD_2 values in all of the groups were found to be statistically unchanged, however, suggests that I/R do not alter acetylcholine-receptor interaction. Therefore, the reduced E_{max} value in I/R may be dependent partly on the change in the regulation of postreceptor processes (ie, excitation-contraction coupling) (18,19). Administration of L-arginine has improved the reduced contractile responses and returned them to sham-control levels, whereas treatment with L-NAME has no ameliorating

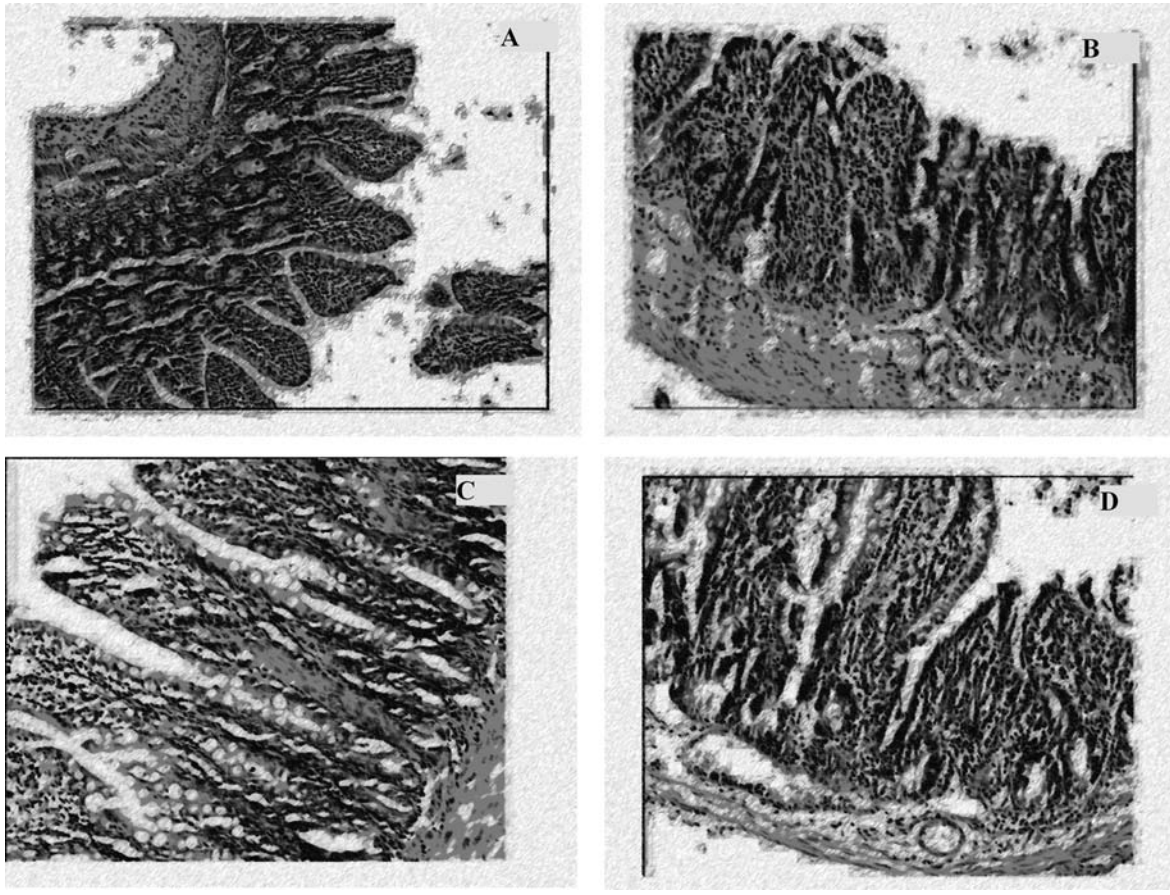

FIG. 2. Light micrographs of rat intestinal tissue. A, Sham-control group; B, I/R group (most extensive morphological changes observed); C, L-arginine-treated group (L-arginine treament ameliorates the histopathological changes after I/R); D, L-NAME-treated group (L-NAME treatment exacerbate mucosal injury). Hematoxylin and eosin stain; original magnification × 200).

effect on ileal contractility. Based on the previous studies, dose for both L-arginine and L-NAME was chosen as 10 mg/kg of rat.

I/R causes such damage as disruption of the electrical activity and contractile response of the ileum (2,8,10). These motility changes eventually would result in bacterial overgrowth and its translocations (12). Implications in the literature suggest that in addition to OFRs (2,8), inflammatory mediators and extravasated leukocytes (6,8), a possible defect in NO metabolism (12,13), could also partly contribute to muscle dysfunction seen after I/R. Therefore, potential strategies intended to prevent I/R injury can be divided into at least 3 different modes: NO supplementation, antioxidant molecules, and approaches to block neutrophil–endothelial cell interaction (22). In the present study, we demonstrated that L-arginine treatment could be protective in I/R-induced impairment of contractile activity as well as reducing postischemic injury of ileal smooth muscle. The protective effects of L-arginine may possibly work through enhancing NO release from the vascular endothelium and reducing lipid peroxidation resulting from an inhibition of superoxide anion ($O_2^{-}\cdot$), whereas inflammation decreases due to decreased neutrophil activation. It is suggested that intestinal mucosal blood flow depends on a balanced release of vasoactive substances from the endothelium (23). In the present study, we also showed that depletion of ileal tissue GSH was restored by L-arginine treatment. Glutathione is an endogenous antioxidant found in all animal cells. It reacts with free radicals and can provide protection from singlet oxygen, hydroxyl radical and $O_2^{-}\cdot$ (24).

Nitric oxide, formed by Ca^{2+}-dependent constitutive NO synthase (ecNOS) (2,12,14,25), has significant roles in maintaining vascular integrity and barrier function under physiological conditions (2,11,14). It is well established that I/R affects endothelium severely, thereby causing dysfunction of endothelium, which is distinguished by a reduction in the release of NO. Accordingly, the loss of endogenous NO generation leads eventually to

endothelial dysfunction and neutrophil-mediated tissue injury (22). Kurose et al reported that intestinal I/R in rats is accompanied by decreased plasma levels of nitrite/nitrate, which are metabolic products of NO (26). It was also demonstrated that plasma levels of L-arginine, substrate for NOS, becomes low in conditions associated with I/R such as necrotisan enterocolitis of infants, a condition caused by mesenteric I/R (7). We administered L-arginine before the reperfusion period because the hypothesis foresees that supplementation of substrate for cNOS during the reperfusion period could reduce lipid peroxidation and enhance inhibition of neutrophil activation.

The exact role of NO during I/R is not only controversial but also a complex issue. Knowledge of the cytoprotective effect of NO originated from the findings that NO generation by endothelial cells decrease after I/R and that NO donors attenuate the increased albumin leakage in mesenteric venules subjected to I/R. NO donors also reduce I/R-induced leukocyte adherence and emigration, and they decrease mucosal injury (7,14–17). Administration of NOS inhibitors (eg, L-NAME) accelerates the time course of capillary leakage as well as postischemic neutrophil activation. NOS inhibitors seem to enhance leukocyte adherence to the mesenteric vascular endothelium as well (7). A beneficial effect of NO is scavenging OFRs such as $O_2^-\cdot$. NO reduces $O_2^-\cdot$ production by neutrophils via acting on membrane subunits of NADPH oxidase. This effect may potentially block the activation of the neutrophils that have already resided within intestine. In addition, NO induces heme oxygenase, an endothelial cell antioxidant, which may provide further protection (7). In the present study, collected data suggested first that elevated lipid peroxidation due to I/R may result in decreased contractility of the ileal smooth muscle and mucosal damage of the ileal wall. Second, the data have implied that administration of L-arginine may help keep the amount of NO in tissue at physiological levels. Because activity of ecNOS presumably decreases as a result of I/R, the protective ability of NO against ischemic insult is consistent with previous studies showing that NO prevents neutrophil activation at least indirectly by blocking the expression of P-selectin (17), thereby reducing inflammation, free radical generation, and production of $O_2^-\cdot$ (7). L-NAME administration appeared to result in disturbance of the contractile response and mucosal injury by elevating lipid peroxidation. Because duration of reperfusion in the present study was 3 hours, administration of L-NAME before reperfusion could further increase tissue injury as a result of inhibiting ecNOS. This may explain why the dose-response curve from the L-NAME-treated group was lower than that from I/R group despite the statistically indistinguishable E_{max} values among the groups.

Our results suggested that L-arginine treatment ameliorated structural and functional damage accompanied with experimental warm I/R by both inhibiting lipid peroxidation and maintaining tissue GSH levels. The beneficial effects of L-arginine treatment can be promising in cold I/R models, particularly for small intestinal transplantation and graft preservation studies. In fact, a recently published study by Fu et al suggests that L-arginine supplementation during cold I/R may act as a useful adjunct to preserve the grafted intestine (27). Increasing NO concentrations with L-arginine administration was beneficial, presumably due to the ability of L-arginine to inhibit phagocyte adherence and its radical scavenging potential. In our study, however, leukocyte activation and infiltration into the tissue have not been measured. Therefore, further studies are needed to evaluate its clinical use and the exact mechanisms responsible for the protective effect of NO.

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