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INTESTINAL ISCHEMIC PRECONDITIONING PROTECTS THE INTESTINE AND REDUCES BACTERIAL TRANSLOCATION

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ABSTRACT—Ischemic preconditioning (IPC) was first demonstrated in the heart, but this protective effect has been also recently described in the intestine. The aim of this study was to determine the effects of intestinal ischemic preconditioning on the morphology of intestine and bacterial translocation. Twenty-four male Wistar rats weighting 250 to 300 g were randomized into three groups. A control group of rats (n = 8) were subjected laparotomy. In an ischemic group (n = 8), laparotomy was performed and the superior mesenteric artery was occluded by an atraumatic clamp for 30 min. In the preconditioned group (n = 8), before the ischemia-reperfusion (I/R) period (as in ischemic group), rats were subjected to an initial 10 min of intestinal ischemia and 10 min of reperfusion. Twenty-four hours later, to evaluate whether the I/R induced intestinal injury and bacterial translocation (BT), tissue and blood samples were collected, and liver, spleen, and mesenteric lymph node specimens were obtained under sterile conditions for microbiological analysis. Samples of ileum were removed for both biochemical and histopathological evaluation. In the I/R group, the incidence of bacteria-isolated mesenteric lymph nodes, spleen, liver, and blood was significantly higher than other groups ($P < 0.05$). IPC prevented I/R-induced BT and it significantly reduced the I/R-induced intestinal injury ($P < 0.05$). Increased inducible nitric oxide (NO) synthase (iNOS) expression observed on the ileal specimens of the I/R group was found to be prevented by IPC. Our data suggest IPC as a key factor that reduces BT and iNOS activation in intestinal I/R. This is the first study showing that intestinal IPC blocks the cascade of events that causes BT and intestinal injury that may lead to sepsis.

KEYWORDS—Ischemia-reperfusion, intestinal injury, nitric oxide, iNOS, multiple organ failure

INTRODUCTION

Postischemic reperfusion injury represents a source of substantial morbidity and mortality in myocardial infarction, trauma, septic shock, and multiple organ failure (MOF). Gut hypoperfusion has been implicated as an initiating event in the development of MOF (1). Because damage to the mucosal barrier can induce bacterial translocation (BT) and the gastrointestinal tract has been defined as the "motor" of MOF, postischemic reperfusion injury in the intestine has gained attention (2, 3).

BT is the passage of viable indigenous bacteria to sterile body sites such as the mesenteric lymph nodes (MLN), spleen, liver, and bloodstream during damage of the mucosal barrier (4, 5). Although rodents and men are different, bacterial overgrowth and alteration of the mucosal barrier integrity promote BT from the intestinal tract (6). The underlying mechanism in the pathophysiology of the mucosal barrier damage is attributed to ischemia/reperfusion (I/R) as mediating systemic inflammatory response sydrome (SIRS), sepsis, MOF states. On the other hand, reactive oxygen species such as superoxide anion, hydrogen peroxide, peroxynitrite, and the highly reactive hydroxyl radical are thought to play a pivotal role in the pathogenesis of I/R injury (7). Thus, it is plausible that oxyradical injury is one of the proposed mechanisms for I/R induced intestinal barrier dysfunction (8).

Ischemic precontioning (IPC) is defined as one or more brief periods of ischemia with intermittent reperfusion that protect against a sustained period of subsequent ischemia. This phenomenon was first demonstrated in the heart (9), but this protective effect has been also recently described in the intestine (10). In its classical description, the primary beneficial effect of preconditioning is a delay in the development of tissue necrosis (11). Prior to intestinal necrosis, at the stage in which there is an ischemia-induced loss of mucosal integrity, BT might occur and this may propagate tissue necrosis. Our hypothesis was that IPC of the intestine will reduce I/R-induced intestinal injury, BT, and inducible nitric oxide (NO) synthase (iNOS) activation. The aim of this study was to determine the effects of transient intestinal IPC on the intestinal mucosal morphology and BT. We also investigated the effects of intestinal IPC on nitrite/nitrate levels and iNOS expression in the ileum.

MATERIALS AND METHODS

The experiments described in this manuscript were performed in accordance with the National Institutes of Health *Guidelines on the Care and Use of Laboratory Animals*, and approval of the ethic committee of Mersin University School of Medicine was obtained prior to study. Twenty-four male Wistar rats (250–300 g) were used in the present study. As a standard protocol, all the rats were housed in a quiet non-stressful environment for 1 week prior to study.

Experimental protocol

After fasting over night, all animals (including control group) were anesthetized with i.m. ketamine (80 mg kg^{-1}) and xylazine (7 mg kg^{-1}) and were placed in a supine position on a heating pad for maintenance of body temperature between 36°C and 37°C. They were randomly divided into three groups. In the first group, which served as control (n = 8), rats were subjected to only laparotomy. In the second group, which served as the ischemic group (n = 8), laparotomy was performed and the superior mesenteric artery was exposed and occluded by an atraumatic micro-

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vascular clamp for 30 min. In the third group, which served as the preconditioned group (n = 8), rats were preconditioned with 10 min of ischemia and 10 min of reperfusion immediately prior to the 30-min ischemic insult. The abdomen was closed with 3/0 silk suture in all of the rats.

Twenty-four hours later, rats were anesthetized with i.m. ketamine (50 mg kg^{-1}) and xylazine (7 mg kg^{-1}). To evaluate the I/R-induced intestinal injury and BT, tissue and blood samples were collected. First, a thoracotomy was performed under aseptic conditions and blood samples were obtained by sterile cardiac puncture. A midline laparotomy was performed and liver, spleen, and MLN specimens were obtained under sterile conditions. Then, samples of ileum were removed for both biochemical and histopathological evaluation.

Microbiologic analysis

Microbiologic analysis was performed as described previously (34). A 1-mL sample of blood from each animal was immediately placed into Bactec Peds Plus/F blood culture medium (Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated at 37°C for 7 days under aerobic conditions in a Bactec 9240 system. Broths were incubated at 35°C until turbid, and the turbidity was adjusted to match that of a 0.5 Mc Farland standart (10^8 CFU/mL). By using normal saline, a 1/100 dilution of the suspension was made to give an adjusted concentration of 10^6 CFU/mL. From all cultures, subsequent subcultures were performed on blood agar, eosin-methylene blue (EMB) agar, and chocolate agar (12). All samples were stained by acridine orange and gram technique. The liver, spleen, and MLN specimens were placed into 2 mL of brain hearth infusion (BHI) broth, weighed, and homogenized. These samples were then placed on blood agar and EMB agar. All cultures were incubated under aerobic and anaerobic conditions and were examined at 24 and 48 h for presence of growth. The identification of bacterial species was performed by standard microbiologic methods. Colonization was expressed as the number of CFU per milliliter of homogenate (CFU/g).

Detection of ileal nitrite and nitrate levels

At the end of the experimental period, ileal segments were obtained for measurements of tissue nitrite and nitrate levels. Samples of terminal ileum were harvested at the end of the experimental period and were stored at −70°C until the assays were performed. Nitrite (NO_2-) and nitrate (NO_3-) were measured using nitrate reductase and Greiss reagent as described previously (5). Equal volumes of homogenizate and potassium phosphate buffer were placed in an ultrafilter and were centrifuged at 4000 rpm for 45 min. The ultrafiltrate was collected and used in the test. Nitrates were quantitatively converted to nitrites for analysis. Enzymatic reduction of nitrate to nitrite was carried out using coenzymes (NADPH and FAD) in the presence of nitrate reductase in step of incubation assay. *N*-1-(naphthyl)ethylenediamine dihydrochloride, sulfanilamide, and incubation solutions were mixed at a ratio of 1:1:2 (v/v). These mixtures were incubated for 5 min at room temperature in dimmed light and were measured at 540 nm. Sodium nitrite of 1.00 mM was used as standard for determination of nitrite, and potassium nitrate of 80 mM was used as standard for determination of nitrate (NO colorimetric assay, 1-756-281, Roche Diagnostics, Mannheim, Germany).

Histopathologic analysis

Sections were obtained from intestinal segments that were fixed in 10% formaldehyde and stained with hematoxylin-eosin. Mucosal injury, inflammation, and hyperemia/hemorrhage were assessed and graded in a blind manner by a pathologist using the histologic injury scale previously defined by Chiu et al. (13). Briefly, mucosal damage was graded from 0 to 5 according to the following criteria: grade 0, normal mucosal villi; grade 1, development of subepithelial Gruenhagen's space at the apex of the villus, often with capillary congestion; grade 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; grade 3, massive epithelial lifting down the sides of villi, possibly with a few denuded tips; grade 4, denuded villi with lamina propria and dilated capillaries exposed, possibly with increased cellularity of lamina propria; and grade 5, digestion and disintegration of the lamina propria, hemorrhage, and ulceration (13, 34).

Immunhistochemical analysis

Tissue samples were fixed in 10% formaldehyde and were embedded in paraffin. After deparaffinization and heat-induced antigen retrieval using a microwave oven (three 5-min cycles in citrate buffer, pH 6.0), endogenous peroxidase was abolished with methanol and H_2O_2, and non-specific background staining was blocked by incubating the sections for 5 min in normal swine serum. Subsequently, sections were stained using a three-step avidin-biotin complex (ABC) technique using rabbit polyclonal anti-iNOS antibody (Transduction Laboratories, Lexington, KY) diluted 1:100 in phosphate-buffered saline (PBS), pH 7.2. Sections were counterstained with hematoxylin-eosin and examinated by a pathologist (blind to the sample identity). The positive cells were counted in 100 adjacent epithelial cells and were repeated in three high-power fields, and the total number of positive cells was expressed as a percentage of 300. Staining was scored as: 0, negative; 1, 1% to 5%; 2, 6% to 25%; and 3, 26% to 100%.

Statistical analysis

All scores are given as mean ± SD except intestinal injury scores, which are given as mean ± SEM. Statistical evaluation for proportional comparisons for cultures of tissues was made using the chi-square test with Yates correction. Comparisons for quantitative culture and ileal nitrite/nitrate levels were analyzed using anlysis of variance (ANOVA). Comparisons for intestinal injury score and iNOS staining score were analyzed using Kruskall-Wallis variance analysis. *P* values less than 0.05 were considered statistically significant.

RESULTS

All animals survived during the experimental protocol. I/R caused severe BT in rats (Table 1) and the incidence of bacteria-isolated MLNs and spleen was significantly higher than the control and IPC groups ($P < 0.05$). I/R caused marked BT in liver, but this increase did not reach statistical significance when compared with IPC group. IPC prevented I/R-induced BT. Animals from the control group demonstrated no bacterial colonization in the harvested tissues. The predominating bacteria was *Escherichia coli*, however, *Klebsiella* and *Proteus vulgaris* were also encountered (data not shown).

Twenty-four hours later, small intestine of the rats was assessed for tissue damage by histologic examination. As shown in Figure 1, I/R caused significant tissue damage. These changes varied from denuded villi with exposed dilated capillaries to significant architectural distortion, lamina propria disintegration, ulceration, and hemorrhage. The histopathologic scores of control group and IPC group were significantly smaller than the I/R group (Fig. 1). IPC significantly reduced

TABLE 1. **Incidence of bacterial translocation in blood and tissue specimens**

	MLNs		Spleen	
Group	Incidence	CFU/g	Incidence	CFU/g
Control (n = 8)	0/8 (0%)	–	0/8 (0%)	–
I/R (n = 8)	8/8 (100%)*,†	822.13 ± 345.69 (850)	8/8 (100%)*,†	300.75 ± 73.90 (297)
IPC (n = 8)	2/8 (25%)	50.13 ± 95.18 (0)	2/8 (25%)	83.25 ± 180.87 (0)
	Liver		**Blood**	
Group	Incidence	CFU/g	Incidence	CFU/g
Control (n = 8)	0/8 (0%)	–	0/8 (0%)	–
I/R (n = 8)	8/8 (100%)*	463.5 ± 196.94 (458)	4/8 (50%)	362.5 ± 223.55 (213)
IPC (n = 8)	4/8 (50%)	80.88 ± 144.23 (16)	0/8 (0%)	–

CFU/g: Mean ± SD of colony forming units per gram of tissue and median values in parenthesis. These values reflect just the samples that contained translocating bacteria. *$P < 0.05$, significantly different from control group. †$P < 0.05$, significantly different from IPC group.

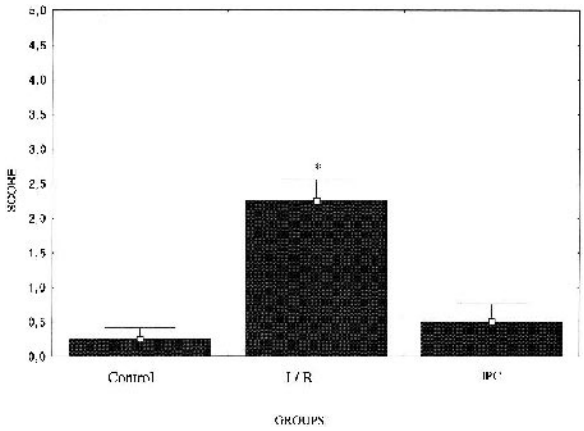

FIG. 1. **Histopathologic scores of the bowel specimens obtained from the study groups.** Results are presented as mean ± SEM. An asterisk represents I/R group with significantly higher injury scores when compared with control and IPC groups (*$P < 0.05$; Kruskal-Wallis variance analysis).

the I/R-induced intestinal injury compared with the I/R group. There was no statistical significant difference between the scores of this group and the control group (Fig. 2, A–C).

The ileal nitrite/nitrate level of I/R group was significantly greater than that of control, and its elevation was suppressed by IPC ($P < 0.05$; Table 2).

Immunohistochemical staining for iNOS was found negative in control group. Increased iNOS expression was observed in the ileal specimens of the I/R group (3 ± 0, $n = 8$, staining in rats) was found to be prevented by IPC (1.125 ± 0.354, $n = 8$, staining in rats; $P < 0.05$; Fig. 3, A–C).

DISCUSSION

In the present study, we demonstrated that IPC abrogates postischemic ileal injury and prevents I/R-induced BT by a mechanism that is initiated by inhibition of iNOS expression in the I/R group. Our results provide evidence that the relationship between BT and iNOS expression in intestinal tissue is crucial. Our data are the first to demonstrate that intestinal IPC reduces BT and iNOS activation in I/R and contributes to the maintenance of intestinal mucosal integrity.

I/R injury to the small intestine causes local production of reactive oxygen species and cytokines that induce endothelial responses that attract circulating neutrophils into the area of local injured tissue (14, 15). This involves complex interaction between intestinal wall integrity and BT. As an antioxidant, iron chelator desferoxamine has been used by Lelli et al. (16) to limit organ-specific oxidant-mediated lipid peroxidation in postischemic injury in immature intestine. At this point, preconditioning gains attention as it is referred as a tissue is rendered resistant to the deleterious effects of prolonged ischemia by previous exposure to brief periods of vascular occlusion (17). In our study, histopathologic scores of the bowel specimens (Fig. 1) revealed that intestinal preconditioning protects against I/R injury of the small intestine. The microbiological data (Table 1) showed that intestinal preconditioning reduces BT.

A growing body of evidence suggests that NO may play a regulatory role in BT (18, 19). Although there is controversy

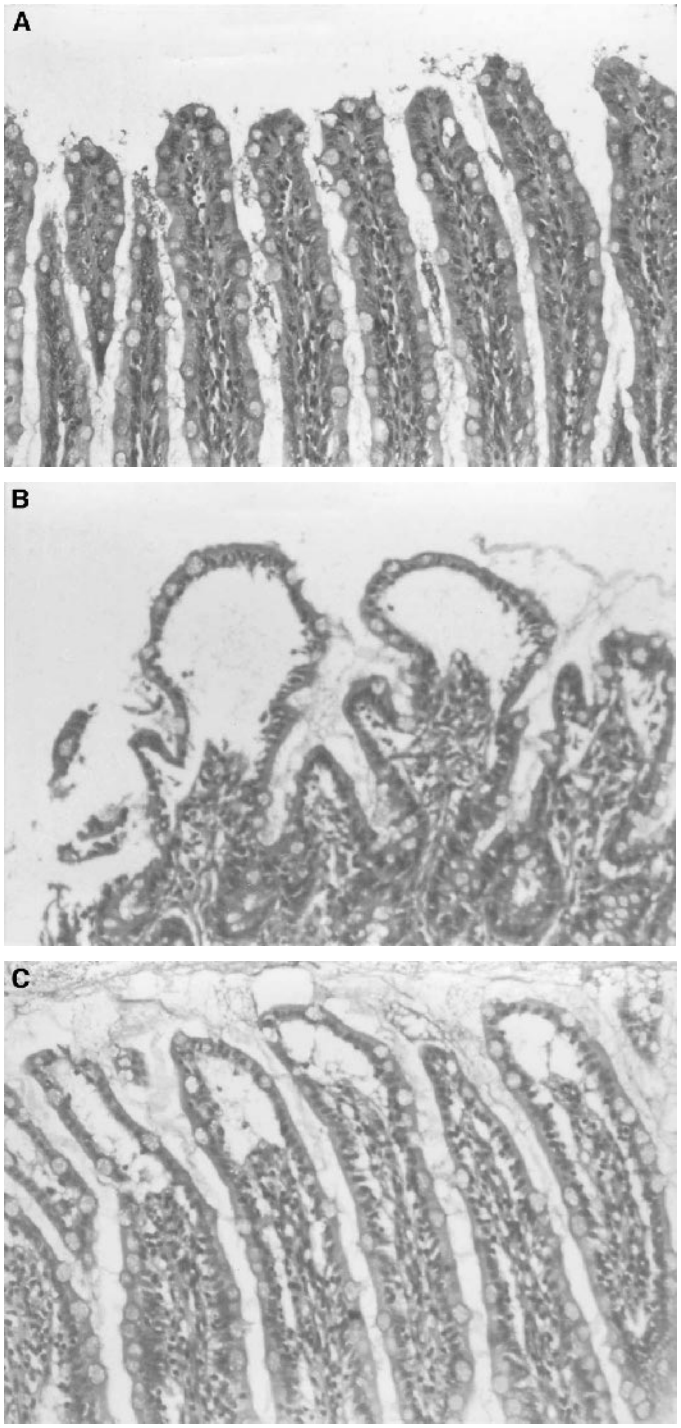

FIG. 2. **Photomicrographs of hematoxylin & eosin-stained sections of the small intestine (×100).** (A) Control group showing normal histopathology. (B) I/R group showing denuded villi and exposed dilated capillaries, and neutrophil infiltration in lamina propria. (C) IPC group showing development of subepithelial Gruenhagen's space at the apex of the villus.

regarding the effects of NO in the intestine, it appears that NO plays a paradoxical role in intestinal physiology. Small amounts of NO may have a cytoprotective effect by enhancing mucosal blood flow. However, there is an increasing body of evidence indicating that overproduction of NO may damage the intestinal integrity, leading to failure of the gut barrier function (20). The most thermodynamically favorable reaction of NO is with the superoxide radical to form peroxynitrite, a

TABLE 2. **Descriptive statistics of ileal nitrite/nitrate levels of the study groups at the end of the experiment**

Groups	Mean ± SD (nmol/g tissue)
Control (n = 8)	22.68 ± 3.91
I/R (n = 8)	74.31 ± 6.19*
IPC (n = 8)	29.23 ± 5.83

Data expressed as mean ± SD nmol/g tissue. *$P < 0.05$, significantly different from other groups.

potent oxidant (21). There is a substantial evidence that NO and/or peroxynitrite can act on the mitochondria to inhibit cellular respiration as a trigger for apoptosis. This process can act to nitrate proteins and such nitrated proteins have been colocalized to the apoptotic enterocytes (18, 22). Shedding of apoptotic enterocytes can result in a transient bare area through which BT can occur. A possible explanation is that IPC reduces apoptosis by regulating gene expression related to the apoptotic pathway (23, 24). We demonstrate in the present study that I/R increased iNOS expression, ileal nitrite/nitrate levels, and intestinal mucosal injury, which were protected by IPC. The proposed mechanisms of intestinal barrier failure during I/R include massive NO production, peroxynitrite formation, and conversion hypoxantine dehydrogenase into xantine oxidase (25). IPC preserved the intestinal integrity by a mechanism that is probably initiated by blockage of iNOS expression in I/R and by decreasing BT.

As a transcriptional regulatory protein, nuclear factor-κB (NF-κB) plays a central role in regulating mediators involved in multiple organ dysfunction associated with I/R injury (26). Excessive activation of NF-κB results in an exuberant inflammatory injury to the organs, and it has been shown that antioxidant treatment suppresses NF-κB activation (27). Kim et al. (28) showed the suppression of iNOS by inhibiting NF-κB activation, and Chen et al. (29) reported inhibition of LPS-induced iNOS and COX-2 gene expression via suppression of NF-κB activation. In addition to these studies, Qu et al. (30) showed that NF-κB regulates the expression of inducible NOS in rat small intestine. Similarly, IPC presents a defense mechanism against a sustained period of subsequent ischemia that may have a role in diminishing the activity of NF-κB in our intestinal I/R model. Furthermore, the changes in NF-κB expression might play a role in iNOS suppression and in the preservation of ileal histology in the present study.

The adverse effects of iNOS in intestinal I/R has been more emphasized recently. Suzuki et al. (31) showed that iNOS knockout mice were more resistant to intestinal I/R-induced mucosal injury than wild-type mice. Banan et al. (32) recently reported that iNOS upregulation mediates oxidant-induced disruption of F-actin and barrier of intestinal cytoskeletal structure. In addition, Tsuruma et al. (33) suggested that one of the mechanisms of the protective effect of heat-shock protein-73 on small intestinal I/R injury in the rat is the inhibition of iNOS expression. The relationship between BT and the NO pathway suggests that iNOS inhibition abrogated the intestinal injury and reduced BT (18, 19). As reported by Szabo et al. (34), the formation of NO and peroxynitrite cause the breakage of DNA strands and activation of poly (ADP-ribose) synthetase (PARS) enzyme in I/R and septic states. As such in our previous study, we found that PARS inhibition prevented intestinal injury and BT in LPS-induced sepsis (35). In the present study, in addition to suppression of iNOS expression, IPC might have inhibited the PARS activation during reperfusion period as well, as recently suggested by Liaudet et al. (36). Considering the mentioned mechanisms above, it seems plausible to propose that the inhibitory effect of IPC in the intestine may be related

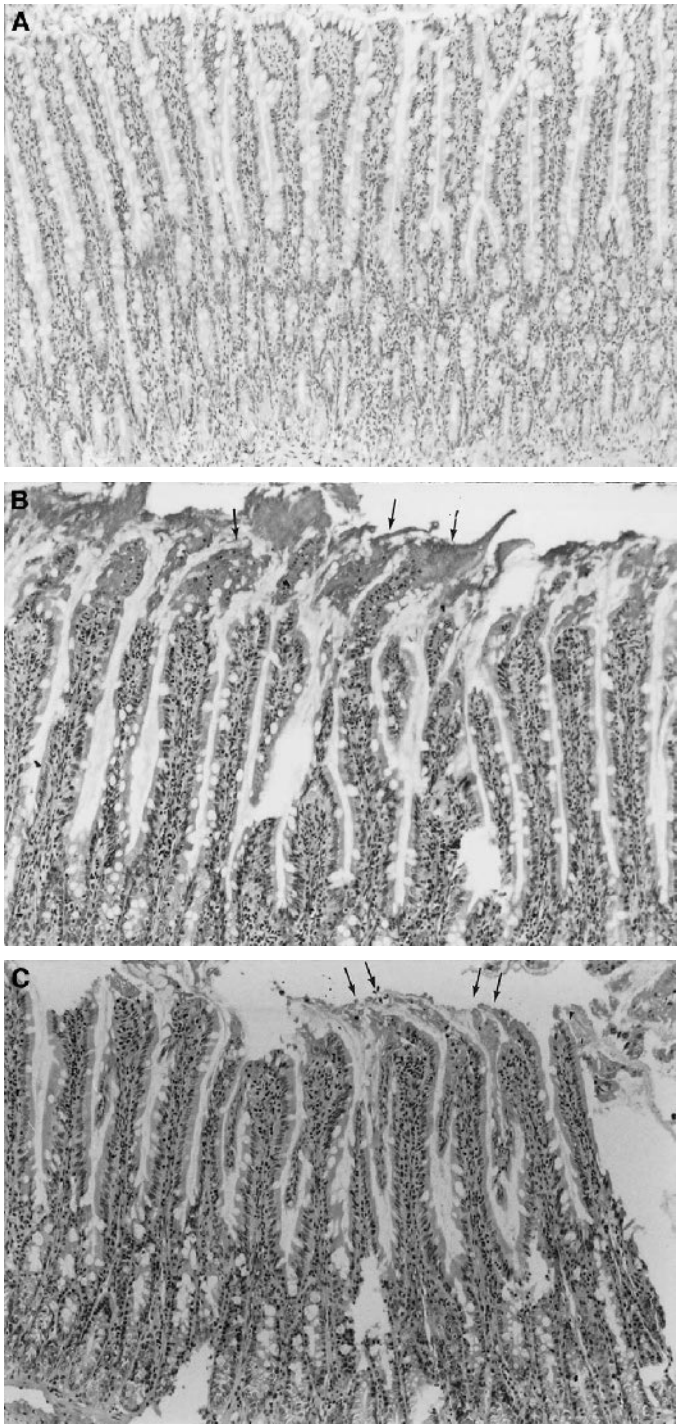

FIG. 3. **Photomicrographs of immunohistochemical study (×100)**. Arrows show areas of staining in all photomicrographs. (A) No staining for iNOS in ileal mucosa from the control group. (B) Increased iNOS expression in ileal mucosa from the I/R group. (C) IPC group prevented the increase in iNOS expression in the I/R group.

to iNOS. It is possible that intestinal IPC might have inhibited iNOS expression via PARS inhibition in the small intestine. Confirming our previous study, IPC protected histopathology of ileum in intestinal I/R model and reduced BT (35).

In summary, IPC abrogated postischemic ileal injury and prevented I/R-induced BT. This is the first study showing that intestinal IPC blocks the cascade of events that causes BT and septic states by regulating iNOS expression.

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