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# LAZAROID U-74500A FOR WARM ISCHEMIA AND REPERFUSION INJURY OF THE CANINE SMALL INTESTINE

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***BACKGROUND:*** **Although lazaroids have been shown to protect various organs from ischemia/reperfusion injury, results obtained in the small intestine have been conflicting.**

***STUDY DESIGN:*** **The canine small intestine was made totally ischemic for 2 hours by occluding the superior mesenteric artery and the superior mesenteric vein with interruption of the mesenteric collateral vessels. A lazaroid compound, U74500A, or a citrate vehicle was given intravenously to each of the six animals for 30 minutes before intestinal ischemia. Intestinal tissue blood flow, lipid peroxidation, neutrophil infiltration, adenine nucleotides and their catabolites, and histologic changes after reperfusion were determined.**

***RESULTS:*** **Lazaroid treatment attenuated decline of the mucosal and serosal blood flow after reperfusion. Accumulation of lipid peroxidation products and neutrophils in mucosal tissues was markedly inhibited by the treatment. Postischemic energy resynthesis was also augmented by lazaroid. Morphologically, mucosal architectures were better preserved with lazaroid treatment after reperfusion, and recovered to normal by postoperative day 3 in the treated group and by postoperative day 7 in control animals.**

***CONCLUSIONS:*** **Lazaroids protect the canine small intestine from ischemia/reperfusion injury by inhibiting lipid peroxidation and neutrophil infiltration. Dogs are tolerant of 2-hour normothermic complete intestinal ischemia. *J. Am. Coll. Surg.*, 1997, 184: 389-396.**

HIGHLY REACTIVE FREE RADICALS derived from the reaction of molecular oxygen with xanthine oxidase have been believed to play a major role in ischemia/reperfusion injury of the small intestine (1). Peroxidation of cellular membranes by oxygen radicals has been shown in postischemic mucosal tissue using malondialdehyde measurement (2), electron-spin resonance, or conjugation diene assay (3). In addition, intestinal injury was prevented by various antioxidants, such as superoxide dismutase (1), catalase (4), allopurinol (1), or α-tocopherol (5). Recently, neutrophil activation (6) and reduced nitric oxide production (7) have also been shown to be associated with intestinal ischemia/reperfusion injury.

Lazaroids, a group of synthetic 21-aminosteroid compounds lacking glucocorticoid and mineralocorticoid actions (8), are potent antioxidants that have been used to protect against ischemia/reperfusion injury of the central nervous system (9), heart (10), lung (11), liver (12), and kidney (13). Results of the use of lazaroids in cases of intestinal ischemia have been conflicting. Chen and coworkers (14), Horton and Walker (15), Stone and colleagues (16), and Katz and associates (17) reported amelioration of mucosal injury with lazaroids, and Park and coworkers (18) and Van Ye and associates (19) found no protection. In the current study, we evaluated the effect of lazaroid U74500A on the canine small intestine undergoing 2 hours of normothermic ischemia.

## MATERIALS AND METHODS

*Animals.* Twelve adult female beagle dogs, weighing 7.2 kg to 11.2 kg, were used. After overnight fasting, the animals were anesthetized with an intravenous injection of 25 mg/kg thiopental-sodium, intubated, and maintained with isoflurane, nitrous oxide, and oxygen by positive pressure mechanical ventilation. During surgery, lactated Ringer's (LR) solution (Baxter, Deerfield, Ill) was given continuously at a rate of 25 to 30 mL/kg per hour through the right jugular vein. Animal body temperature was maintained with a hot water blanket connected to a heat therapy pump (Gaymar, Orchard Park, NY).

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Heart rate, femoral arterial pressure, and esophageal temperature were continuously monitored by a portable patient monitor (Model 514, Spacelabs, Redmond, Wash).

*Operative procedures.* Two-hour warm ischemia of the small intestine was induced by clamping both trunks of the superior mesenteric artery (SMA) and the superior mesenteric vein (SMV). Isolation of the small bowel was performed by the technique originally described by Lillehei and associates (20) and modified later by us for the procurement of intestinal grafts (21). In brief, through a midline laparotomy, the entire small bowel, except short segments near the ligament of Treitz and the ileocecal valve, was isolated on a vascular pedicle consisting of the SMA and the SMV. Lymph nodes and connective tissues surrounding both vessels were carefully dissected and ligated. Immediately before vascular occlusion, the proximal end of the intestine was clamped with atraumatic forceps and the distal end of the intestine was transected. Complete intestinal ischemia was induced 5 minutes after injecting 5 U/kg of sodium heparin (Upjohn, Kalamazoo, Mich) by occluding both the SMA and the SMV with vascular clamps. Ischemia was maintained for 2 hours. During intestinal ischemia, the abdominal wound was temporarily closed to avoid excessive water and heat loss. After collecting ileal tissues for biochemical and histologic studies, distal intestinal continuity was restored by end-to-end anastomosis. One gram of cephamandole nafate (Eli Lilly, Indianapolis, Ind) was given intraoperatively.

The dogs were given standard kennel food the morning after surgery. Ten milligrams per kilogram of intramuscular ampicillin (Fort Dodge Laboratories, Fort Dodge, Iowa) and 500 mL of LR solution with 5 percent dextrose were administered daily for 7 days after the operation. The dogs were brought back to the operating room on the third and seventh postoperative days, anesthetized, and reexplored to collect ileal tissue specimens. On the third postoperative day, the ileum was reanastomosed and the dogs were returned to the animal facility. On postoperative day 7, the dogs were sacrificed after ileal tissue specimens were collected.

*Experimental groups.* Lazaroid U74500A, supplied by the Upjohn Company, was dissolved in a citrate buffer vehicle (pH 3.0) at a concentration of 2 mg/mL. The agent (lazaroid group: n=6), 5 mg/kg, or the vehicle (control group: n=6), 2.5 mL/kg, was given to the dogs through a peripheral vein continuously for 30 minutes before intestinal ischemia. The investigators were blinded to which dogs received lazaroid treatment.

*Intestinal tissue flow.* Tissue blood flow of the ileal mucosa and serosa were measured with a laser doppler flowmeter (ALF21, Advance Co., Tokyo, Japan) before intestinal isolation, after drug administration, 60 minutes and 120 minutes after the onset of ischemia, and 5 minutes, 15 minutes, 30 minutes, and 60 minutes after reperfusion. The measurement was repeated three times on each surface at the antimesenteric site of the terminal ileum.

*Histopathology and biochemistry.* A portion of the distal end of the small intestine, 5 cm long, was resected before ischemia; at the end of 2 hours of ischemia; 15 minutes, 30 minutes, and 60 minutes after reperfusion; and on postoperative days 3 and 7 for biochemical and histologic studies. For histopathologic analysis, tissues were fixed with 10 percent formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The severity of morphologic abnormality was evaluated blindly and scored according to the Park's classification system (22) by a single pathologist. For biochemical analysis, the mucosal layer was quickly scraped with a glass slide, transferred to liquid nitrogen, and stored at −70 degrees C until levels of lipid peroxide products (LPOP), myeloperoxide (MPO), maltase, adenine nucleotides, and purine catabolites were measured. Lipid peroxide product levels in the tissue were estimated as the sum of 4-hydroxy-2(E)-nonetal (4-HNE) and malondealdehyde (MDA) using spectrophotometric kits (LPO-586, Bonnevil Sur Marne, Cedex, France) (23). The supernatant of 10 percent homogenate with Tris-HCl buffer (pH 7.4) was mixed with N-methyl-2-phenilindol and methanesulfonic acid. The mixture was incubated for 40 minutes at 45 degress C, and then placed in an ice water slurry for 10 minutes. After centrifugation, the supernatant was decanted and its absorbance was read at 586 nm. Myeloperoxide was measured using the fluorospectrophotometric method of Krawisz and associates (24). One unit of MPO activity was defined as the concentration that caused a 1.0 change in optical density at 460 nm for 1 minute at 22 degrees C. Adenine nucleotides and purine catabolites were measured using a Waters HPLC system (Waters Chromatography Division, Millipore Corp., Milford, Mass; Model 510 pumps, Model 484

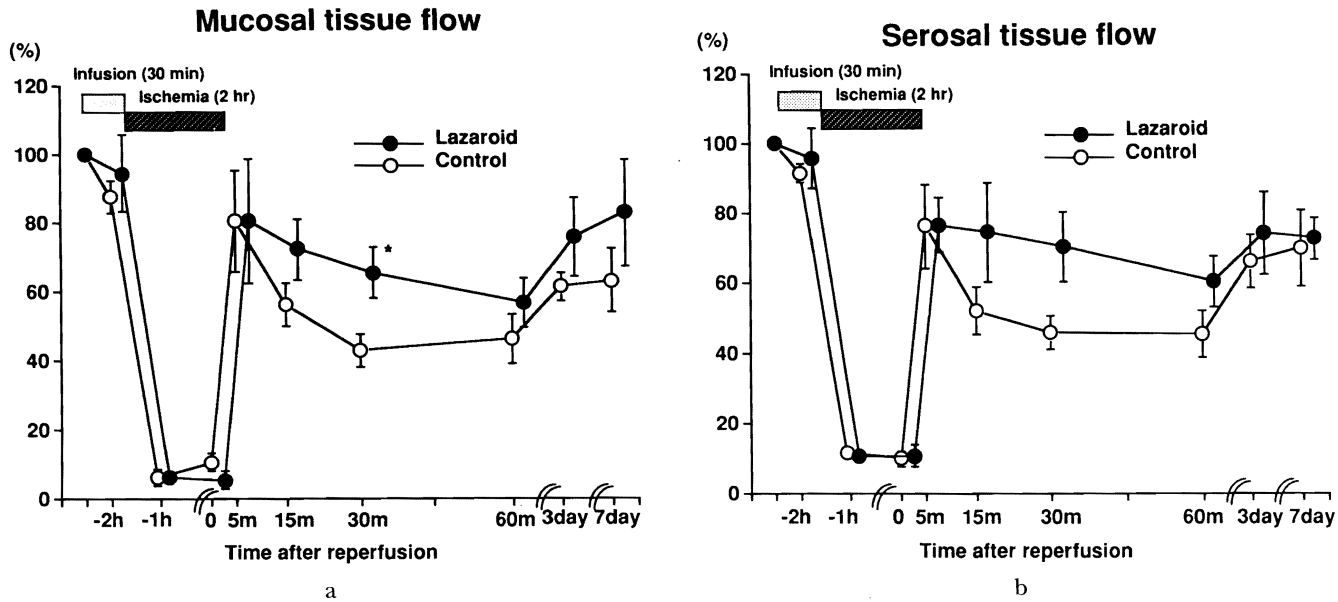



FIG. 1. a, Changes in mucosal tissue flow, and b, serosal tissue flow. *p<0.05 compared with controls. *min,* Minutes; *hr* and *h,* hour; and *m,* month.

absorbance module, and Model 717 WISP system) (25). The concentrations were monitored at 254 nm (Waters 484, Tunable Absorbance Detector). Protein contents in the homogenates were measured using the Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif) (26).

*Statistics.* Data are expressed as mean plus or minus standard error of the mean. The Mann-Whitney U-test was used for comparison between the lazaroid and control groups at the same point in time. The Wilcoxon signed rank test was used for comparison with pre-ischemic values in each group. A p value of less than .05 was considered significant.

## RESULTS

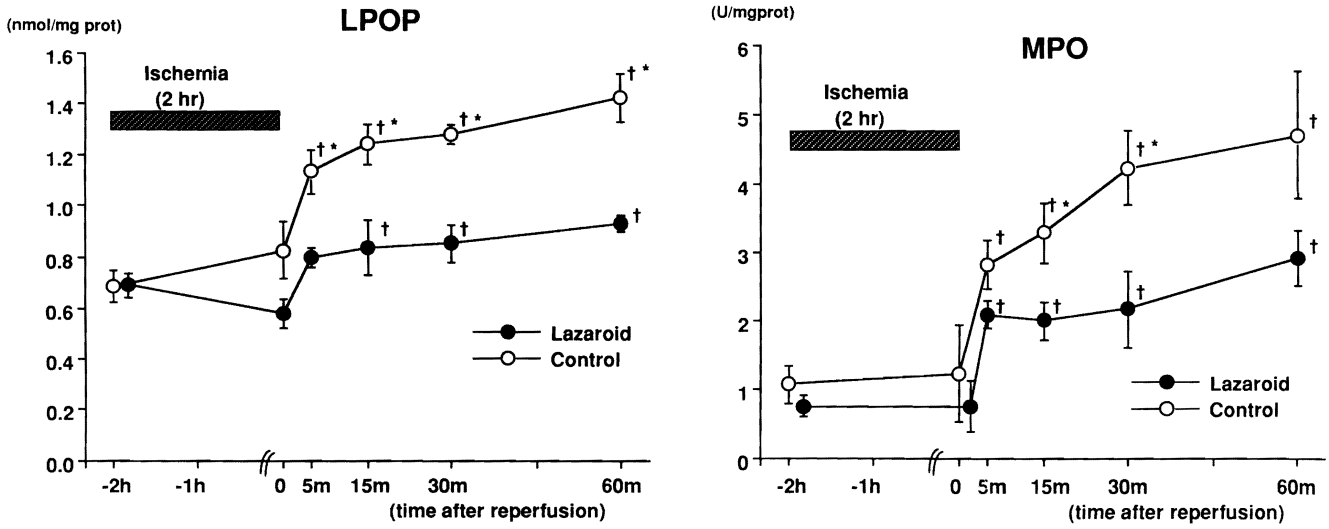
*Clinical course.* No significant hemodynamic changes occurred during U74500A or vehicle administration. In spite of the use of a heating blanket and temporary closure, there was a gradual decline in esophageal body temperature during the experiment in both groups. In both groups, five of six animals survived the 7-day follow-up period. One dog in each group died of intussusception on postoperative day 3. Mild diarrhea developed postoperatively in all surviving dogs, but the dogs remained active during the follow-up period.

*Intestinal tissue blood flow.* Drug administration caused no changes in blood flow at mucosal and serosal tissues (Fig. 1). After reperfusion, however, tissue blood flow was significantly suppressed at each measurement site in the control group compared to the pre-ischemic values. Lazaroid treatment significantly attenuated the decrease of mucosal blood flow at 30 minutes ($p<.035$). Tissue blood flow recovered to normal levels 7 days after surgery in both groups.

*Lipid peroxidation.* Reperfusion of the control group intestine after 2 hours of warm ischemia caused a significant accumulation of LPOP in the mucosal tissue. Lipid peroxide product levels increased immediately after reperfusion and continued to increase, reaching twice the pre-ischemic level by 60 minutes (Fig. 2a). Lazaroid treatment abolished the early increase in LPOP, and markedly suppressed further lipid peroxidation. The difference was highly significant between the two groups.

*Myeloperoxide.* Lazaroid inhibited neutrophil infiltration in the intestinal mucosa after reperfusion (Fig. 2b). Tissue activity of MPO in the control group increased from 1.06±1.9 U/mg protein to 4.22±2.7 U/mg protein at 30 minutes after reperfusion; that of the lazaroid group remained at 2.16±0.5 U/mg protein ($p<.025$).

*Adenine nucleotides and purine catabolites.* Two-hour warm ischemia induced a significant decline in energy charge (27), adenosine triphosphate (ATP), and adenosine diphosphate in both groups. An expected increase in adenosine monophosphate and hypoxanthine (HX) was also seen during ischemia. Reoxygenation allowed partial restoration of the high energy phosphates




a b

FIG. 2. a, Changes in lipid peroxidation products (LPOP: sum of 4-hydroxy-2(E)-nonenal and malondealdehyde), and b, myloperoxidase activity (MPO). *p compared with lazaroid group. †p<0.05 compared with pre-ischemia value in each group. *prot,* protein; *hr,* and *h,* hour; and *m,* month.

in both groups, but ATP and total adenosine nucleotides at 30 minutes were significantly higher in the lazaroid group than in the no-treatment group (p<.037 and p<.043, respectively).

*Histopathology.* At the end of the ischemic period, focal separation of the epithelium from its underlying lamina propria was more prominent in the control group (Fig. 3). The lamina propria was typically edematous, and mucosal capillaries were dilated and congested, but an active inflammatory component was present. After reperfusion, these alterations were superseded by

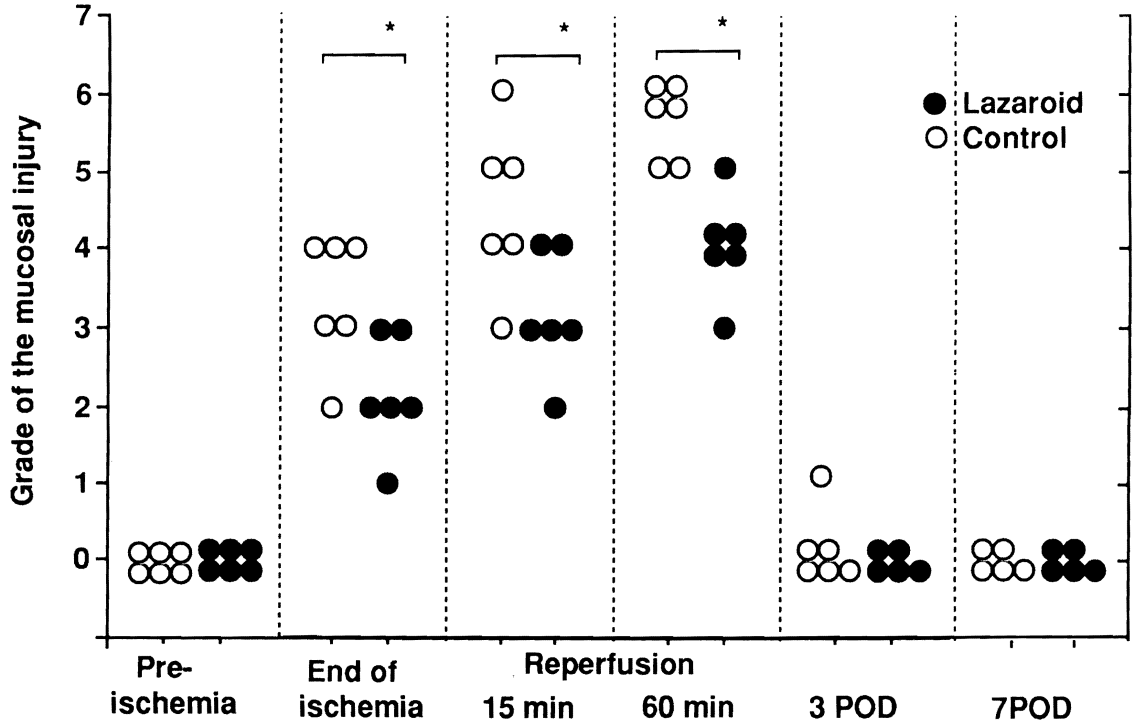
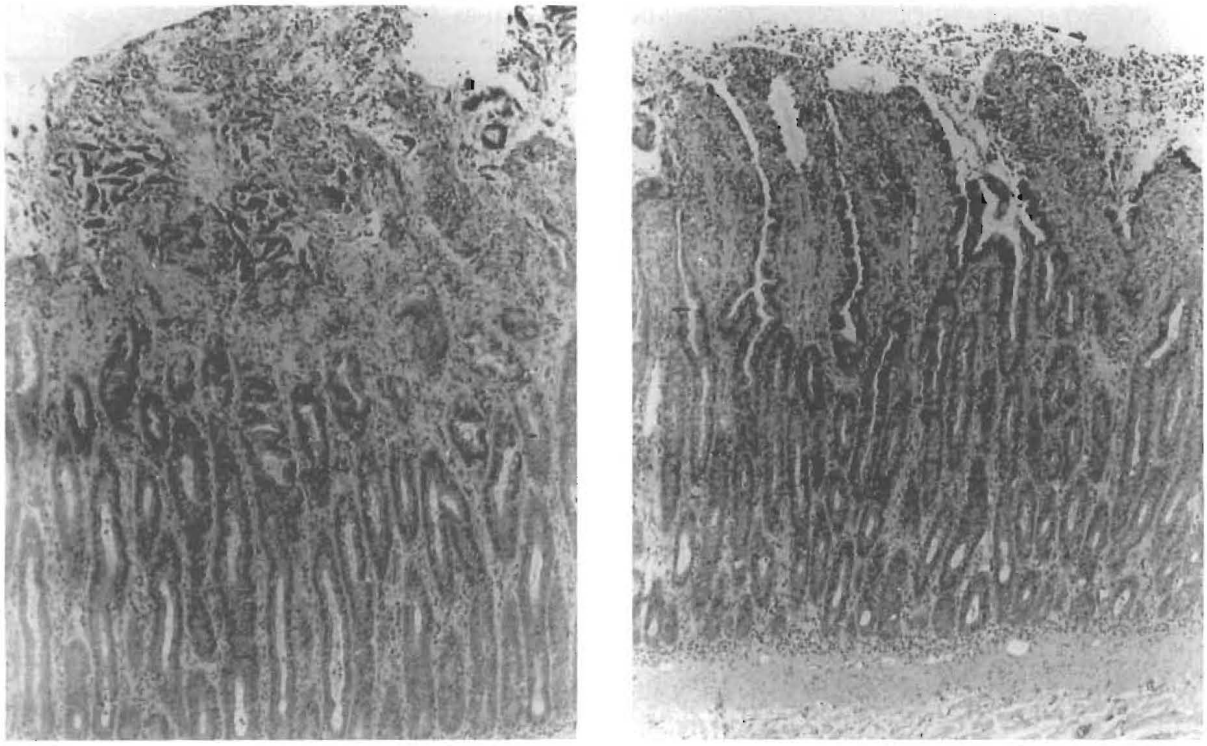



FIG. 3. Histologic extent of the mucosal injury expressed using the grades described by Park and associates (22). *p<.05. *min,* Minutes; and *POD,* postoperaive day.



a b

FIG. 4. a, Mucosa in one control group animal at 60 minutes after reperfusion. There is complete disruption and loss of the villi with focal involvement of crypt layer (Park's level 6). These areas are replaced by necrotic cellular debris, hemorrhage, and early inflammatory exudate. The crypt bases are intact but the crypt epithelium shows prominent reactive changes. Hematoxylin-eosin, original magnification, ×100. b, Mucosa in one of the lazaroid group animals at 60 minutes after reperfusion. The villi are preserved, although they are largely depleted of their epithelium (Park's level 4). The luminal surface contains sparse necrotic material and inflammatory cells. The crypts, although showing reactive alteration, are intact. Hematoxylin-eosin, original magnification, ×100.

greater mucosal injury. Focal or patchy destruction of the mucosal structure developed in the superficial region and involved deeper mucosa in some instances. Neutrophilic infiltrates became evident, as well as the presence of luminal exudate of necrotic debris and an inflammatory exudate. More severe mucosal injury developed 60 minutes after reperfusion, showing extension of the necrotic area in the control group (Fig. 4a). These changes were significantly ameliorated in the lazaroid group (Figs. 3 and 4b). Mucosal alterations were mostly resolved by the third postoperative day, and the mucosa had normal architecture with no residual abnormalities by postoperative day 7 (Fig. 3).

## DISCUSSION

This study demonstrated that normothermic 2-hour ischemia of the canine intestine caused severe mucosal damage after reperfusion, causing a decline in mucosal tissue flow and sloughing of the mucosal layer. The treatment of animals with lazaroid U74500A before intestinal ischemia attenuated histologic damage from ischemia/reperfusion by inhibiting lipid peroxidation and neutrophil infiltration in the mucosal tissues. Irrespective of the treatment, the dogs tolerated 2-hour intestinal ischemia rather well, and the mucosal structure recovered to normal by postoperative day 3 in the treated group and by postoperative day 7 in the control group.

Ischemia/reperfusion injury of the small intestine has been explained by the generation of superoxide anions produced from the hypoxanthine-xanthine oxidase system (located in the enterocytes of the villus tip and in endothelial cells in the microvasculature) and the NADPH oxidase system in neutrophils. Once generated, superoxide anions promote production of reactive oxygen metabolites, such as hydrogen peroxide and hydroxyl radicals. Hydroxyl radicals extract a hydrogen atom from polyunsaturated fatty acids

TABLE I.—MUCOSAL LEVELS OF ADENINE NUCLEOTIDES AND PURINE CATABOLITES

| | *Before ischemia* | *End of ischemia*† | *Reperfusion* | | | |
|---|---|---|---|---|---|---|
| | | | *5 min* | *15 min* | *30 min* | *60 min* |
| EC* | | | | | | |
| Lazaroid | 0.69±0.03 | 0.13±0.02 | 0.55±0.05† | 0.57±0.02† | 0.57±0.05† | 0.05±0.04† |
| Control | 0.65±0.03 | 0.16±0.02 | 0.56±0.06 | 0.50±0.05† | 0.50±0.05† | 0.55±0.06† |
| TAN (nmol/mg prot) | | | | | | |
| Lazaroid | 47.3±2.4 | 32.9±4.4 | 27.7±3.2† | 25.2±2.7† | 32.8±1.2†‡ | 27.5±2.3‡ |
| Control | 46.1±1.9 | 26.8±4.9 | 27.0±3.2† | 23.2±3.4† | 25.9±2.7‡ | 21.8±2.0† |
| ATP (nmol/mg prot) | | | | | | |
| Lazaroid | 26.0±2.1 | 1.6±0.6 | 10.9±1.3† | 10.3±1.4† | 13.9±3.4†‡ | 9.0±1.0† |
| Control | 23.2±1.4 | 1.6±1.1 | 10.9±2.0† | 7.9±1.7† | 8.6±4.0† | 9.0±1.0† |
| ADP (nmol/mg prot) | | | | | | |
| Lazaroid | 13.2±0.8 | 4.6±0.6 | 8.7±1.0† | 8.3±1.3† | 96±0.7† | 9.0±0.7†‡ |
| Control | 13.0±0.7 | 4.9±0.8 | 7.8±1.0† | 6.9±0.9† | 8.0±0.4‡ | 5.9±0.6† |
| AMP (nmol/mg prot) | | | | | | |
| Lazaroid | 8.2±1.0 | 26.7±4.8 | 8.4±1.0 | 6.5±0.5 | 9.4±1.7 | 9.6±1.8 |
| Control | 10.0±1.4 | 20.3±4.0 | 9.4±1.6 | 6.5±0.5 | 9.2±2.2 | 7.1±1.9 |
| HX (nol/mg prot) | | | | | | |
| Lazaroid | 0.94±0.13 | 4.58±0.40 | 2.00±0.30† | 1.34±0.20† | 1.58±0.28† | 1.29±0.21 |
| Control | 1.07±0.13 | 3.75±0.75 | 1.49±0.26 | 1.59±0.28 | 1.43±0.17 | 1.26±0.24 |

*Energy charge (EC=(ATP+1/2ADP)/(ATP+ADP+AMP)).
†P<.05 compared with pre-ischemia values in each group.
‡P<.05 compared with controls.
All data are expressed as mean ±SEM.
min, Minutes; TAN, total adenine nucleotides (TAN=ATP+ADP+AMP); prot, protein; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; and HX, hypoxanthine.

(PUFA) to form lipid radicals (L), which in turn react with oxygen to form lipid peroxyl radicals (LOO), lipid hydroperoxide (LOOH), and lipid alkoxyl radicals (LO). They all react with PUFA to propagate more lipid peroxidation (28). Peroxidation of cellular membranes leads to impairment of cellular function and, finally, to cell death.

In this study, a significant increase in lipid peroxidation in the mucosa of intestines in the control group after reperfusion was confirmed by a new spectrophotometric assay method (23). In ischemia/reperfusion experiments, many investigators used a thiobarbituric acid (TBA) reaction for the measurement of one of the LPOP, MDA. But because the TBA method has been reported to be nonspecific and often provides inconsistent results (29), a new assay method was applied in this study. Using this new method, both 4-HNE and MDA levels were measured without the use of TBA. Recently, 4-HNE, another LPOP, was found to be more cytotoxic than MDA (30) and a potent chemotactic factor for neutrophils (31).

Administration of lazaroid U74500A before intestinal ischemia inhibited accumulation of LPOP in mucosal tissue after reperfusion. Many studies have described the inhibition of ischemia/reperfusion injury by lazaroids in the central nervous system (9, 32), heart (10), lung (11), liver (12), and kidney (13) in experimental animals and in clinical trials. As we have reported, amelioration of ischemia/reperfusion injury by lazaroid U74389G, another lazaroid compound, allowed successful 48-hour preservation and transplantation of canine livers (33). Lazaroids exert their protective effect largely from the inhibition of toxic hydroxyl radical production by chelating ferrous ions ($Fe^{2+}$) in Harber-Weiss reaction or in Fenton reduction of the free radical cascade. Lazaroids also inhibit superoxide production (34) and degranulation of neutrophils (35). Neutrophils are a particularly important factor in ischemia/reperfusion injury of the small intestine. Neutrophils are recruited in the microvasculature of the postischemic tissues by enhanced expression of adherence molecules on the surfaces of neutrophils and endothelial cells (6). Once activated, neutrophils release various cytokines and proteolytic enzymes, which further augment tissue injury initiated by ischemia/reperfusion. Administration of CD11/CD18 antibody or p-selectin antibody ameliorates ischemia/postreperfusion injury. In this study, MPO activity in the mucosal tissue increased in the control group, reflecting neutrophil infiltration after reperfusion (24). Lazaroid treatment significantly suppressed MPO levels in the mucosal tissue. Because free radicals increase levels of leukotrien B4 (35), a potent chemotactic substance of neutrophils, lazaroid U74500A appears to inhibit neutrophil infiltration by its antioxidant properties.

Previously, the effects of lazaroids on ischemia/reperfusion injury of the small intestine have been examined only in rat models (14-19). According to these studies, lazaroids inhibit the increase in intestinal permeability after 20-hour

cold, 60-minute warm (14), and 20-minute warm ischemia (15). Stone and associates (16) showed the attenuation of villus abnormalities after 60-minute warm ischemia by an infusion of 3 mg/kg lazaroid U74006F or U78715G prior to reperfusion. Katz and colleagues also reported that infusion of U74389G to the donor (6 mg/kg) and recipient rat (3 mg/kg) minimized morphologic damage after 18-hour cold preservation and transplantation (17). But, Park and coworkers (18) and Van Ye and associates (18) found no protective effect from U74006F (6 mg/kg) against histologic damage in 60-minute warm or 5-hour cold ischemia (18), or in a 10-minute warm ischemia model (19). These inconsistencies might be caused by differences in ischemic time, experimental model, lazaroid compound, and the timing and method of drug administration. The lazaroid compound and dose (lazaroid U74500A at a dose of 5 mg/kg) was chosen because it was found to be more protective in a 2-hour canine liver ischemia model than other lazaroid compounds (36).

Although the intestine is susceptible to insult from ischemia/reperfusion (37), the canine small bowel appears to be more resistant to ischemia than is the rat small bowel. While Hill and associates (38) reported an 80-percent mortality rate after 1-hour normothermic intestinal ischemia in rats, Lillehei and associates (20) described eight of ten dogs that survived 2-hour intestinal ischemia. The markedly high activity of xanthine oxidase in the rat intestine may explain the difference (39). Although the canine intestine is rather tolerant of normothermic ischemia (40, 41), a potent antioxidant, lazaroid U74500A, could not prevent ischemia/reperfusion injury completely. While mucosal damage was less severe, treated animals still showed histologic derangements, a gradual decrease in mucosal tissue flow, and slow accumulation of neutrophils.

Our results suggest that the mechanism of intestinal ischemia/reperfusion injury is multifactorial, involving not only reactive oxygen metabolites, but also luminal proteolytic enzymes, neutrophils, nitric oxide (7), endothelin, prostaglandins, and other agents. Delineation of the mechanism and invention of a new therapeutic strategy for protection against intestinal ischemia/reperfusion injury, which are currently under study at our laboratory, will serve to improve intestinal preservation and patient treatment.

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