Effects of the 5-Lipoxygenase Inhibitor A-64077 on Intestinal Hypothermic Organ Preservation Injury

MARTIN J. MANGINO, JANICE E. MANGINO, BHARAT KOTADIA and MAREK SIELCZAK

Department of Critical Care Medicine, Miami Children’s Hospital, Miami, Florida and Division of Pulmonary Medicine, Dept. of Research, University of Miami School of Medicine at Mt. Sinai Medical Center, Miami Beach, Florida

Accepted for publication January 21, 1997

ABSTRACT

The effects of the orally active selective 5-lipoxygenase inhibitor Zileuton (A-64077, (N-(1)-benzo[b]thien-2-ylethyl)-N-hydroxyurea) were studied in a canine model of hypothermic intestinal organ ischemia-reperfusion (I/R) injury (transplant preservation injury). Forty-eight hours of hypothermic intestinal ischemia utilizing Collin’s flush, followed by 1 hr of reperfusion (transplantation) in A-64077-treated animals, resulted in a 3-fold increase in intestinal oxygen uptake and blood flow relative to the untreated controls. The postreperfusion movement of fluid from the microcirculation into the intestinal lumen significantly increased in the control animals at reperfusion, and A-64077 treatment dramatically exacerbated this phenomenon. Mucosal neutrophil infiltration, or the processes leading to infiltration, significantly increased after 48 hr of cold ischemia and 1 hr of normothermic reperfusion in the untreated animals. A similar response was observed in A-64077-treated dogs, but the absolute levels of MPO were 10-fold less relative to untreated animals, including intestinal tissue obtained before I/R. Hypothermic I/R injury in this model resulted in severe histologic injury. A-64077-treated dogs, however, demonstrated significant improvements in histologic injury. Mucosal synthesis of LTB4 rose significantly after cold I/R injury and was abrogated by A-64077 treatment. The synthesis of PGE2 significantly increased after cold I/R in both untreated and A-64077-treated dogs. The increase in PGE2 production after hypothermic I/R in the A-64077-treated animals was higher relative to the untreated control animals. In conclusion, this study indicates that arachidonic acid metabolism via the 5-lipoxygenase pathway plays a significant role in the pathophysiology of hypothermic intestinal I/R injury. Furthermore, the 5-lipoxygenase inhibitor A-64077 possesses favorable pharmacologic and biologic responses in this intestinal injury and should be considered in the clinical amelioration of intestinal transplantation preservation injury.

Intestinal I/R injury at normothermic conditions (37°C) results in severe morphologic injury characterized by loss of villus epithelial cells, capillary congestion, focal necrosis and neutrophilic inflammation (Mangino et al., 1989; Granger et al., 1986; and Parks and Granger, 1986). Intestinal I/R injury also causes a breakdown of the microvascular endothelial and villus epithelial barrier. This results in massive movements of fluid and the convective solvent drag transfer of macrovascules from the capillary into the interstitium and through the villus epithelial barrier into the intestinal lumen (Mangino et al., 1989; Toledo-Pererya and Granger, 1993). Furthermore, mucosal tissues subjected to I/R injury synthesize significant quantities of arachidonic acid metabolites, including prostaglandins, thromboxanes, leukotrienes and HETE regiosomers (Mangino et al., 1989). The 5-lipoxygenase pathway that produces leukotrienes and 5-HETE plays a significant role in intestinal I/R-induced pathophysiology. Inhibition of 5-lipoxygenase with the orally available and specific enzyme inhibitor A-64077 (Zileuton, Abbott Laboratories, Chicago, IL) significantly improves reperfusion intestinal blood flow and VO2 and abolishes the I/R-induced increase in mucosal neutrophil infiltration in normothermic I/R injury. (Mangino et al., 1994).

Clinical organ transplantation typically involves harvesting of the organ and intravascular flushing with cold preservation fluids followed by simple cold storage at 4°C before transplantation into the recipient donor (Belzer and Southard, 1988). The cold ischemia times vary, case by case and are carefully kept to a minimum, but they may result in hypothermic ischemia times of several hours or days, depending on organ-sharing strategies and tissue cross-matching times (Clark, 1992). Thus, prolonged hypothermic ischemia results in I/R injury at reperfusion (transplantation) in the small intestine, especially because this organ is extremely sensitive to ischemia, even at hypothermic temperatures (Mangino et al., 1994). Clearly, a parallel exists between normothermic

ABBREVIATIONS: I/R, ischemia-reperfusion; HETE, hydroxyeicosatetraenoic acid; LTB4, leukotriene B4; MPO, myeloperoxidase; VO2, oxygen consumption.
and hypothermic intestinal ischemia-reperfusion injury in the small intestine. A recent study by this laboratory has characterized intestinal mucosal arachidonic acid metabolism in intestines subjected to prolonged cold ischemia (48 hr) and subsequent short-term reperfusion injury (Mangino et al., 1996b). These results indicate significant increases in the tissue production of prostaglandins, thromboxanes and 5-lipoxygenase products after 48 hr of hypothermic intestinal ischemia and 1 hr of normothermic reperfusion after transplantation. Although the synthesis of many arachidonic metabolites significantly increased after cold I/R (Mangino et al., 1996b), the 5-lipoxygenase metabolites (leukotrienes and 5-HETE) were synthesized 1000-fold higher than the cyclooxygenase metabolites. The 5-lipoxygenase metabolites detected were LTB\textsubscript{4} and 5-HETE in the \textit{delta} lactone molecular configuration. Similar responses in 5-lipoxygenase metabolism in the normothermic and hypothermic intestinal I/R models have been observed, and selective 5-lipoxygenase inhibitors have been shown to be protective in normothermic intestinal I/R (Mangino et al., 1989; Mangino et al., 1994). Therefore, this study was designed to evaluate the effects of the 5-lipoxygenase inhibitor A-64077 in hypothermic intestinal organ preservation I/R injury.

Materials and Methods

Surgical procedures. All experiments \(n = 12\) were conducted in adult mongrel dogs. Animals were anesthetized with halothane-nitrous oxide, and a segment of the distal ilium (about 75 g) was isolated on its vascular pedicle, removed and quickly flushed with 4°C Euro-Collin’s solution under aseptic conditions. Each segment was flushed with 200 ml of Collin’s solution through the single artery perfusing the segment and with 500 ml of Collin’s solution through the intestinal lumen. One group of dogs \(n = 6\) served as untreated controls, and the other group \(n = 6\) was treated with the 5-lipoxygenase inhibitor A-64077 (5 mg/kg p.o.) given 2 hr before removal of the intestinal grafts and 2 hr before autotransplantation. Both groups of intestinal grafts were stored at 4°C for 48 hrs in cold Collin’s solution. The ends of the remaining intestine were anastomosed end to end to restore continuity of the bowel, and the abdominal incision was closed. The animals were then allowed to awaken spontaneously. After 48 hr of hypothermic intestinal ischemia, the segments were reperfused \textit{in situ} for 60 min. The single artery perfusing the segments and the single vein draining the segments were anastomosed to the femoral artery and vein, respectively. A sample of the venous outflow and a reference arterial sample of blood were directed to separate cuvettes of an arterio-venous oxygen content difference analyzer (A-VOX Systems, San Antonio, TX). A blood flow probe was placed in the arterial circuit (Transonics, 2 mm) for measurement of arterial blood flow. The blood flow signal and the A-VO\textsubscript{3} signal were recorded on a grass polygraph. Oxygen consumption was computed as the product of blood flow and the A-VO\textsubscript{2} difference. At the end of the 60-min reperfusion period, all bowel secretions were harvested for measurement of fluid flux into the lumen. Ileal tissues were obtained before ischemia, after 48 hr of ischemia and after 48 hr of ischemia and 1 hr of reperfusion in both untreated and A-64077-treated animals for measurement of neutrophil content, lipid mediator synthesis and histology.

Biochemical determinations. MPO activity was measured and used as an index of tissue neutrophil content (Grisham et al., 1986). About 1 g of tissue was homogenized in buffer and centrifuged at 2000 \(\times\) g, and the supernatant was discarded. The remaining pellet was rehomogenized in phosphate-buffered saline (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide and 0.01 M EDTA. This material was subjected to three freeze/thaw cycles and centrifuged. The remaining supernatant was assayed for MPO by measuring the \(\text{H}_2\text{O}_2\)-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. The rate of oxidation, and therefore MPO activity, was monitored and quantitated by the change in light absorbance at 655 nM in a Shimadzu 1601 Doublebeam UVVIS Spectrophotometer. One unit of MPO activity was defined as the amount of MPO necessary to change light absorbance at 655 nM by 1.0 AU/min.

Mucosal production of LTB\textsubscript{4} and PGE\textsubscript{2} was assessed by measuring the release of eicosanoid into tissue incubation media \textit{via} competitive binding radioimmunoassay. About 200 mg of mucosal tissue was incubated in 2.5 ml Krebs’ buffer at 37°C for 30 min under an atmosphere of 95% O\textsubscript{2} and 5% CO\textsubscript{2} in a Dubanoff shaking metabolic incubator. After the 30-min incubation period, the incubation media were harvested, frozen and later assayed for LTB\textsubscript{4} and PGE\textsubscript{2}. Equal quantities of sample media, specific antibody for the eicosanoid being measured (Perceptive Diagnostics, Cambridge, MA) and tritium-labeled tracer (New England Nuclear, Boston, MA) were combined and allowed to achieve equilibrium at 4°C for 18 hr. After this equilibration period, the unbound eicosanoid in the sample was removed with a charcoal-dextran solution. After centrifugation and pelleting of the unbound eicosanoid, the antibody-bound material was quantitated by liquid scintillation spectroscopy. All experimental values were obtained by comparison with standard curves performed with each assay using authentic eicosanoid standards (Sigma Chemical Company, St. Louis, MO). Data were normalized to 100 mg tissue weight and represent the total analyte produced over a 30-min period. The PGE\textsubscript{2} antiserum was provided by Dr. Aubrey Morrison, Washington University School of Medicine, St. Louis, MO.

Serum A-64077 levels were determined at the time of reperfusion of the cold stored intestines, and this time-point represents 2 hr after the last p.o. dose of A-64077. Serum proteins were precipitated with one volume of cold methanol and centrifugation. The resulting supernatant was injected directly into a Shimadzu LC-10AD tertiary gradient liquid chromatograph with the output directed through a Varian 9050 UVVIS Spectrophotometer. The output of the spectrophotometer was analyzed by a Hewlett-Packard HP 3395 integrator.

The HPLC was operated in the reverse phase using a C-18 
\textit{Bondapak 300 \times 3.0-mm} column, 10-μ particle size (Waters), with a mobile phase of methanol/water/acetic acid, 500:500:0.4 delivered isocratically at a flow rate of 1.0 ml/min. A-64077 was detected and quantitated by UV absorbance at 231 nM and compared with a standard curve produced with known quantities of A-64077. The elution time of A-64077 on this system was about 8 min.

Histology. Samples of intestine were obtained before ischemia, after 48 hr of hypothermic ischemia and after 48 hr of reperfusion and were placed in 10% neutral buffered formalin. Tissue was embedded in paraffin, sectioned at 4 μ, stained with hematoxylin and eosin and examined by light microscopy in a blinded manner. The overall histologic damage was assigned a grade according to the following criteria: (Mangino et al., 1989) \textit{Grade 0}, no specific pathologic changes could be seen. Villus architecture, fibrovascular core distributions and goblet cells appear normal. \textit{Grade 1}, mild damage. Villus height is normal, but the tips of some villi are denuded of epithelium, and the tips are dilated by eosinophilic proteinaceous liquid. The crypts appear distorted and irregular with mild vascular congestion. The lamina propria and muscle layers are normal. \textit{Grade 2}, moderate damage. Specifically, loss of villus height with lymphangiectasis of eosinophilic fluid is visible. Focal erosions, depletion of goblet cells and increased numbers of inflammatory cells, mostly polymorphonuclear leukocytes in crypts and in the lamina propria, are seen. Vascular congestion, edema and focal hemorrhages are present. Lamina and muscle layers are normal. \textit{Grade 3}, severe damage. More than 50% of the villi are gone, and the ones remaining are severely denuded of epithelium. Focal necrosis is seen with granulomatous material replacing the villus epithelia. Neutrophil infiltration in crypt epithelia (cryptitis) and invasion of neutrophils into the crypt lamina (crypt abscesses) are seen. Plaque cells, eosinophils, basophils and lymphoid aggregates are present with polymorphonu-
clear leukocytes in the capillaries. Focal necrosis of the lamina is seen. Submucosa and muscle layers are normal.

**Statistical analysis.** Statistical significance was set at \( P < .05 \). Most data were analyzed using analysis of variance with Dunnett’s post-test. Histology scores were analyzed by the Kruskal-Wallis non-parametric ANOVA with Dunn’s multiple-comparison post-test. All data except histology scores followed a Gaussian distribution frequency.

**Results**

Figure 1 shows mucosal LTB\(_4\) synthesis from mucosal intestine before ischemia, after 48 hr of hypothermic ischemia and after 1 hr of reperfusion from untreated animals and from animals treated with the selective 5-lipoxygenase inhibitor A-64077. Hypothermic ischemia and short-term reperfusion significantly doubled mucosal LTB\(_4\) synthesis relative to nonischemic intestine. The I/R-induced increase in LTB\(_4\) synthesis was abolished in A-64077-treated animals. Also, the absolute tissue levels were reduced 100-fold in treated animals.

Figure 2 illustrates PGE\(_2\) synthesis by intestinal mucosa before ischemia, after 48 hr of cold ischemia and after reperfusion in untreated and A-64077-treated animals. PGE\(_2\) synthesis significantly rose in intestinal mucosa in both A-64077-treated and untreated animals after 48 hr of hypothermic ischemia and 1 hr of reperfusion, a result that indicates enzymatic specificity of this agent for 5-lipoxygenase inhibition (fig. 1 vs. fig. 2). Also, the PGE\(_2\) synthesis by intestinal mucosal tissue after hypothermic I/R was significantly higher in the 5-lipoxygenase inhibitor-treated animals relative to the untreated controls. This may indicate a reorientation of arachidonic acid metabolism toward the cyclooxygenase pathway and away from the 5-lipoxygenase metabolic pathway via a mass-action substrate shift (Needleman et al., 1986).

Figure 3 demonstrates the dramatic effects of the 5-lipoxygenase inhibitor A-64077 on intestinal blood flow after 48 hr of hypothermic I/R. Intestinal blood flow at reperfusion was 1- to 5-fold higher relative to the blood flow values obtained in the untreated controls. The 5-fold increases in blood flow levels in the 5-lipoxygenase inhibitor-treated dogs were observed from 30 to 60 min after reperfusion, but blood flow in the treated animals was significantly higher at all of the measured time-points.

Figure 4 illustrates the differences in VO\(_2\) after 48 hr of cold ischemia and during short-term reperfusion in untreated animals and in those treated with the selective 5-lipoxygenase inhibitor A-64077. Clearly, A-64077 treatment...
significantly increases intestinal VO₂ during the reperfusion period, especially after 30 min of reperfusion.

Figure 5 shows the transmucosal flux of fluid from the microcirculation at 1 hr of reperfusion in control and A-64077-treated animals. Fluid flux in intestines before ischemia was negative, indicating net fluid absorption, which is normal. Fluid movement after reperfusion in the untreated controls and the A-64077-treated dogs significantly increased relative to intestinal fluid flux before ischemia. Furthermore, the transvascular movement of fluid into the intestinal lumen was significantly higher in the 5-lipoxygenase-inhibited animals, as compared with the untreated animals. Intestinal blood flow, fluid flux and protein flux were not influenced by A-64077 in nonischemic intestines relative to untreated controls (Mangino et al., 1994).

Figure 6 indicates significant increases in mucosal MPO levels after 48 hr of hypothermic ischemia and 1 hr of reperfusion relative to mucosal MPO levels measured before ischemia or after 48 hr of cold ischemia in both untreated and A-64077-treated dogs. Although MPO levels significantly increased after I/R in the A-64077-treated animals, the absolute MPO values that were measured were 10-fold less than those measured in the untreated animals. This 10-fold decrease in MPO levels was observed in intestinal mucosa obtained before ischemia, after 48 hr of hypothermic ischemia and after 1 hr of reperfusion, relative to the respective tissues in the untreated group. These data suggest that this agent is capable of depleting resident tissue or adherent MPO-producing cells, as well as attenuating I/R-induced increases in neutrophil infiltration into the intestinal mucosa or adherence to the intimal lining.

Figure 7 shows the histologic injury suffered by intestinal tissue subjected to 48 hr of cold ischemia and 1 hr of normothermic reperfusion in both untreated and 5-lipoxygenase inhibitor-treated animals. Forty-eight hours of hypothermic intestinal ischemia and short-term reperfusion (1 hr) result in significant tissue histologic ischemia, as assessed by light microscopy. Specifically, intestinal cold I/R injury in this model causes massive villus shortening, loss of villi epithelial cells, vascular congestion and inflammatory cell infiltration. Administration of the selective 5-lipoxygenase synthesis inhibitor A-64077 significantly reduced the degree of histologic I/R injury compared with that commonly observed in the untreated control animals.

Figure 8 depicts representative histologic photomicrographs of normal intestinal tissue (panel A) and intestinal

Fig. 4. Intestinal VO₂ during reperfusion in untreated and A-64077-treated dogs. ** P < .05 between the two groups at the respective time intervals; n = 6 in each group.

Fig. 5. Intralumenal fluid flux after 1 hr of reperfusion in intestinal segments before ischemia, after 48 h of cold ischemia in untreated dogs and after 48 hr of cold ischemia in A-64077-treated dogs. n = 6 in each group, ** P < .05 relative to before ischemia. # P < .05 relative to control reflow.

Fig. 6. Mucosal myeloperoxidase activity from intestines obtained before ischemia, after 48 hr of hypothermic ischemia and after 1 hr of in situ reperfusion in untreated animals and in animals treated with the 5-lipoxygenase synthesis inhibitor A-64077. All values are mean ± S.E.M.; n = 6.

Fig. 7. Average histologic grade of ischemic injury by light microscopy of intestinal sections before ischemia, after 48 hr of cold ischemia and after 1 hr of reperfusion in untreated and A-64077-treated animals. The grading scale is from 0 (normal) to 3 (severe ischemic injury); n = 6.
tissue at various stages of hypothermic ischemia reperfusion injury (panels B–D, histologic grades 1–3, respectively; see “Materials and Methods”). Serum blood levels of A-64077 in the treated animals was measured 2 hr before reperfusion of the hypothermically stored intestinal grafts. High-performance liquid chromatography (HPLC) revealed that the average A-64077 blood levels were 15.9 ± 4.4 μM. These concentrations have been shown to abolish LTB₄ production in vitro (Summers et al., 1987) and to abolish intestinal I/R-induced mucosal LTB₄ synthesis in vivo (Mangino et al., 1994). Thus the dosing of this inhibitor in this study results in historically efficacious blood levels. This contention is substantiated by the abolished mucosal LTB₄ levels in the A-64077-treated dogs after cold I/R (fig. 1).

**Discussion**

Forty-eight hours of hypothermic intestinal ischemia and 1 hr of reperfusion in the canine model result in characteristic injury, including microcirculatory breakdown, neutrophil infiltration and leukotriene synthesis. Administration of the selective 5-lipoxygenase synthesis inhibitor A-64077 abolished I/R-induced leukotriene synthesis, tripled postperfusion blood flow and VO₂, prevented postperfusion neutrophil infiltration and improved histologic signs of ischemic injury.

A significant increase in mucosal leukotriene synthesis occurs in normothermic intestinal I/R injury (Mangino et al., 1989) and in intestines subjected to 48 hr of cold ischemia and subsequent short-term reperfusion (Mangino, 1996b et al., and Mangino et al., 1994). Furthermore, the selective 5-lipoxygenase synthesis inhibitor A-64077 significantly improves postperfusion intestinal function and abrogates cellular inflammatory responses in the normothermic model of intestinal I/R injury. (Mangino et al., 1994 and Zimmerman et al., 1990). These data suggest that arachidonate 5-lipoxygenase synthesis plays a significant role in both normothermic and hypothermic intestinal I/R injury.

The selectivity and efficacy of this agent are essential if this drug is to be used as a pharmacologic tool for testing the hypothesis of this study. Serum blood levels obtained before autotransplantation of the intestinal segments indicate a concentration of 15 μM A-64077 (see “Results”). This drug level has been shown significantly to inhibit mucosal 5-lipoxygenase (Mangino et al., 1994), and 15 μM abolishes ex vivo ionophore-stimulated canine blood LTB₄ synthesis (Personal Communication, Dr. George Carter, Abbott Laboratories, Chicago, IL). More important, figure 1 shows significant increases in mucosal LTB₄ synthesis after hypothermic I/R in untreated animals (nanogram quantities), whereas LTB₄ levels in A-64077-treated dogs were abolished (low picogram quantities). Selectivity of A-64077 for the 5-lipoxygenase enzymatic pathway is important in interpreting these data, and selectivity has been verified in this study; the results are shown in figure 2. Mucosal synthesis of the cyclooxygenase metabolite PGE₂ significantly increases after hypothermic I/R relative to nonischemic mucosa, and this observation also occurred in A-64077-treated animals. In fact, PGE₂ levels were significantly higher in A-64077-treated animals after cold I/R relative to untreated controls, which suggests a possible reorientation of endoperoxide metabolism by A-64077 away from the 5-lipoxygenase pathway into the cyclooxygenase pathway. The possibility that A-64077 may inhibit the 12-lipoxygenase or 15-lipoxygenase pathway and therefore account for the observed functional and immunologic effects in this study cannot be definitively ruled out. However, preliminary unpublished HPLC data indicate no inhibition of 12-HETE synthesis in A-64077-treated animals after cold I/R relative to the untreated controls. Therefore, it appears that the observed functional, metabolic, and cellular inflammatory changes in the A-64077 treated animals is most likely attributable to inhibition of arachidonic acid metabolism via
the 5-lipoxygenase pathway or a consequence of such inhibition.

Intestinal blood flow, and therefore O₂ delivery, significantly increased 2- to 5-fold during reperfusion after 48 hr of hypothermic ischemia in A-64077-treated dogs relative to the untreated animals (fig. 3). This massive vasorelaxation in A-64077-treated animals may be due to inhibition of 5-lipoxygenase products formed at reperfusion after prolonged cold ischemia. The possible 5-lipoxygenase metabolites include LTB₄, the thiol ether leukotrienes (LTC₄, LTD₄, LTE₄ and LTF₄) and 5-HETE. Of these metabolites, only the thiol ether leukotrienes possess significant vasoconstrictive properties (Needleman et al., 1986), but this class of leukotrienes does not increase after 48 hr of hypothermic mucosal I/R (Mangino, 1996B). Therefore, the vasorelaxation observed after cold I/R in 5-lipoxygenase inhibitor-treated animals probably is not the result of direct inhibition of 5-lipoxygenase products. However 5-HETE in the delta lactone molecular configuration is produced in dramatically large amounts (nanogram quantities) after cold I/R (Mangino et al., 1996B). The direct vasoconstrictive properties of 5-HETE are considered minimal (Needleman et al., 1986), whereas to the best of our knowledge, the vasoactive properties of 5-HETE in the delta lactone configuration are unknown. Therefore, the possibility that delta lactone 5-HETE synthesis inhibition may be responsible for the dramatic increases in intestinal blood flow at reperfusion in A-64077-treated subjects cannot be excluded. Perhaps, the significantly increased synthesis of the vasodilatory prostaglandin PGE₂ at reperfusion in A-64077-treated dogs causes the observed postreperfusion vasorelaxation. Experiments using cyclooxygenase inhibitors should address this hypothesis. Also, reduced cellular inflammation or the synthesis of other vasoactive mediators with selective 5-lipoxygenase inhibition in this model may account for the observed increased reperfusion blood flow.

Reperfusion intestinal VO₂ after 48 hr of hypothermic ischemia was dramatically higher in A-64077-treated animals than in the untreated controls (fig. 4). There are three mechanisms that might account for this observation: 1) increased cellular oxidative metabolism caused by the drug or a metabolite altered by the drug, 2) enhanced capillary exchange capacity of oxygen from the microcirculation to the mucosal parenchyma and 3) increased numbers of viable aerobic parenchymal cells resulting from A-64077 treatment. The direct enhancement of O₂ metabolism by A-64077 or by altered metabolites resulting from A-64077 treatment seems unlikely, because eicosanoids themselves have not demonstrated such a metabolic effect (Needleman et al., 1986). The possibility that inhibition of the synthesis of 5-lipoxygenase during reperfusion after prolonged cold ischemia enhances the capillary exchange capacity of O₂ from the capillary to the local mucosal cells should be considered. Specifically, enhanced PGE₂ with A-64077 may relax precapillary sphincters, allowing increased capillary recruitment, decreasing the capillary-to-cell diffusion distance and thereby enhancing O₂ exchange to the ischemic parenchymal cells. A similar capillary recruitment may result from the A-64077-induced abolition of neutrophil infiltration (fig. 6), thereby reducing capillary plugging by neutrophils, i.e., inhibiting the no-reflow phenomenon. Finally, increased intestinal VO₂ at reperfusion may simply be the result of larger numbers of O₂ utilizing mucosal parenchymal cells in the A-64077-treated animals relative to the untreated controls. This contention is supported by the significant histologic benefit afforded to A-64077-treated animals after cold I/R (fig. 7). Larger numbers of dead necrotic cells in untreated animals would simply reduce the tissue O₂ demand even with the possibility of equal O₂ delivery in both treated and untreated animals, thereby reducing VO₂ in the untreated controls. Probably a combination of increased oxygen delivery (blood flow and capillary exchange capacity) and an increased O₂ demand (larger numbers of viable O₂-utilizing mucosal cells) contributes to the 5-fold increase in intestinal VO₂ observed in A-64077-treated dogs relative to the untreated dogs.

Hypothermic intestinal ischemia, like normothermic intestinal I/R injury, is characterized by microvascular-mucosal barrier breakdown, resulting in significant transvascular and transepithelial movement of fluid from the microcirculation into the intestinal lumen (Mangino et al., 1989 and Mangino et al., 1994). Figure 5 reproduces these results in hypothermic I/R intestinal injury. Normal intestines exhibited negative fluid flux, indicating a typical net fluid absorption, whereas untreated intestines subjected to 48 hr of cold ischemia and 1 hr of in situ reperfusion produced significant increases in the positive movement of fluid into the intestinal lumen. Intestinal fluid flux in the A-64077-treated animals was 3-fold higher after reperfusion than in the untreated controls. The mechanism for this cannot be conclusively deduced from these data, but it may be attributable to the dramatic increases in intestinal blood flow at reperfusion in the A-64077-treated subjects. Specifically, an increase in total intestinal blood flow, combined with increases in capillary recruitment, may result in elevated capillary hydrostatic pressures, thereby pushing intracapillary fluid across the capillary endothelium and raising interstitial fluid pressures. Assuming that the mucosal epithelial barrier in A-64077-treated animals after cold I/R injury is dysfunctional (figs. 7 and 8), then the accumulated interstitial fluid would be free to cross the mucosal epithelial barrier and enter the intestinal lumen, resulting in the observed enhanced fluid flux in the A-64077-treated subjects relative to the fluid flux observed in the untreated controls. However, this remains strictly hypothetical because capillary pressures were not measured in this study. Also, the possibility of energy-dependent fluid secretion via chloride ion transport in the treated group cannot be excluded. Mechanically reducing blood flow at reperfusion in A-64077-treated animals to levels observed in untreated animals and then observing lumenal fluid flux would be one way to investigate these proposed mechanisms.

Neutrophil infiltration (as assessed by tissue MPO levels) into intestinal mucosa after normothermic and hypothermic I/R has been established (Zimmerman et al., 1990; Mangino et al., 1994; Mangino et al., 1996a). The data in this study reproduce the former results in the untreated animals (fig. 6). Although a minor statistically significant increase in MPO levels was also observed in the A-64077-treated dogs after I/R, the absolute levels of tissue neutrophils were reduced 10-fold relative to the untreated animals. Remarkably, this reduction in tissue neutrophil content was observed in normal intestinal mucosa from animals treated with A-64077. These data suggest that arachidonate 5-lipoxygenase metabolites are involved in the I/R-induced mucosal neutrophil infiltration as well as the resident neutrophil levels.
present in normal intestinal mucosa. Perhaps, the local generation of 5-lipoxygenase metabolites by normal intestine is involved in mucosal immune function by maintaining the observed polymorphonuclear leukocytes (PMNL) population in noninflammatory intestinal mucosa. In fact, LTB₄ is one of the most powerful chemoattractant molecules for neutrophils (Needleman et al., 1986), and these data suggest that 5-lipoxygenase metabolites also maintain the normal resident mucosal neutrophil population in noninflammatory states. The observation of neutrophil depletion by 5-lipoxygenase synthesis inhibitors in the normal intestine may have significant ramifications in diseases associated with mucosal immunity, such as inflammatory bowel disease.

Hypothermic intestinal I/R injury in this model results in characteristic mucosal lesions that are typically observed in normothermic intestinal I/R. Specifically, loss of villus height, denudation of villus epithelial cells, goblet cell deformation and focial laminal necrosis with PMNL and lymphocyte inflammation occur (Mangino et al., 1989 and Parks and Granger, 1986). These findings are depicted in figure 7. Although the A-64077-treated and untreated animals showed significant histologic injury relative to normal nonischemic intestines, the A-64077-treated animals demonstrated significantly less morphologic injury. This reduction in morphologic damage in the 5-lipoxygenase-treated dogs probably results from the observed increase in tissue O₂ delivery (fig. 3), enhanced O₂ uptake (fig. 4) and abolished I/R-induced tissue neutrophil infiltration. The reduced cellular inflammation is probably the result of inhibition of LTB₄ and 5-HETE, which are powerful chemotactic mediators and cause degranulation of neutrophils (Stenson et al., 1980a and Stenson et al., 1980b). The enhanced synthesis of the cytoprotective and anti-inflammatory prostaglandin PGE₂ may also contribute to the observed histologic improvements. Similarly, local PGE₂ in untreated animals has been shown to potentiate the microvascular effects of leukotrienes (Needleman et al., 1986), and this cooperative inflammatory effect would be abrogated with leukotriene synthesis inhibition in the treated animals.

In conclusion, this study demonstrates that the putative 5-lipoxygenase synthesis inhibitor A-64077 selectively inhibits intestinal mucosal leukotriene synthesis, dramatically improves postreperfusion mucosal oxygenation and VO₂, abolishes I/R-induced cellular infiltration (PMNL), and significantly ameliorates histologic signs of I/R injury. The use of this agent in clinical intestinal hypothermic preservation before transplantation should be strongly considered.

References


