Role of Changes in Tissue Nucleotides on the Development of Apoptosis during Ischemia/Reperfusion in Rat Small Bowel

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The aim of this study was to evaluate whether xanthine and adenosine, substances modified proportionally to the duration of ischemia, can determine cell demise (apoptosis/necrosis) during intestinal ischemia/reperfusion (I/R) and to determine the role of nitric oxide (NO) during this process. The following experimental groups were studied: I, cold ischemia; I+X, effect of xanthine; I+T, effect of adenosine (blocking its receptor by theophylline); I+A, effect of excess adenosine; I+T+X, effect of xanthine alone, and I+T+ spermine NONOate (NONOs), I+A+NONOs, I+X+NONOs, role of NO. DNA fragmentation, xanthine/adenosine levels, caspase-3 activity, NO generation, and histological analysis were measured in tissue samples. The rats treated with xanthine or adenosine showed increased levels of caspase-3 activity and DNA fragmentation. In contrast, theophylline-treated rats showed decreased levels of DNA fragmentation and tended to show lower mean values of caspase-3 activity. Administration of xanthine or NONOs to theophylline-treated rats reversed these effects. The results of histological evaluation were in agreement with these previous results. In conclusion, the present study indicates that xanthine and adenosine induced an apoptotic response during cold ischemic preservation of rat small intestine. In particular, the action of adenosine on apoptotic events was mediated by NO. We consider that identification of the role of these factors may help to define the best conditions of tissue preservation before intestinal transplantation. (*Am J Pathol* 2002, 161:1839–1847)

Intestinal transplantation has become an accepted therapy for intestinal disease in patients who depend on total

parenteral nutrition.¹ Because of the recent success of isolated intestinal grafts, and the mortality and morbidity associated with the development of advanced liver disease because of total parenteral nutrition, patients at risk for this complication should be identified and should receive isolated small bowel grafts before the onset of end-stage hepatic failure. The results of small bowel transplantation have dramatically improved throughout the last few years but rejection, infection, preservation, and reperfusion injury resulting in primary small intestine nonfunction continues to be a major obstacle.^{2,3}

Tissue injury related to ischemic preservation can compromise the viability of the organ during transplantation by destroying the mucosal cell layer and increasing permeability.^{4–7} Limited progress has been achieved in improving current techniques for preserving small bowel grafts by modifying cold storage solutions. Thus, Euro-Collins or University of Wisconsin solution (UW) has been used for small bowel preservation with less success than that reported for the preservation of other organ grafts.⁸

The intestinal mucosa is probably one of the most sensitive tissues to ischemia and ischemia/reperfusion (I/R).^{9,10} Two distinct modes of cell death—apoptosis and necrosis—are involved in the destruction of rat small intestinal epithelial cells during I/R but the former was found to be the major mode.¹¹ In contrast to necrosis, apoptosis is an active process of gene-directed cellular self-destruction characterized by morphological changes such as cell shrinkage with remaining intact organelles, condensation of chromatin into crescent caps at the nuclear periphery, and eventual fragmentation of the nucleus and cytoplasm to form apoptotic bodies.¹² Apoptosis in the rat small intestine is exacerbated during reperfusion after warm ischemia,¹² and apoptosis after preservation (cold ischemia) and reperfusion was also shown in transplantation of rat intestinal grafts.¹³ Nevertheless, the factors that modulate apoptosis in the intestinal tissue during I/R are unknown up to date.

Tissue nucleotides such as xanthine and adenosine are modulators of I/R injury that show changing levels according to the time of ischemia. Adenosine may en-

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hance nitric oxide (NO) release, and xanthine, which acts as a substrate for xanthine oxidase (XO), enhances the generation of superoxide during I/R.¹⁴ Reactive-oxygen species (ROS) and NO have also been implicated as signaling molecules for apoptosis.¹⁵ Previous studies show that exogenous nucleotides alter the cellular turnover of human small intestinal epithelium: the addition of exogenous adenosine monophosphate (AMP) results in increased apoptosis in villus cells.¹⁶ However, no data are available on the role of adenosine and xanthine in apoptotic damage during intestinal I/R. The present study was undertaken to elucidate this question as well as to assess whether this apoptotic induction could be mediated by NO. Determination of the role of these factors may help to define the best conditions for tissue preservation before intestinal transplantation.

Materials and Methods

Animals

This study was performed in male Wistar rats (Ifa Credo, Barcelona, Spain) weighing 250 to 300 g. All animals were fasted for 12 hours before surgery, anesthetized with urethane 10% (10 ml/kg, i.p.) and placed in a supine position. Body temperature was maintained between 36°C and 37°C. All procedures were conducted under the supervision of our institution's Research Commission and followed the European Union guidelines for the handling and care of laboratory animals.

Surgical Technique

The jejunum was isolated according to the technique described by Dubois and colleagues¹⁷ and modified by Minor and colleagues.¹⁸ Briefly, the abdomen was opened by a midline incision with bilateral subcostal extensions. The colon was isolated and removed after ligation of the right and middle colic vessels. The portal vascular pedicle was prepared by transecting all pancreatic, gastric, and splenic vessels. The superior mesenteric artery was exposed by isolating it from the neighboring structures. The intestinal lumen was flushed with 10 ml of UW preservation solution at 4°C. Then, the superior mesenteric artery was cannulated with a PE tube (PE 50) and immediately perfused with 5 ml of UW. The portal vein was cannulated with another PE tube (PE 150), and the jejunum was freed from the retroperitoneal tissue. The apertures of the intestinal lumen were cannulated with a PE tube (PE 250). Before ischemic storage, one part of the segment was excised after the vessels were tied to keep the pedicle vascular intact in the other part. Both parts were placed in the same recipient with 25 ml of UW at 4°C for 3 hours.

Perfusion System

After ischemic storage, the tissue freed from the pedicle was divided into different parts to obtain the different samples. The cannulated part of the jejunum was rinsed

with 5 ml of saline solution at 37°C via the superior mesenteric artery and the specimen was placed into a recipient with 100 ml of saline solution kept at 37°C. The intestinal lumen was perfused at a rate of 0.5 ml/min with warm saline solution by a syringe infusion pump (Harvard Apparatus sp, Southnatick, MA). The mesenteric artery was perfused with a modified Krebs-Henseleit buffer containing 200 mg/dl of glucose (Sigma Chemical Co., St. Louis, MO) at a constant rate of 3 ml/min by a peristaltic pump (Gilson Medical Electronics, Villiers-le-Bel, France). The perfusate was kept at 37°C and oxygenated with 95% O₂ and 5% CO₂. After 1 hour of reperfusion, the tissue was processed.

Experimental Groups

To select a suitable period of ischemia in which we could detect alterations in apoptotic parameters, we performed pilot studies in which tissular nucleotide levels, lactate dehydrogenase release, and DNA fragmentation were measured. From these previous studies, a time of 3 hours of ischemia was chosen.

The effect of xanthine during intestinal ischemia and reperfusion was evaluated by administering this substance before and during ischemia. Theophylline, a competitive antagonist of adenosine receptors,¹⁹ was added to the preservation solution during the same period to evaluate the effect of suppressing adenosine function. The effect of excess adenosine was assessed by addition of adenosine into the preservation solution. Moreover, the NO donor, spermine NONOate (NONOs), was also added to the preservation solution in the other experimental groups to evaluate whether the effect of adenosine could be mediated by NO or, alternatively, whether it was able to potentiate the effect of adenosine or xanthine. Animals were randomly assigned to the following five study groups ($n = 5$ each).

Group 1 (I)

Group 1 had 3 hours of ischemia and 1 hour of reperfusion.

Group 2 (I+X)

Group 2 had 3 hours of ischemia and 1 hour of reperfusion plus xanthine. Xanthine was dissolved previously in saline solution (pH 7.4) and then added to UW solution (total volume used per animal, 40 ml) at a concentration of 0.62 mmol/L. Thus, the UW flushed in the vascular and intestinal lumen and the UW used during ischemia were rich in xanthine.

Group 3 (I+T)

Group 3 had 3 hours of ischemia and 1 hour of reperfusion plus theophylline. The administration of theophylline (2.8 mmol/L) followed the same pattern as that of

xanthine, except that it was dissolved directly to the 40 ml of UW solution.

Group 4 (I+ T+ X)

Group 4 had 3 hours of ischemia and 1 hour of reperfusion plus theophylline and xanthine. Both substances, theophylline and xanthine, were administered at the same time by dissolving them into the 40-ml UW solution at the concentrations described above.

Group 5 (I+ X+ NONOs)

Group 5 had 3 hours of ischemia and 1 hour of reperfusion plus xanthine and NONOs. Xanthine was administered as described above. NONOs (16 mg/kg) was resuspended in 300 μ l of phosphate-buffered saline (PBS) (pH = 7.4) 30 minutes before administration. The substance was dissolved in 1 ml of UW solution just before the beginning of ischemia and was administered into the mesenteric artery at the end of flushing.

Group 6 (I+ A)

Group 6 had 3 hours of ischemia and 1 hour of reperfusion plus adenosine. Adenosine (0.5 mmol/L) was dissolved directly into the 40-ml UW solution.

Group 7 (I+ T+ NONOs)

Group 7 had 3 hours of ischemia and 1 hour of reperfusion plus theophylline and NONOs. Theophylline and NONOs were administered as described above.

Group 8 (I+ A+ NONOs)

Group 8 had 3 hours of ischemia and 1 hour of reperfusion plus adenosine and NONOs. Adenosine and NONOs were administered as described above.

Tissue samples were obtained at the end of the preservation period to evaluate nucleotides, NO production, and histological analysis. DNA fragmentation and caspase-3 activity were determined in mucosal samples.

Biochemical Analyses

Determination of Nucleotides and Nucleosides

Intestinal samples, weighing ~100 to 150 mg, were placed in 1.0 ml of 3.6% perchloric acid solution and then immediately homogenized (S25N-8G; IKA, Labortechnik, Stauffen, Germany). After homogenization, the tissues were extracted for 30 minutes at 0.5°C and were centrifuged at 850 $\times g$ for 15 minutes. Supernatants were neutralized with a solution of potassium carbonate 6 N/potassium hydroxide 6 N:1/1 to a pH of 6.0 to 6.5 and centrifuged at 13,000 $\times g$ for 2 minutes. Then, 50 μ l of the supernatant were directly injected into a Waters⁷¹⁷ plus autosampler liquid chromatograph (Waters, Milford, MA).

Nucleotide profiles were obtained using a reversed-phase Spherisorb ODS column (C18 5- μ m particle size, 15 $\times$ 0.4 cm; Teknokroma, Sant Cugat, Spain) coupled to a 600 high-performance liquid chromatography system equipped with a Waters 996 photodiode array detector. The absorbance was monitored at 254 nm. Nucleotide separation was allowed to proceed in an isocratic manner with 100 mmol/L of ammonium phosphate (pH 5.5) until adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), hypoxanthine/xanthine, and AMP were separated. At this point, a mixture of methanol:water (4:96) was introduced into the column, eluting inosine. A mixture of methanol:water (40:60) was introduced after the inosine to elute adenosine. Calibration chromatographs for standards ATP, ADP, AMP, adenosine, inosine, hypoxanthine, and xanthine were generated by injecting 50 μ l of a mixture of known concentrations. The profiles were processed by a Millennium³² system (Waters Millipore, Bedford, MA).

DNA Fragmentation

The extent of DNA fragmentation was determined by a modification of the method reported by Ikeda and colleagues.¹¹ Samples of intestinal mucosa were vigorously pipetted in 500 ml of PBS, and harvested by centrifugation at 3000 rpm for 5 minutes. Pellets were lysed with 0.4 ml of lysing buffer (10 mmol/L Tris, 10 mmol/L ethylenediaminetetraacetic acid, pH 8.0) containing 0.5% Triton X-100, and the lysates were centrifuged at 15,000 rpm for 20 minutes to separate intact DNA from fragmented chromatin. The supernatant, containing fragmented DNA, was placed in a separate microfuge tube and both fractions were precipitated for 30 minutes at 4°C in 0.4 ml of 1 N perchloric acid. Precipitates were sedimented at 15,000 rpm for 20 minutes. Perchloric acid (0.1 ml 1 N) was added to the pellets by vigorously pipetting and samples were boiled for 5 minutes to hydrolyze DNA. DNA was quantified by using diphenylamine reagent.²⁰ The percentage of DNA fragmentation was defined as the ratio of the DNA content of the supernatant to the total DNA in the lysate.

Caspase-3 Activity

Caspase-3-like activity was determined by measuring proteolytic cleavage of the specific substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC; Biomol, Plymouth Meeting, PA). Intestinal mucosa was homogenized and sonicated in assay buffer (50 mmol/L HEPES, 10% sucrose, 0.1% CHAPS, 5 mmol/L oxidized glutathione, 5 mmol/L dithiothreitol). We used 25 μ g of protein of each sample and 12 μ mol/L DEVD-AMC to perform the assay. The AMC released was quantified for 1 hour at 37°C by fluorospectrophotometry using 380-nm excitation and measurement of 450-nm emission.

NO Production

NO production in intestinal tissue was determined by tissue accumulation of nitrate and nitrite. Frozen samples

were homogenized in 1 ml of PBS (pH = 7.4) at 4°C. Protein concentration was determined from these homogenates. The homogenates were centrifuged at 40,000 × *g* for 60 minutes and 450 μl of the supernatants were filtrated through 10,000 molecular weight cutoff microcentrifuge filters (Ultrafree-MC, Millipore). Nitrite assay was performed following the Cayman Chemical Nitrate/Nitrite assay kit (Cayman Chemical Co., Ann Arbor, MI). To allow conversion of nitrate into nitrite, 80 μl of the sample filtrates were incubated with nitrate reductase and enzyme cofactors for 3 hours at room temperature. At the end of the incubation period, Griess reagents were added to the samples and allowed to stand at room temperature for 10 minutes. Finally, the samples were read at 540 nm with a plate reader and nitrite contents were calculated using a nitrate standard.

Histopathological Analyses

Intestinal tissue was embedded in 4% formalin-fixed paraffin and cut into 4-μm sections and stained with hematoxylin and eosin. Three blinded independent observers evaluated the histological sections by using Park's and colleagues classification.²¹ The degree of apoptosis was assessed according to the number of apoptotic bodies observed in the intestinal crypts after reperfusion and was scored as: 1 (less than three apoptotic bodies per crypt), 2 (three to five apoptotic bodies per crypt) and 3 (more than five apoptotic bodies per crypt).

Statistical Analyses

Data are expressed as means ± SEM. The means of different groups were compared using one-way analysis of variance. Student-Newman-Keuls test was used to evaluate significant differences between groups. The Mann-Whitney rank sum test was used in the histopathological analysis. Significant differences were assumed when *P* was <0.05.

Results

Figure 1 shows the tissular levels of xanthine after ischemia. As expected, xanthine was significantly accumulated in the intestinal tissue of the groups administered exogenous xanthine (groups I+X, I+T+X, and I+X+NONOs). Adenosine levels significantly increased after 3 hours of ischemia in groups administered exogenous adenosine or xanthine (Figure 2). Nevertheless, no significant differences were found in ATP+ADP+AMP levels between the different groups studied (Figure 3).

Figure 4A shows DNA fragmentation in the jejunal mucosa after I/R. The percentage of DNA fragmentation significantly increased when exogenous xanthine was administered (group I+X). In contrast, when theophylline was administered (group I+T) the percentage of DNA fragmentation decreased to significantly lower values than those found in group I. This effect was reverted

Figure 1. A and B: Intestinal xanthine levels after 3 hours of ischemia. I, ischemia; X, xanthine; T, theophylline; NONOs, spermine NONOate; A, adenosine. +, *P* < 0.05 versus group I.

Figure 2. A and B: Intestinal adenosine levels after 3 hours of ischemia. I, ischemia; X, xanthine; T, theophylline; NONOs, spermine NONOate; A, adenosine. +, *P* < 0.05 versus group I.

Figure 3. A and B: ATP, ADP, and AMP intestinal levels after 3 hours of ischemia. I, ischemia; X, xanthine; T, theophylline; NONOs, spermine NONOate; A, adenosine.

when xanthine was administered concomitantly with theophylline (group I+T+X). The administration of xanthine plus NONOs (group I+X+NONOs) also increased the percentage of DNA after reperfusion compared with that in group I.

Administration of adenosine alone (group I+A) or in combination with NONOs (group I+A+NONOs) significantly increased DNA fragmentation (Figure 4B). Moreover, the administration of NONOs plus theophylline (group I+T+NONOs) reversed the effect of theophylline on DNA fragmentation.

Caspase-3-like activity was used as an earlier indicator of apoptosis. Caspase-3-like activity showed a tendency to decrease when theophylline was administered (group I+T) (Figure 5A). When xanthine was administered alone (group I+X), with theophylline (group I+T+X), or with NONOs (group I+X+NONOs), caspase-3 activity was significantly higher than that found in group I. Administration of adenosine (I+A) also significantly increased caspase-3 activity at the end of ischemia (Figure 5B). The effect of theophylline was reversed when NONOs was administered concomitantly with theophylline (group I+T+NONOs): caspase-3 activity significantly increased again. Administration of adenosine concomitant with NONOs also increased caspase-3 activity.

Figure 6A shows intestinal generation of NO, evaluated as nitrite and nitrate production. The group administered exogenous xanthine showed a non-significant tendency to increase NO accumulation. As expected, when the adenosine receptor was blocked by theophylline admin-

Figure 4. A and B: DNA fragmentation (%) in the jejunal mucosa after 3 hours of ischemia and 1 hour of reperfusion. I, ischemia; X, xanthine; T, theophylline; NONOs, spermine NONOate; A, adenosine. +, $P < 0.05$ versus group I.

istration, there was little accumulation of NO: the levels found were significantly lower than those in group I. The addition of xanthine to theophylline (group I+T+X) had no effect: NO production remained lower. However, NO production was significantly increased when NONOs was administered concomitant with xanthine (group I+X+NONOs).

Figure 6B shows that adenosine administration significantly increased NO generation compared with that found in group I after ischemia. As expected, when a NO donor was co-administered with theophylline (group I+T+NONOs) or with adenosine (group I+A+NONOs), higher levels of NO production were detected.

Finally, Table 1 and Figure 7 show the degree of histological injury according to Park's classification after 3 hours of ischemia and the degree of apoptosis in the intestinal crypts after 1 hour of reperfusion. The degree of histological injury showed no differences when theophylline was administered (group I+T) compared with that found in group I. In contrast, tissue damage was slightly higher in the groups administered xanthine or adenosine (groups I+X, and I+A), or those administered NONOs. The greatest histological injury was found in the group concomitantly administered theophylline and xanthine (group I+T+X).

Regarding the degree of apoptosis in the intestinal crypts after reperfusion, most treatments increased apoptotic bodies compared with those in group I: only the group treated with theophylline (group I+T) showed a

Figure 5. A and B: Caspase-3-like activity in the jejunal mucosa after 3 hours of ischemia and 1 hour of reperfusion. I, ischemia; X, xanthine; T, theophylline; NONOs, spermine NONOate; A, adenosine. +, $P < 0.05$ versus group I.

decrease. The highest number of apoptotic bodies was found in the groups administered NONOs (group I+T+NONOs, I+X+NONOs, and I+A+NONOs) and in the group treated with adenosine (group I+A).

Discussion

This is the first study to show a relationship between nucleotide levels in small intestinal tissue and the development of apoptosis during I/R injury. Apoptosis is implemented by a regulatory cascade that has primarily been conserved from the nematode *Caenorhabditis elegans*, in which it was first studied, to human beings.²² The molecular basis of apoptosis is complex but its regulatory pathways converge on a common effector mechanism of cell death orchestrated by a family of cysteine endoproteases called caspases.²³ Recent synthesis of specific caspase inhibitors has enabled controlled molecular manipulation of apoptotic cell death. Therefore, study of the effect of mediators of I/R injury on apoptosis activation could offer attractive therapeutic perspectives aimed at improving the viability of organs during conservation procedures before transplantation.

Cells can die via two pathways, necrosis or apoptosis (programmed cell death). Necrosis is a pathological form of cell death caused by physical, chemical, or osmotic damage with consecutive disruption of internal and external membranes, leading to cell swelling and lysis with release of cytoplasmic material. This will often trigger an

Figure 6. A and B: Nitrate and nitrite intestinal production after 3 hours of ischemia. I, ischemia; X, xanthine; T, theophylline; NONOs, spermine NONOate; A, adenosine. +, $P < 0.05$ versus group I.

inflammatory response. In contrast, during apoptosis, the nucleus and cytoplasm are condensed and fragmented, yielding so-called apoptotic bodies, which are rapidly phagocytosed by macrophages or neighboring cells. This orderly packaging and removal prevents presentation of cytoplasmic cell content by antigen-presenting cells, thus avoiding subsequent inflammatory and/or autoimmune reactions. Consequently, apoptosis can be used for homeostatic purposes.^{24–26} Previous studies of intestinal ischemia show that induction of apoptosis occurs during the ischemic phase but continues into the early reperfusion phase.^{11,12} Moreover, DNA fragmentation reaches a peak 1 hour after reperfusion and returns

Table 1. Histological Lesions Observed in the Intestinal Mucosa after 3 Hours of Ischemia and Apoptosis in the Intestinal Crypts after 1 hour of Reperfusion

Groups	Tissue damage (Park's classification)	Apoptosis (reperfusion)
I	1	2
I+X	1–2	2–3
I+T	1	1–2
I+T+X	2–3	2–3
I+X+NONOs	1–2	3
I+A	1–2	3
I+T+NONOs	1–2	3
I+A+NONOs	1–2	3

I, ischemia; X, xanthine; T, theophylline; NONOs, spermine NONOate; A, adenosine.

Figure 7. Histological sections of the rat small bowel corresponding to different groups in this study. **A:** Normal architecture and length of the villi (Park's classification 0-1), representative of groups I and I+T. **B:** Extended Gruenhagen space is observed along the half length of the villi (Park's classification 2), representative of groups I+X, I+X+NONOs, I+A, I+T+NONOs, and I+A+NONOs. **C:** Total lifting of the villi epithelium (Park's classification 3) representative of group I+T+X group. **D:** Intestinal crypt showing occasional apoptotic bodies (arrow) representative of the I+T group. **E:** Intestinal crypt showing numerous apoptotic bodies in the epithelium, lumen, and lamina propria (arrows), corresponding to group I+T+NONOs. H&E; original magnifications, ×100 (A-C); ×400 (D, E). (Registered User Page 25 07/19/02.)

to baseline values by 6 hours. These data suggest that the time of reperfusion used for sampling in the present study (1 hour) was suitable. Thus, induction of intestinal apoptosis and mucosal recovery are rapid processes and fewer additional tissular complications (ie, inflammation, immune response, and so forth) can be expected with apoptosis than with necrosis.¹²

During ischemia, cellular consumption of ATP leads to accumulation of adenosine and to increases in the level of extracellular adenosine; this accumulation of adenosine is believed to confer cytoprotection to the ischemic tissue.^{27,28} In another experimental model, adenosine and related nucleosides were able to induce apoptosis during the initial stages of neuronal growth and development and to prevent or delay apoptosis in more mature sympathetic neurons subjected to nerve growth factor

deprivation in culture. Both the induction and prevention of apoptosis were independent of receptor activation and totally dependent on the intracellular accumulation and subsequent phosphorylation of adenosine.²⁹ However, in our case the administration of an adenosine receptor antagonist—theophylline—before I/R decreased DNA fragmentation and apoptotic morphologically detectable damage in small intestinal tissue. A plausible direct effect of theophylline on apoptosis cannot be excluded. In this sense, activation of apoptosis by theophylline in chronic lymphocytic leukemia cells has previously been described.¹⁹ In addition, theophylline may inhibit phosphodiesterase¹⁹ leading to an increase in cyclic adenosine monophosphate (cAMP), which has been described as effective in minimizing the development of apoptosis in cold stored liver grafts when administered in the form of

dibutyl-*c*AMP,³⁰ or in improving postischemic recovery in cold preserved intestines.³¹ Although a nonspecific action of theophylline cannot be ruled out, the finding that adenosine administration increased DNA fragmentation as well as caspase-3 activity points to a role of adenosine in the development of apoptosis. Adenosine may promote cytoprotection through activation of different types of receptors in different tissues,³² but few data are available on the role of the different adenosine receptors in mediating cytoprotection in intestinal tissue. Thus, the specific role of adenosine or adenosine receptors on the activation of several members of the caspase family remains to be elucidated and constitutes an interesting field for the development of future studies.

NO is an important molecule with diverse roles. Currently, there is great interest in studying NO because of its role in the modulation of apoptosis. NO can promote or inhibit apoptosis depending on the cell type and co-existing metabolic or experimental conditions. Overall, NO has an apoptosis-inhibiting effect mediated through the inhibition of caspases by S-nitrosylation, by inhibiting Fas-induced apoptosis or by increasing anti-apoptotic proteins such as Bcl-2.¹⁵ Several studies suggest that NO may be an important protective molecule against intestinal I/R injury.^{33,34} Other studies suggest that NO may participate in the breakdown of intestinal mucosa after I/R insult.³⁵ We found that tissue NO generation increased during I/R when adenosine was added to the preservation solution, but decreased when adenosine receptors were blocked by theophylline, even in the presence of exogenous xanthine. These data suggest that adenosine is related to NO production during I/R and are in agreement with previous studies showing that adenosine induces NO generation during ischemia in other organs such as the liver.²⁸ The present data also suggest a direct role of NO in the activation of apoptosis during I/R in rat small intestine. Adenosine administration led to an increase in NO generation that was concomitant with an increase in caspase-3-like activity and DNA fragmentation. This finding, together with the effect of NO donor administration (NONOs) (ie, reversal of the effect of blocking adenosine receptors resulting in higher DNA fragmentation and caspase-3-like activity) indicates a role for NO as an inducer of apoptosis. This finding contrasts with the NO inhibition of apoptosis previously found in rat gastric mucosa cells,³⁶ but agrees with previous results in which NO seemed to be required for the activation of apoptosis^{34,37} and does not exclude the possibility that NO could protect the tissue by a different way.^{34,38} Indeed, NO did not induce damage in the small intestinal tissue according to Park's classification (Table 1). Interestingly, NO administration to adenosine- or xanthine-treated rats increased caspase-3-like activity without modifying DNA fragmentation compared with treated animals without NONOs administration; this finding suggests that NO could require longer reperfusion periods to exert its effect when the NO dose is excessive, although a threshold for NO or for the fragmentation technique cannot be totally excluded.

We found that the addition of exogenous xanthine increased caspase-3-like activity, DNA fragmentation and

morphologically recognizable apoptotic injury in small intestinal tissue. The protective effects of blocking adenosine function by theophylline were reversed by further addition of xanthine. Moreover, the administration of NONOs to the xanthine-treated group induced the same effect on apoptotic parameters as that produced by the administration of theophylline; this finding indicates a NO-independent effect of xanthine on apoptosis. A large body of evidence links the production of ROS to the pathophysiology of tissue damage associated with I/R. One of the major *in vivo* sources of ROS is XO.³⁹ Xanthine dehydrogenase (XD), the precursor of XO, catalyzes the conversion of hypoxanthine to xanthine and subsequently to uric acid, coupled with the reduction of NAD⁺ to NADPH.⁴⁰ XD to XO conversion occurs during ischemia. XO preferentially uses molecular oxygen to NAD⁺ as the electron receptor and thereby generates superoxide. Thus, XD to XO conversion seems to play a role in reperfusion injury.⁴¹ However, to date no study has determined whether the XO system and subsequent ROS production can influence the development of apoptosis during I/R injury. The present findings suggest a role of xanthine in the development of apoptosis during intestinal I/R because the addition of exogenous xanthine as XO substrate produced apoptotic tissue injury even when adenosine receptors were blocked.

In conclusion, changes in the tissular levels of adenosine and xanthine were related to the development of apoptosis during I/R in the rat small bowel. Adenosine administration induced apoptosis and blockage of adenosine receptors by theophylline-inhibited apoptosis. This effect was mediated by NO. Xanthine increased apoptosis and caspase-3 activity through a pathway different from that found for adenosine receptors. This pathway was related to ROS production by XO when xanthine was used as substrate. We suggest that all these factors should be taken into account to reduce apoptotic events when improving the available preservation solutions for small intestine transplantation.

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