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Xanthine Oxido-reductase Activity in Ischemic Human and Rat Intestine

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We measured time course and extent of xanthine dehydrogenase (XD) to xanthine oxidase (XO) conversion in ischemic human and rat intestine. To model normothermic no-flow ischemia, we incubated fresh biopsies for 0, 2, 4, 8 and 16 h. At $t = 0$ h, XO was less in humans than in rats ($P < 0.0004$), while XD was essentially the same ($P =$ NS). After 16 h incubation at 37°C, there was no appreciable XD-to-XO conversion and no change in neither XO nor XD activity in human intestine. In contrast, the rat intestine had XO/(XO + XD) ratio doubled in the first 2 h and then maintained that value until $t = 16$ h. In conclusion, no XO-to-XD conversion was appreciable after 16 h no-flow normothermic ischemia in human intestine; in contrast, XO activity in rats increased sharply after the onset of ischemia. An immunohistochemical labelling study shows that, whereas XO + XD expression in liver tissue is localised in both hepatocytes and endothelial cells, in the intestine that expression is mostly localised in epithelial cells. We conclude that XO may be considered as a major source of reactive oxygen species in rats but not in humans.

Keywords: Free radicals; Xanthine oxidase; No-flow ischemia; Reperfusion

INTRODUCTION

Abdominal aortic aneurysm surgery involves aortic clamping and no-flow normothermic ischemia of the inferior hemisoma, mainly the large bowel. Readmission of oxygenated blood at the end of the intervention induces structural and functional alterations that not only affect the intestine, but also propagate distally with potential evolution in multiple organ failure, an important cause of death.[1] A consistent part of the injury is attributable to the reactive oxygen species (ROS) released during the reperfusion of ischemic intestine, similarly to what is observed in cells exposed to hypoxia-reoxygenation.[2] Doubts remain, however, on the major source(s) of ROS formed during ischemia-reperfusion.

The reaction of xanthine oxidase (XO, EC 1.1.3.22), which catalyses the conversion of hypoxanthine into xanthine and of xanthine into urate with production of H_2O_2, has been supposed as a major ROS contributor during reperfusion of ischemic tissues since 1981.[3] As a matter of fact, both the substrate (hypoxanthine or xanthine) and the enzyme (XO) are expected to occur in large amounts in ischemic tissues. The phosphorylation potential fall enhances ADP catabolism with increased production of hypoxanthine and xanthine. At the same time, intracellular proteases, stimulated by Ca^{++} overload and acidosis, typical features in ischemic tissues, convert xanthine dehydrogenase (XD, EC 1.1.1.204) into XO.[4] Both XO and XD use either hypoxanthine or xanthine as substrates, but whereas in the XD reaction NAD^+ is the final electron acceptor with formation of NADH, in the XO reaction O_2 is transformed into H_2O_2,[4] which rapidly transmutes into ROS, thereby leading to tissue dysfunction.[5] Whereas the intracellular accumulation of hypoxanthine and xanthine during ischemia is unquestionable,[6] the role of XD conversion to XO is controversial. In the rat intestine, the XD-to-XO conversion was reported to range from <1 min[4] to several hours at 37°C,[7] with prolonged times observed in rat liver, kidney, heart and lung.[8]

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In humans, most studies were performed using polyclonal or monoclonal antibody techniques, indicating relatively high expression of XD and XO in liver and intestine (reviewed in Ref. [9]). However, it is difficult to differentiate the XD and XO activities with those techniques.[10] Furthermore, part of the XO activity and expression found in human failing hearts were attributed to the presence of inflammatory cells rather than to intrinsic processes in the myocytes[11] and no XO activity change was observed in diseased human livers.[12]

The aim of this study was to test the hypothesis that XO represents a major source of free radicals during intestine ischemia and reperfusion. To this aim, we compared the time course and extent of the XD-to-XO conversion in ischemic human and rat intestine. To simulate ischemia with reproducibility in the two species, we incubated biopsies at 37°C in a humidified environment. In addition, to help comparison with previous studies, we performed parallel determinations in rat liver biopsies. These measurements are accompanied by an immunohistochemical study aimed at assessing the cell localisation of XO + XD expression.

MATERIALS AND METHODS

Chemicals

All chemicals, except otherwise stated, were provided at the highest available purity degree from Sigma-Aldrich (St.Louis, MO, USA). Catalase was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). The buffer was 50 mM KH_2PO_4, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol and 2000 IU catalase, pH 7.8 at 37°C.

Biopsies

Eight-week old (270 g body weight) male Sprague-Dawley rats were obtained from Harlan (Udine, Italy, $n = 6$/group). Fasting (24 h) rats were anaesthetised by i.p. injection of heparinised sodium thiopental (100 mg kg^{-1}), the abdomen was opened and intestine (colon) and liver were quickly excised. Human intestine biopsies were obtained during surgery for intestinal tumour removal. Whereas part of the fresh samples was incubated at 37°C (baseline), an aliquot was rapidly immersed in liquid nitrogen and maintained at −80°C until analysis.

To simulate normothermic ischemia, 250–300 mg aliquots of the biopsies were incubated for 2, 4, 8 and 16 h at 37°C in humidified petri dishes covered by an aluminum fold. At the end of the incubation, the samples were frozen in liquid nitrogen and maintained at −80°C until analysis.

All frozen biopsies were extracted at ice temperature. A 250–300 mg aliquot was weighed, homogenised (Ultraturrax T8, IKA Labortechnik, Staufen, Germany) in 4 ml g^{-1} buffer and centrifuged (19,000*g* for 30 min). The supernatant was removed and eluted through a 5×1.44 cm^2 Sephadex G-25 column equilibrated with the buffer. The eluate was fractionated into 1 ml fractions, which were individually tested for XO and XD activity. The protein concentration was measured from the absorbance at $\lambda = 280$ nm. The protein concentration is expressed as mM tryptophan, assuming $\varepsilon = 5$ mM^{-1} cm^{-1}.

Determination of XO and XD Activities

According to,[12] for the assay of XO, we monitored the formation of urate, assuming the reaction xanthine + O_2 → urate + H_2O_2. For the assay of XD, we monitored NADH formation in the presence of 140 μM NAD^+, assuming the reaction xanthine + NAD^+ → NADH + urate (Fig. 1). The reactions, measured simultaneously in two cuvettes, were started by adding 0.1 ml tissue homogenate to 1 ml buffer with 100 μM xanthine at 37°C.

To test the accuracy of XO determination, we performed ten consecutive determinations in a sample with fixed known XO activity. To test recovery we added known amounts of XO to the reaction buffer. In both cases, we used a commercially available source of XO (Sigma-Aldrich) and assumed the nominal activity for comparison.

To calculate the concentration of urate and NADH in the reaction mixture, we measured the absorbance at $\lambda = 260$, 291 and 341 nm every 30 s for 10 min by spectrophotometry (Uvikon 901, Kontron Instruments, Milan, Italy). Absorbance data were solved in a 3×3 square matrix to yield the concentration of the parent species, whose spectra are shown in Fig. 2. The XO or XD activity was calculated from either the urate or NADH vs. time plots, respectively, assuming a pseudo-first order approximation. The activity is expressed as μM urate or NADH/min/mM tryptophan. To express XD-to-XO conversion, we used the ratio XO/(XO + XD) vs. time.

Immunohistochemistry

Frozen specimens embedded in OCT medium (Leica Instruments, Nussloch, Germany) were cut into 5 μm-thick sections in a cryomicrotome (Leica CM1510) and placed on silanized glass slides. After drying at room temperature for 5 min, sections were fixed for 45 min in 4% cold buffered formalin, rinsed three times (5 min each) in cold PBS (pH 7.4), post-fixed for 5 min with 2:1 (vol/vol) ethanol:acetic acid at −20°C, rinsed twice in PBS and boiled for

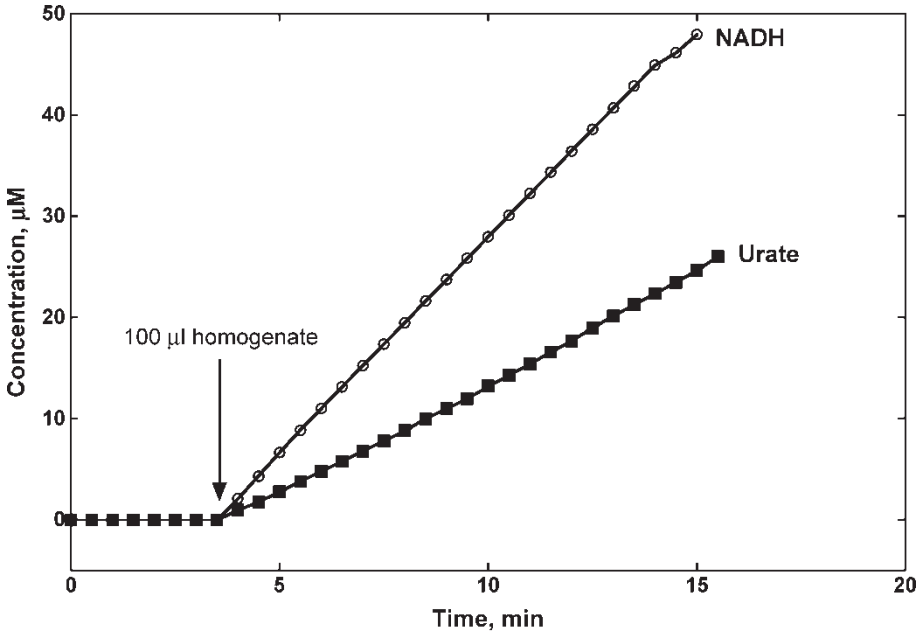

FIGURE 1 Typical determination of the activity of xanthine oxidase (squares) and xanthine dehydrogenase (circles) at 37°C, assuming the reaction schemes described in "Materials and Methods" section. The concentration of urate and NADH are calculated by solving for each point the matrix derived from the simultaneous determinations of absorbance at $\lambda = 260$, 291 and 341 nm. Initially, only xanthine is present, with no appreciable non-enzymatic oxidation rate. At $t = 4$ min, 0.1 ml homogenate is added, either in absence (squares) or in the presence (circles) of 140 µM NAD^+. The activity is calculated from the maximal slope of the curves assuming pseudo-first order kinetics.

10 min in 10 mM citrate buffer (pH 6.0). After cooling, the slides were washed three times in distilled water and one time in PBS. To block endogenous peroxidase, the sections were exposed for 5 min to 3% H_2O_2 and rinsed with distilled water and PBS. The slides were blocked with 10% normal goat serum (Chemicon International, Temecula, CA) for 1 h under gentle agitation.

For labelling, we used a primary antibody (Ab), rabbit polyclonal raised against xanthine oxidoreductase (Rockland, Gilbertsville, PA) diluted in 1:10000 ratio in 1.5% normal goat serum in PBS, which cannot distinguish between XO and XD. The sections were incubated under mild agitation in a humidified chamber for 1 h at room temperature and overnight at 4°C. After three 5 min washes

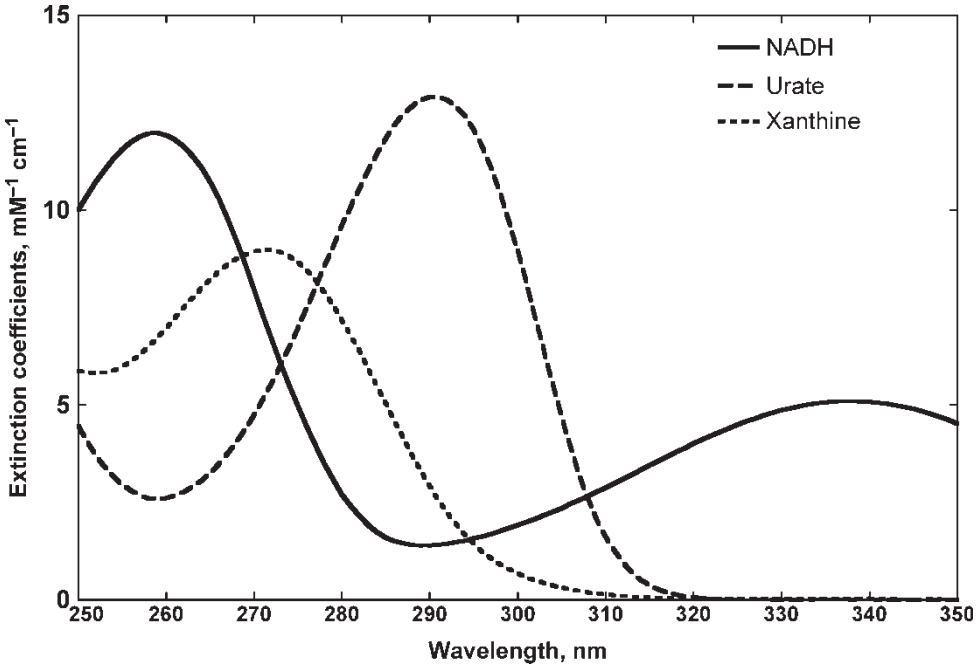

FIGURE 2 UV/Vis spectra of xanthine, urate and NADH.

in PBS, the slides were treated for 45 min at room temperature with the secondary Ab (peroxidase-conjugated goat anti-rabbit IgG, Sigma) diluted 1:800 in 1.5% normal goat serum and rinsed four times for 5 min each with PBS. To detect peroxidase activity, the sections were incubated for 3 min at room temperature with 1 mg ml^{-1} diaminobenzidine (Dako, Carpinteria, CA), counterstained with Gill's haematoxylin and mounted in 9:1 glycerol:PBS. Negative controls were prepared for every specimen by substituting the primary antibody with 1.5% normal goat serum. Images were acquired at 40 × magnification in an Axiolab E microscope (Zeiss, Germany) equipped with a colour Camera (CCD C4200, Hamamatsu Photonics KK, Hamamatsu City, Japan).

Statistics

Data are expressed as mean ± SEM. The significance of the differences was evaluated by the ANOVA test, followed by the Fisher's post-test if significant. The significance level was set to $P = 0.05$. The coefficient of variation was calculated as 100 SD/mean. To test accuracy, we performed the Lord test according to the formula: [(mean value of the determinations) − (nominal activity of the enzyme)]/(range of the measured values). This test, that allows determining the incidence of casual errors on the deviation from the mean, was calculated using 10 independent observations of XO on the same sample. A value <0.23 corresponds to $P < 0.05$ and excludes systemic deviations.

RESULTS

The pooled coefficient of variation for the determination of the activity of XD and XO was 4.9% ($n = 10$ each). The Lord test yielded 0.20 and the recovery of XO activity was 95.6 ± 4.2%.

Figure 3 shows baseline data obtained in fresh biopsies. The activity of XD was essentially similar in the three groups (ANOVA P = NS). In contrast, the activity of XO was markedly higher in rat intestine as compared to both rat liver ($P < 0.05$) and human intestine ($P < 0.01$). The ratio XO/(XO + XD) was higher in rat intestine than both rat liver ($P = 0.05$) and human intestine ($P < 0.01$).

To assess XD-to-XO conversion during no-flow ischemia, we determined the activity of both enzymes during biopsies incubation for up to 16 h at 37°C (Fig. 4). In rat intestine and liver, XO activity increased markedly at the beginning of simulated ischemia, but reached equilibrium after 2–4 h. By contrast, XD in rat intestine decreased immediately after starting simulated ischemia, but in rat liver this decrease was less pronounced. As a consequence, XO/(XO + XD) in rat liver doubled within 2 h of simulated ischemia and remained constant for up to 16 h. In human intestine, there was no appreciable XD-to-XO conversion and no change in the activity of either XD or XO for up to 16 h.

To address XO + XD expression localisation, Fig. 5 shows peroxidase immunoreactivity in fresh tissue and after 4 h incubation at 37°C. In fresh rat liver, staining was most evident in the cytoplasm of both hepatocytes and endothelial cells (arrows). Simulated ischemia did not change appreciably the pattern. In fresh rat intestine, staining was mainly localised in epithelial and goblet cells (arrow). Although tissue disruption in specimen incubated at 37°C for 4 h prevented univocal localisation, simulated ischemia appeared to increase XO + XD expression in the epithelial cells (arrow). Little staining was observed in both fresh and incubated human intestine, in agreement with the low activity of XO and XD.

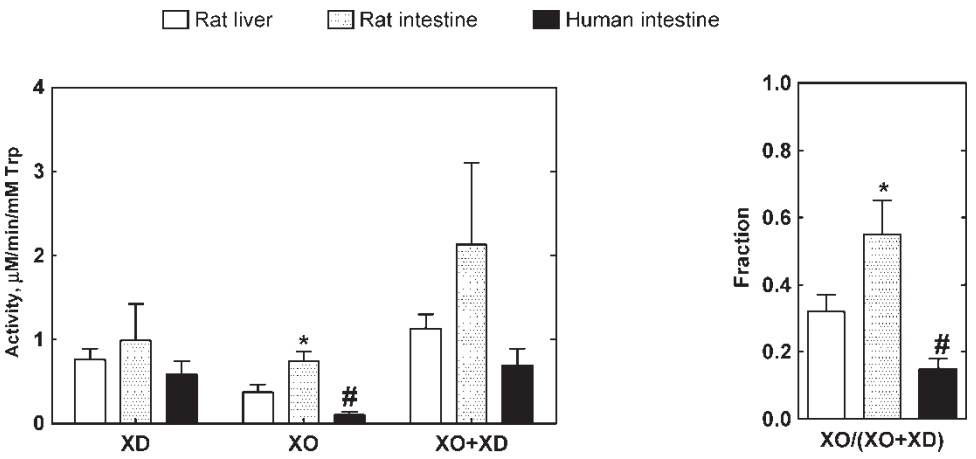

FIGURE 3 Activity data in fresh biopsies. ANOVA test yielded P = NS, $P = 0.0015$, P = NS and $P = 0.005$ for the activity of xanthine dehydrogenase (XD), xanthine oxidase (XO), XO + XD and the ratio XO/(XO + XD), respectively. *, $P < 0.05$ vs. rat liver; #, $P < 0.05$ vs. rat intestine.

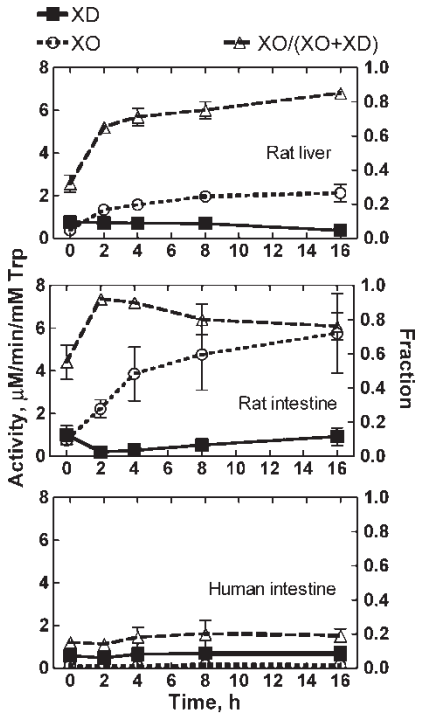

FIGURE 4 Activity of xanthine dehydrogenase (XD, solid lines, left axis), xanthine oxidase (XO, dashed lines, left axis) and ratio XO/(XO + XD) (long-dashed lines, right axis) after incubation of biopsies from rat liver, rat intestine and human intestine at 37°C for up to 16 h. The scale of the vertical axes is the same in all panels to help comparison.

DISCUSSION

In this study, we observed that XO activity is blunted in human non-ischemic intestine with respect to both rat intestine and liver, whereas XD activity is almost the same. When intestine biopsies were subjected to a condition analogue to normothermic no-flow ischemia, no changes were observed in human biopsies, whereas in the rat the activity of XO increased sharply in the first 2 h, followed by a plateau for up to 16 h. In rat liver, the pattern was essentially similar to rat intestine, but with 40–60% reduction in the activity of both XO and XD with respect to intestine.

We selected spectrophotometric enzyme activity measurements in the place of monoclonal or polyclonal antibody determination because our aim is to compare the time course and extent of the XD-to-XO conversion in ischemic human and rat intestine. According to,[13] we added catalase to the reaction mixture to enhance the conversion of H_2O_2, formed in the XO reaction, into H_2O and O_2, thereby preventing damage to enzymes. We choose to assess XD activity from NAD^+ reduction to NADH, rather than from differential measurement of urate formed in the absence and in the presence of NAD^+, due to potential interference by intermediates between the XO and XD forms.[11]

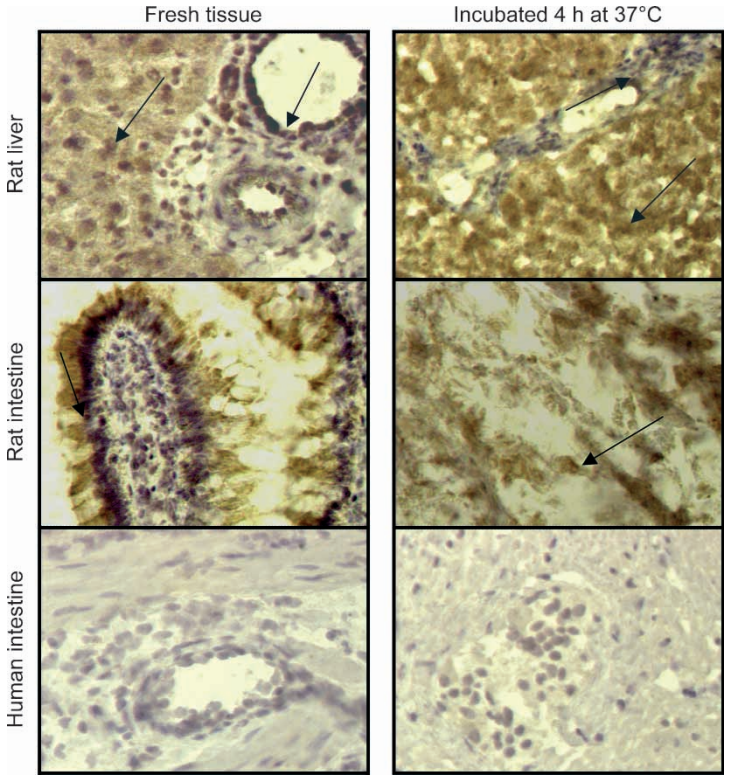

FIGURE 5 Immunohistochemical localisation of xanthine oxidase (XO) and xanthine dehydrogenase (XD) in rat liver, rat intestine and human intestine (from top to bottom), in fresh tissue and in tissue incubated 4 f at 37°C (left and right columns, respectively). XO + XD is marked with polyclonal antibody against xanthine oxido-reductase and peroxidase-conjugated anti-rabbit IgG, stained with diaminobenzidine, which yields a deep brown colour (arrows as explained in the text). Magnification 40 ×.

As reported,[9,10] the total (XO + XD) activity is markedly lower in human than rat intestine. The lower XO activity (about 1/8th as that measured in the rat) might be explained in part with the presence in humans of a protein synthesis repressor specific for XD.[9] However, from our data, it appears that human XD should be present in a more stable form than rat XD, but to our knowledge there are no data supporting this feature. The XO induction in the rat could be due to the activation of XD by post-translational modification, which involves the substitution of the sulphur atom with oxygen at the molybdenum centre.[9,14] This observation fits into a model where O_2, e.g. a non-ischemic condition, inactivates post-translationally XO and XD activities as it was observed in cell cultures.[15,16]

The immunohistochemical study was performed using both fresh tissue and tissue incubated for 4 h at 37°C, which was taken as representative of the situation occurred following the XD to XO conversion (in tissues able to do such conversion). This study could not differentiate XD from XO and we are unable to exclude cross reactions with other proteins such as albumin and vitamin-D binding protein.[17] Nevertheless, peroxidase immunoreactivity clearly shows that, whereas in liver tissue XO + XD is localised in both hepatocytes and endothelial cells, in intestine tissue XO + XD is mainly localised at the level of epithelial cells.

The role of XO as a major source of ROS during ischemia/reperfusion has recently been questioned.[18–23] Mitochondrial uncoupling may be a valid alternative for ROS production in hypoxic cells.[20,24,25] Indeed, ROS-induced ischemia-reperfusion injury is evident in rabbit hearts, which lack XO,[26] and in intestine of XO-deficient rats.[27] Furthermore, XO is not necessarily involved during ischemia-reperfusion injury in dog small intestine.[28] In this report, we show that the effect of normothermic no-flow ischemia on XO activity is negligible in human intestine, in contrast to what is observed in rat intestine and liver. The lack of appreciable response after 16 h no-flow ischemia at 37°C in human intestine complies with the hypothesis that ischemia-reperfusion in humans involves ROS originated from sources other than XO. Thus, XO does not appear to be a major ROS source during reperfusion in humans, but in rat intestine and liver XO activity increases sharply after the onset of normothermic ischemia, thereby representing a potential source of ROS in this species.

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