Abstract

AIM: To determine the effects of allopurinol, an inhibitor of xanthine oxidase, and apocynin, an inhibitor of NADPH oxidase, on oxidant stress and liver injury caused by hepatic ischemia/reperfusion (I/R) procedure in mice.

METHODS: Mice were pretreated with a xanthine oxidase inhibitor, allopurinol, or NADPH oxidase (NOX) inhibitor, apocynin before the hepatic I/R procedure. Then treated or untreated mice underwent the hepatic I/R procedure. The effects on hepatic injury and superoxide anions were determined after starting reperfusion.

RESULTS: A standard warm hepatic I/R procedure led to a marked increase in superoxide anion production as indicated by a superoxide anion tracer, MCLA. At the same time, the procedure caused profound acute liver injury, as indicated by elevated serum alanine aminotransferase and tumor necrosis factor-α levels, reduced liver glutathione levels and elevated malondialdehyde contents, as well as a high apoptotic cell count. All these changes were reversed by the use of apocynin or allopurinol prior to the hepatic I/R procedure.

CONCLUSION: Allopurinol and apocynin exerted protective effects on hepatic ischemia/reperfusion injury. The protection is associated with blocking the generation of superoxide anions during the hepatic I/R procedure by inhibiting xanthine oxidase and NADPH oxidase activity.

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Key words: Ischemia/reperfusion; Reactive oxygen species; Allopurinol; Apocynin; NADPH oxidase; Xanthine oxidase

Peer reviewer: Paul E Sijens, PhD, Associate Professor, Department of Radiology, UMCG, Groningen 9713GZ, The Netherlands

INTRODUCTION

Excessive reaction oxygen species (ROS) cause tissue damage and cell death by binding and altering cellular macromolecules, including DNA, proteins and lipids, and affect their function. One main chemical source which has been shown to contribute significantly to overall pronounced oxidant stress during hepatic ischemia/reperfusion (I/R) procedure is xanthine oxidase (XO), which generates superoxide anions (O$_2^-$) during the conversion of hypoxanthine to xanthine. It is known from many studies that much of the sustained injury during organ transplantation, including the liver, is triggered by ROS via activated XO, because allopurinol, an XO inhibitor, provided some protection against the hepatic I/R-induced injury$^{[1]}$.

NADPH oxidase (NOX), using NADPH as the source of electrons, catalyzes one electron reduction of molecular oxygen to generate O$_2^-$$^{[3]}$, which is a central and initial ROS molecule and may convert to more active and toxic ROS, such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^-$), or peroxide nitrite (ONOO$^-$) in the presence of H$^+$, H$_2$O$_2$, and nitric oxide (NO$^-$)$^{[9]}$. These O$_2^-$-derived ROS participating in the inflammatory process, are thought to be key mediators for the activation of Kupffer cells$^{[6]}$ and thus, are crucial in the apoptotic and/or necrotic cell death of the parenchymal cells and sinusoidal endothelial cells (SEC) in the liver$^{[5]}$. The generation of superoxide anions by NADPH oxidase serves as a host defense mechanism.
against invading microorganism infection and the enzyme is present in phagocytic cells, such as monocytes and neutrophils. Although the evidence exists that ROS generated by NOX participate in many cellular responses, and may be involved in many injury processes, few studies are available that investigate the role of NOX in the contribution to pronounced oxidant stress during the ischemia/reperfusion-induced hepatic injury. Apocynin is a naturally occurring methoxy-substituted catechol that effectively inhibits NADPH oxidase through preventing the assembly of its multi-subunits. Thus, we used this inhibitor to explore the role of NADPH oxidase in the generation of superoxide anions during the hepatic I/R procedure and investigate whether apocynin confers any protection against the injury in a mouse model of warm ischemia/reperfusion-induced acute liver injury.

MATERIALS AND METHODS

Animals and treatments

ICR mice, from Charles River Laboratory, Wilmington, MA, were fed a pellet diet and water ad libitum and maintained on a 12 h-light/dark cycle. The animal experiment was performed according to a protocol approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). The protocol was prepared in accordance with the National Institutes of Health animal use guidelines. Mice were pretreated with either an XO inhibitor, allopurinol (50 mg/kg, i.p. from Sigma Chemical Co. St. Louis, MO) or a NOX inhibitor, apocynin (3 mg/kg, i.p. from Acros Organics, Geel, Belgium) one day and one hour before the hepatic I/R procedure. A warm hepatic I/R procedure was performed as reported previously by us. In brief, mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). Laparotomy was made with a middle incision to expose the lobes of the liver. Following surgical exposure of the portal vein, mice were injected with heparin (100 unit/kg) via tail vein to prevent the formation of blood clot during the ischemia duration. The portal vein and hepatic artery were occluded for 30 min with a microaneurysm clamp to induce hepatic ischemia. Then, the clamp was removed to allow blood to flow through the liver again (reperfusion).

A total of six groups (6 mice in each group) were included in the experiment. Group A: sham-operated group, in which mice received normal saline (N.S.), and underwent a sham operation without I/R procedure; Group B (N.S. control group) in which mice underwent hepatic I/R procedure plus N.S. injection; Group C (allopurinol treatment group), in which animals received prior allopurinol injections and subsequent hepatic I/R procedure; Group D (apocynin treatment group), in which animals received prior apocynin injections and subsequent hepatic I/R procedure. Six and twenty four hours after starting the reperfusion, blood samples were collected from the vena cava before sacrifice. The liver was rapidly excised after drawing blood from the vena cava. Portions of liver tissue were fixed in 10% neutralized formalin for histological evaluation or snap frozen in liquid nitrogen, and maintained at -80°C until homogenization for the various biochemical assays.

Serum alanine aminotransferase assay

Serum levels of alanine aminotransferase (ALT) served as an indicator of liver injury and were analyzed using a commercially available diagnostic kit (Catachem Inc., Bridgeport, Connecticut), and expressed as unit/L.

Measurement of ROS generation in liver homogenates

2-Methyl-6-((P-methoxyphenyl)-3,7-dihydroimidazo(1,2-α)pyrazine-3-one (MCLA) enhanced chemiluminescence was used to determine O2· generation, as reported previously. Approximately, 10 mg of frozen liver tissue was homogenized on ice in 1 mL of homogenization buffer containing 20 mmol/L of N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and 10 mmol/L ethylene diamine tetraacetic acid (EDTA). The homogenate was subjected to low speed centrifugation (1000 g) for 10 min to remove debris. Luminometer vials containing 2 mL of prewarmed Krebs-HEPES buffer (99 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1 mmol/L KH2PO4, 1.9 mmol/L CaCl2, 25 mmol/L NaHCO3, 11.1 mmol/L glucose, and 20 mmol/L HEPES, pH 7.4) with 1.0 μmol/L of MCLA were placed in the dark for at least 20 min. After the dark adaptation, background readings were recorded in a luminometer (Lumat LB; Berthold Technologies, BmbH &Co. KG, Germany), and then 20 μL of homogenate supernatant was added to each vial containing MCLA. Chemiluminescent emission in relative light units (RLU) was recorded during a plateau phase of each recording period, and corrected by subtracting the background reading. The calculated value was used to express the integrated values of chemiluminescence (RLU/second/μg protein).

Assays of liver glutathione and malondialdehyde content

Levels of the reduced form of glutathione (GSH) and the lipid peroxidation product, malondialdehyde (MDA) in liver tissue were measured spectrophotometrically 6 h and 24 h after starting reperfusion by commercially available kits (OXIS Research, Portland, OR). The levels of GSH and MDA are expressed as nanomoles per milligram of protein.

Histological examination

Fixed liver specimens were embedded in paraffin, sectioned in 4 μm thickness, and stained with hematoxylin and eosin (HE) for the evaluation of liver injury. Photomicrographs were taken with a digital camera under a microscope. In addition, frozen sections of the livers were stained with the terminal deoxyribonucleotidyl transferase (TdT) for detecting of apoptotic cells by an in situ Apoptosis Detection Kit according to the manufacturer’s instructions (Chemicon Inc., Temecula, CA), and reported previously by us.

Determination of serum tumor necrosis factor-α levels

Serum tumor necrosis factor-α (TNF-α) levels after the hepatic I/R procedure were determined with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) and expressed as nanograms per milliliter.
Statistical analysis
Most data were expressed as mean ± SD of the mean (SEM), and evaluated with an ANOVA analysis and Newman-Keuls tests for multiple comparisons among groups. A P value less than 0.05 was considered statistically significant.

RESULTS
Amelioration of I/R-induced acute liver injury
As shown in Figure 1E, the hepatic I/R procedure caused a marked increase in serum ALT levels 6 h to 24 h after starting the reperfusion. Prior intraperitoneal injection of either allopurinol or apocynin led to a marked decrease in serum ALT levels compared to animals with I/R plus N.S. injection at both 6-h and 24-h points (P < 0.01). It appeared that either allopurinol or apocynin tended to exert an improved protection to the same extent, and that the differences were not statistically significant at both time points between these two groups. These findings were consistent with the histological findings as shown in Figure 1A-D. The hepatic I/R procedure led to marked necrosis in the central lobule (Zone III) with significant inflammatory infiltration (Figure 1B). A significant reduction in necrosis was found in the livers of animals receiving injections of allopurinol or apocynin (Figure 1C and D) compared to the control group (Figure 1A). For controls, additional animals were injected with allopurinol or apocynin without the hepatic I/R procedure. Serum ALT levels in animals receiving apocynin (65 ± 17 unit/mL, n = 3) or allopurinol (57 ± 14 unit/mL, n = 3) were similar to levels in N.S. controls (46 ± 27 unit/mL, n = 3). These data indicate that neither apocynin nor allopurinol is toxic to the liver.

Enhanced apoptosis in hepatic I/R-induced liver injury
Apoptotic cells in liver sections were detected by in situ staining using TUNEL assay. As shown in Figure 2, 6 h after starting reperfusion, profound apoptotic cells with positive TUNEL red fluorescent staining in nuclei were...
visualized in animals with the hepatic I/R procedure (Figure 2B) compared to sham-operated controls (Figure 2A). Prior treatment of allopurinol or apocynin prevented apoptotic cell death caused by the hepatic I/R procedure (Figure 2C and D).

### Enhanced superoxide anion release during the hepatic I/R procedure

We employed MCLA, which is proportional to levels of \( \text{O}_2^- \) and singlet oxygen, to investigate whether the hepatic I/R procedure enhanced the chemiluminescent emission from the liver homogenates. We found that light emission from the liver homogenates was elevated 3-fold 6 h after starting the perfusion, compared to animals with sham operation. The enhanced chemiluminescent emission, caused by the I/R procedure, was markedly inhibited by prior administration of allopurinol or apocynin (Figure 3).

This finding provides convincing evidence that NADPH oxidase or xanthine oxidase is the equally important source of superoxide anions, which contributes to pronounced oxidant stress during the hepatic I/R procedure.

### Restored GSH and reduced MDA levels by allopurinol or apocynin treatment

Liver GSH and MDA levels were determined as indicators of oxidant stress and lipid peroxidation during the hepatic I/R procedure. As shown in Figure 4, both allopurinol or apocynin led to a restoration of decreased GSH levels and a reversion of elevated MDA levels in the liver \((P < 0.05-0.01)\). The GSH levels in the treated groups were close to controls 6 h after starting the reperfusion.

### Serum TNF-\( \alpha \) level

Serum TNF-\( \alpha \) was determined using an ELISA kit 6 and 24 h after starting the reperfusion. It is clear that the hepatic I/R procedure caused an elevation of serum TNF-\( \alpha \) levels, and that the prior administration of either allopurinol or apocynin reduced the TNF-\( \alpha \) levels when compared to the I/R+N.S. controls \((P < 0.05-0.01, \text{Figure 5})\).

### DISCUSSION

In the present study, we demonstrated that prior administration of either allopurinol or apocynin prevented the hepatic I/R-induced acute liver injury in mice; the protection was due to a blockage of xanthine oxidase (XO) and NADPH oxidase (NOX) by allopurinol and apocynin, respectively. The findings indicate that both XO and NOX play a critical role in the generation of superoxide anion and superoxide anion-derived ROS during the hepatic I/R procedure. Thus, our findings further confirm the involvement of NOX activation in the hepatic I/R-induced acute injury.

Our previous study showed that pronounced oxidant stress is the major cause of the hepatic I/R injury, and the delivery of either extracellular superoxide dismutase or...
Hepatic I/R-induced depletion of reduced form of glutathione and enhanced lipid peroxidation, and effects of apocynin and allopurinol. A: Effect of apocynin or allopurinol on liver GSH levels with the hepatic I/R procedure. Liver GSH content was determined spectrophotometrically and expressed as nanomoles per milligram of protein of the tissue; B: Effects of apocynin or allopurinol on liver MDA levels with I/R procedure. Liver MDA content was determined spectrophotometrically and expressed as nanomoles per milligram of tissue protein (mean ± SEM). \( ^{\alpha}p < 0.01 \) vs Sham controls; \( ^{\beta}p < 0.05, ^{\gamma}p < 0.01 \) vs the hepatic I/R procedure plus N.S. at 6 h time point (n = 6 in each group).

In conclusion, the findings in the present study demonstrate that the pretreatment of both allopurinol and apocynin prevented ischemia/reperfusion-induced acute liver injury and that the protection is associated with the blockade of either xanthine oxidase or NADPH oxidase...
activity. The activation of both enzymes contributes to the generation of superoxide anions and related reactive oxygen species during the hepatic ischemia/reperfusion-induced liver injury. This study implies a new pharmacological intervention approach by blocking either xanthine oxidase or NADPH oxidase, or in combination to improve the donor organ quality and survival after the transplantation in recipients.

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COMMENTS
Background
Ischemia/reperfusion-associated donor liver damage is inevitable during the harvest, preservation, transportation and implantation to a recipient. Enhanced oxidative stress has been linked to the donor organ damage; however, the source of reactive oxygen species (ROS) for enhanced oxidative stress has not been well defined. Superoxide anion generated from the reaction of hypoxanthine to xanthine catalyzed by xanthine oxidase (OX) has been thought to be one source of ROS, but an intrinsic source of ROS, via the activation of NADPH oxidase (NOX), has not been well studied in a hepatic ischemia/reperfusion model system.

Innovations and breakthroughs
We found that there was a marked increase in superoxide anion release during the ischemia/reperfusion procedure, and that the use of apocynin, a specific inhibitor of NADPH oxidase, effectively minimized the superoxide anion release, oxidative stress and liver damage caused by a hepatic ischemia/reperfusion procedure in mice.

Applications
The study implies a new pharmacological intervention approach by blocking either xanthine oxidase or NADPH oxidase, or in combination to improve the donor organ quality, function and survival after the transplantation in recipients.

Peer review
This study of the beneficial effects of allopurinol and apocynin on the healing of ischemia/reperfusion-induced liver injury is original. It is a well-designed and well-written paper. Allopurinol and apocynin exerted protective effects on hepatic ischemia/reperfusion injury. The protection is associated with blocking the generation of superoxide anions during the hepatic I/R procedure by inhibiting xanthine oxidase and NADPH oxidase activity.

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