Significance of xanthine oxidase in the production of intracellular oxygen radicals in an in-vitro hypoxia-reoxygenation model

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Abstract

Background/Purpose. The peroxidation of membranous phospholipids induced by ischemia reperfusion was inhibited in Cu/Zn superoxide dismutase (SOD) overexpressing mice, suggesting a detrimental role for intracellular reactive oxygen species (ROS) in reoxygenated cell injury. To ascertain the in-vitro relevance of this hypothesis, the present study examined the participation of intracellular ROS in reoxygenation injury.

Methods. This examination was done in two experimental models: Cu/Zn-SOD transgenic (Tg) mice that underwent hypoxia-reoxygenation in vitro and normal mice pretreated with a specific inhibitor of xanthine oxidase, BOF-4272, followed by in vitro hypoxia-reoxygenation.

Results. The release of aspartate aminotransferase (AST) and the peroxidation of phospholipids were both ameliorated in hepatocytes from the Tg mice compared with findings in hepatocytes from normal mice. Similar findings were seen in the BOF-4272-pretreated cells, in which there was a decrease in AST and phospholipid peroxides.

Conclusions. These results support the pivotal role of intracellular ROS generated by xanthine oxidase in reoxygenated cell injury, and suggest the viability of using an intracellular antioxidative therapy for reperfusion injury of the liver.

Key words Hepatic ischemia-reperfusion injury · Superoxide · Lipoperoxidation · Superoxide dismutase · Xanthine oxidase

Introduction

Ischemia-reperfusion (I/R) injury of the liver is considered to be an important issue because it significantly affects the postoperative results in hepatic resection and hepatic transplantation. Reactive oxygen species (ROS) generated by reoxygenation have been implicated in the occurrence of this injury. Many reports have demonstrated ROS production in various reperfusion injuries, but it remains unclear whether ROS is responsible for the initiation of I/R injury of the liver. In our previous study, we attempted to clarify the participation of ROS production in I/R injury of the liver by demonstrating an increase in phosphatidylethanolamine hydroperoxide (PCOOH) in the liver tissue phospholipid membrane following I/R injury. We further showed that the increase of tissue PCOOH was inhibited in the liver of transgenic mice overexpressing Cu/Zn superoxide dismutase (SOD), with a 1.8-fold increase in SOD activity in the cytoplasmic fraction compared with that in normal mice. In addition, we also demonstrated a specific and sensitive correlation between PCOOH levels in hepatic tissue and the extent of I/R injury. The change in the plasma PCOOH concentration following hepatic I/R occurred in a time-dependent manner and was found to be a useful and sensitive parameter reflecting actual membrane injury. Thus, although ROS production is an undeniable effect of I/R injury of the liver, the cells and the enzymatic systems whereby the ROS are being produced are still unknown.

Hepatocytes have several intrinsic ROS production systems. It is presumed that ROS are enzymatically produced in the cytoplasm by the xanthine oxidase (XO) system including cytochrome C distortion of the mitochondrial electron transmission system, and production by p450 in the microsome fraction. Extracellular sources of oxygen radical production from other cells, such as migrating neutrophils, can also produce ROS. In the latter phase of I/R injury, neutrophils and vascular endothelial cells bind to each other via cellular adhesion molecules induced by the reoxygenation of both cell types, causing neutrophils to generate superoxides by NADPH oxidase. Kupffer cells, the resident macrophages of the liver, are also regarded as a source of extracellular ROS in the early phase of reperfusion. Furthermore, Kupffer cells produce cytokines that play an important role in the pathophysiology of the inflam-
mation seen in I/R injury. We paid close attention to the XO system for ROS production for a short time period immediately after reperfusion. However, whether or not the intracellular XO actually serves as a true source of ROS and whether or not the generated ROS can cause cell damage are questions which still need to be answered. Thus, by subjecting hepatocytes to hypoxia-reoxygenation, we may be able to clarify the detailed mechanism whereby phospholipid peroxidation and ROS formation occur.

The purpose of this study was to ascertain the relevance of the ROS scavenging system and its effects on hepatic cytoplasmic ROS production as a possible preventive or treatment method against hepatic I/R injury. We used hepatocytes cultured from Cu/Zn-SOD transgenic mice and performed in-vitro hypoxia-reoxygenation to activate the intracellular ROS scavenging system. This model allowed us to look at the direct effects of hepatocellular oxygen radical production, and excluded the cytotoxic effects of Kupffer cell-derived cytokines and inflammatory mediators generated in the cellular surroundings.

Allopurinol is an XO inhibitor used to block the superoxide production generated by the XO system. The conventional theory, that allopurinol can attenuate superoxide production generated by the XO system, has been undermined by the finding that allopurinol itself can eliminate superoxide and hydroxyl radicals. Therefore, we also examined the effect of a specific inhibitor of XO, BOF-4272, which has no ROS scavenging function, on hypoxia-reoxygenation in a normal hepatocyte culture.

Materials and methods

Experimental animals

Human (h) sod 1 gene-expressing transgenic mice, prepared by Epstein et al. were subcultured at the Department of Gastrointestinal Surgery, Tohoku University Postgraduate School, and mated with ICR mice. Male F1 mice, 8 weeks of age (weighing from 35 to 40 g), were examined by a polymerase chain reaction (PCR) method to verify the presence of the transgene and divided into two groups, a transgenic group (Tg group), expressing the h-sod1 gene; and a control group (C group), without the gene, and subjected to the following experiments. There were no differences in bodyweight and lifespan between the two groups of mice and no obvious occurrence of disease was observed.

Isolation and primary culture of hepatocytes

Hepatocytes were isolated from both transgene (Tg) and control (C) mice according to the method proposed by Seglen. Cells with more than 90% viability were plated at 5 × 10⁶ cells/ml and incubated in a 5% CO₂-containing humidified atmosphere at 37°C. The medium was prepared using Williams Medium E (GIBCO, Carlsbad, USA) containing 10% fetal calf serum (FCS), 10⁻⁸M insulin, 10⁻⁸M dexamethasone, 100,000 units/l penicillin, and 100 mg/l streptomycin. After 3 h, dead cells were removed, and the medium was changed to a serum-free medium. Cells were incubated for 21 h and used for various experiments.

Preparation of the in-vitro hypoxia-reoxygenation model and sample collection

The 24-h-cultured hepatocytes in each group were anaerobically incubated for 3h, using an anaerobic box devised by Kamiya et al. of Kansai Medical University, followed by aerobic culture. For both the Tg group and the C group, hepatocytes and the cultured supernatant were collected immediately before anaerobic culture (prehypoxia), immediately after anaerobic culture (hypoxia), and at 24h after reoxygenation.

Experiment 1: significance of biomembrane phospholipid peroxidation and the intracellular oxygen radical production system in the in-vitro hypoxia-reoxygenation model

Using the above hypoxia-reoxygenation model, I/R injury was reproduced in vitro. Hepatocytes were collected before anaerobic culture (prehypoxia), immediately after anaerobic culture (hypoxia), and at 2, 4, 6, and 12 h after reoxygenation for both groups of animals. The cultured supernatant was collected before anaerobic culture (prehypoxia), immediately after anaerobic culture (hypoxia), and at 2, 4, 6, and 12 h after reoxygenation. The hepatocellular ATP level was determined as an index of hypoxia. The aspartate aminotransferase (AST) level in the cultured supernatant and the hepatocellular PCOOH level were determined as indices of cell damage after reoxygenation.

Experiment 2: examination of the intracellular oxygen radical production system in the in-vitro hypoxia-reoxygenation model

The cytoplasmic XO blocking experiment was conducted by using a specific inhibitor of XO/xanthine dehydrogenase, BOF-4272 (sodium-8-(methoxy-4-phenylsulfonylphenyl) pyrazol [1,5-a]-1,3,5-triazine-4-olate monohydrate (BOF; Otsuka Pharmaceutical, Tokyo, Japan). Isolated hepatocytes were incubated for 24 h, BOF was added to the medium at a concentration of 2µg/ml, and incubation was performed for an addi-