Inhibition of Heme Oxygenase Ameliorates Sepsis-Induced Liver Dysfunction in Rats

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Abstract
Purpose. The disintegration of heme produces carbon monoxide (CO), a known vasodilator, which is catalyzed by heme oxygenase (HO). This study aimed to clarify the effect of HO inhibition on septic rat livers using two types of HO inhibitors; Sn-protoporphyrin (Sn-PP) and Zn-protoporphyrin (Zn-PP).

Methods. Sepsis was induced in male Sprague-Dawley rats by cecal ligation and puncture (CLP). Either NaOH or HO inhibitors were injected intraperitoneally; first 18 h prior to CLP, then immediately after CLP. The animals were killed 12 and 24 h after CLP and the liver tissue and plasma were harvested.

Results. Using Northern blotting, we found that mRNA of the stress-inducible isozyme, HO-1, was dramatically induced 12 h after CLP. Administering the HO inhibitors, Sn-PP and Zn-PP (5 µmol/kg), induced a significant inhibition of the elevation of aspartate aminotransferase plasma levels, the elevation of cyclic guanosine monophosphate (cGMP) in the liver tissue, and the increase in the sinusoidal space ratio, 24 h after CLP. Both Sn-PP and Zn-PP decreased the mortality rate 24 h after CLP compared with normal saline.

Conclusions. CO produced by excessively induced HO-1 after CLP promotes an immoderate dilation of the sinusoidal space through the up-regulation of cGMP, resulting in liver dysfunction. Therefore, administering HO inhibitors at appropriate doses could be beneficial for the amelioration of sepsis-induced liver dysfunction.

Key words Sepsis · Heme oxygenase-1 · Sinusoid · Carbon monoxide

Introduction
Sepsis still often causes multiple organ failure, despite advances in intensive care medical support. An important function of the liver lies in its role in energy metabolism, and maintaining good hepatic function is considered one way to improve the survival rate of patients with sepsis. The morphopathological lesions of the liver in the late stages of canine endotoxin shock show sinusoidal dilatations with an accumulation of red cells and leukocytes, and damage of the sinusoidal lining.1 It is not certain whether these changes are a result of the liver injury, or of an active reaction of the sinusoidal lining cells.

We assume that carbon monoxide (CO), which has recently attracted great interest as a gaseous mediator of vasodilatation, plays an important role in this sinusoidal dilatation.2,3 CO activates soluble guanylate cyclase4 to produce cyclic guanosine monophosphate (cGMP), which relaxes the vascular smooth muscle and causes vasodilatation.5 CO has also been reported to function as an endogenous modulator of hepatic sinusoidal perfusion through a relaxing mechanism involving Ito cells.6 Most CO is produced from heme which is then converted to biliverdin and iron (Fe). The enzyme that catalyzes this break-down of heme is heme oxygenase (HO).7,8 Two isozymes of HO exist, namely, HO-1 and HO-2, both of which have been found to be the products of two distinct genes. The HO-2 isoform is constitutively expressed, while the HO-1 isotype is a protein induced by stress following hypoxia, ultraviolet radiation, endotoxin, and heat shock. Cyclic DNA clones for rat heme oxygenase have been isolated9 and identified as the major 32-kD-a heat shock protein, HSP32.10,11

It was reported that HO-1 induced in response to these stresses contributes to mediating protection through the antioxidative effects of products such as biliverdin and bilirubin,12 and through the vasodilative effect of CO.13 However, the role of HO in the liver in...
sepsis has not yet been well clarified. We conducted the following experiment in an attempt to clarify whether dilation of the sinusoidal space in sepsis caused by CO induction can maintain hepatic function, or in contrast, if it causes further deterioration of liver function. We also investigated the role of HO and CO, which are considered to control these histological or functional phenomena.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 250–300 g were used for all experiments. Rats were acclimated for 10 days in the animal care facility and allowed free access to water and food.

Cecal Ligation and Puncture Model (CLP)

The rat model of sepsis resembled human sepsis caused by postoperative peritonitis in that it was induced by cecal ligation and puncture. This procedure was designed by Wichterman et al. In summary, under diethyl ether anesthesia, a 1-cm midline incision was made in the abdomen, and the cecum was exposed. After ligation distal to the ileocecal valve with 3-0 silk, the cecum was punctured twice using an 18-gauge needle.

Administration of HO Inhibitors

The HO inhibitors, Sn-protoporphyrin (Sn-PP) and Zn-protoporphyrin (Zn-PP), were dissolved in 0.05 M NaOH and administered intraperitoneally to rats twice; first, 18 h before CLP, then immediately after CLP. Depending on the treatment received, animals were divided into the Sn group and the Zn group, respectively. As a control group, we used rats injected intraperitoneally with 1.5 ml of 0.05 M NaOH according to a similar schedule. The animals were killed 18 h after drug administration (pre-CLP) or 12 and 24 h after CLP, and the liver and blood were harvested. The doses of Sn-PP were 2.5, 5, 10, and 50 µmol/kg, and those of Zn-PP were 2.5, 5, and 10 µmol/kg. For each dose given at each treatment time, 4–6 animals were used.

Aspartate Aminotransferase (AST) Activity and Alanine Aminotransferase (ALT) Activity

Whole blood samples were collected in heparinized syringes by aortic puncture. The samples were centrifuged and plasma was collected. AST and ALT activity was determined using a prepared kit (Spotchem II, Menarini Diagnostics, Barcelona, Spain).

Assay for cGMP Levels in Hepatic Tissue

Cyclic GMP measurement was performed on frozen tissue samples using an enzyme-linked immunosorbent assay kit (Cayman Chemicals, Ann Arbor, MI, USA). Hepatic tissue samples were snap-frozen in liquid nitrogen immediately after harvesting, as the half-life of cGMP is very short. The frozen samples were weighted, then homogenized in 10% trichloroacetic acid (TCA)-phosphate buffer (PB) solution. The supernatant was decanted after centrifugation at 1500 g for 10 min, and the remaining TCA was extracted with water-saturated diethyl ether. This extraction was repeated five times. All ether was removed by heating the sample tubes to 70°C for 5 min. The competitive reaction between the sample cGMP and a known amount of cGMP tracer was exposed by immunochemical techniques, and products were read at 415 nm on a microplate spectrophotometer.

Calculation of Volume Percentage of the Sinusoidal Space to the Liver (Point-Counting Method)

Morphometric analysis of the liver tissue was performed as described previously. Briefly, after the liver tissue was fixed with 10% formalin and embedded in paraffin, 3-µm-thick sections of each sample were stained with hematoxylin–eosin (H&E). A 10 × 10 millimeter was mounted to the eyepiece and the section was observed under a microscope at a magnification of ×400, and the number of dots overlying the blood space was counted. This counting was performed in five visual fields for every animal, and the sinusoidal space ratio was calculated as the fraction of points overlying the blood space per total number of points counted.

Microsomal Heme Oxygenase

The liver was excised and repeatedly washed with ice-cold saline solution. All further operations were carried out at 0–4°C. The liver was homogenized in a Potter-Elvehjem-type glass homogenizer in three volumes of ice-cold potassium phosphate buffer (50 mM, pH 7.4). The homogenate was centrifuged at 20000 × g for 15 min, and the supernatant was further centrifuged at 168000 × g for 40 min. The microsomal pellet obtained was resuspended in potassium phosphate buffer (50 mM, pH 7.4), and the supernatant obtained was resuspended in the same buffer as biliverdin reductase.

Assay of Microsomal Heme Oxygenase

Heme oxygenase activity was determined as described elsewhere, with some modifications. Briefly, 1 mg of fresh liver microsomes was added to a reaction mixture containing nicotinamide adenine dinucleotide phos-