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Role of Bilirubin as an Antioxidant in an Ischemia–Reperfusion of Rat Liver and Induction of Heme Oxygenase

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The anti-oxidative effect of bilirubin was investigated in an ischemia–reperfusion model of rat liver. The rat portal vessel and liver artery were ligated with a vascular clip, and after reperfusion the urine was collected at intervals. The amount of biopyrrins, the oxidative metabolites of bilirubin, in rat urine reached a maximum 4 hours after reperfusion. Biotripyrrin-a and -b which are biopyrrins isolated from human urine were included in urinary bilirubin oxidative metabolites of rats after reperfusion. The hepatic mRNA level of heme oxygenase-1 (HO-1), the rate limiting enzyme of bilirubin biosynthesis, reached a maximum after 4 hours. Furthermore, the hepatic activity of HO began to rise 4 hours after treatment and remained high until 24 hours posttreatment. These findings suggest that bilirubin acts as a physiological antioxidant *in vivo* in ischemia–reperfusion and that bilirubin biosynthesis is evoked by oxidative stress.

Bilirubin has been considered both harmful and useless *in vivo.* However, bilirubin was recently shown to scavenge reactive oxygen species produced by oxidative stress *in vivo,* and the oxidative metabolites of bilirubin, biopyrrins are excreted into urine (1,2). In clinical settings, the blood bilirubin level was slightly increased after laparotomy although liver dysfunction did not occur in some cases (3,4). The biopyrrin level in the urine of postoperative patients measured by the enzyme-linked immunosorbent assay (ELISA) using an anti-bilirubin monoclonal antibody (24G7)(5–7) was markedly increased (1). Bilirubin has been suggested to act as an antioxidant to scavenge reactive oxygen species, and be oxidized itself into various types of biopyrrin (1,2). Biopyrrin in the urine was detected not only in postoperative patients but also in healthy humans. Changes in its level with age have also been observed including greater diurnal variation in younger people and lower level in elderly people (8).

In this study, we prepared ischemia-reperfusion model of liver (9,10), in which reactive oxygen species are highly produced, and examined the antioxidant activity of bilirubin based on changes in the urinary biopyrrin level. In addition, we focused on the hepatic activity and gene expression of heme oxygenase (HO-1), a rate-limiting enzyme of bilirubin biosynthesis, which is known as a typical protein induced by oxidative stress.

MATERIALS AND METHODS

Preparation of Ischemia–Reperfusion Model of Liver

Six-week-old male Sprague-Dawley (SD) rats each weighing about 200 g (Sankyo, Tokyo, Japan) were used to prepare ischemia-reperfusion model in the liver. Laparotomy was performed under general anesthesia with ether. The hepatoduodenal ligament including the portal vessels and liver arteries was ligated by the Pringle method using a vascular clip (Bear's Inc., 120g)(10), and then compressed to block the blood flow, causing complete liver ischemia. After 15 minutes, the clip was removed for reperfusion. In addition, simple laparotomy models (sham operation) were prepared as a control.

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Enzyme-Linked Immunosorbent Assay (ELISA)

The procedure, described previously (6), was slightly modified to obtain more precise data. The anti-bilirubin monoclonal antibody (24G7) used characteristically has an epitope in the dipyrrole region of bilirubin (7). Therefore, the biopyrrin level, which cannot be measured by the diazo method, a common method of measuring bilirubin in clinical settings, can be quantified by ELISA using this antibody.

High-Performance Liquid Chromatography (HPLC)

(a) Partial purification using Sep-Pak C18 ENV. The urine collected 1–10h after treatment was mixed with an equal amount of acetonitrile and the solution was then centrifuged at 10,000 × g for 20 minutes. After the supernatant was lyophilized immediately, it was dissolved in 10 ml of purified water, and separated into 8 fractions using a Sep-Pak C18 ENV (Waters)(Fr. 1, 0%; Fr. 2, 10%; Fr. 3, 20%; Fr. 4, 30%; Fr. 5, 35%; Fr. 6, 40%; Fr. 7, 50%; Fr. 8, 100%: acetonitrile/5% acetic acid, v/v). After the separated fractions (Fr. 1–Fr. 8) were lyophilized and redissolved in 2 ml of purified water, the biopyrrin level in each fraction was determined by ELISA for both the treatment group and the control group.

(b) Analysis of biopyrrin by HPLC. Among the fractions separated using a Sep-Pak C18 ENV, Fr. 4 showed a large amount of biotripyrrin-a and biotripyrrin-b and was analyzed by HPLC. Analysis was performed using a Waters 600E-LC system with a spectrophotometric detector (Waters Model 440 Absorbance Detector). A μBondasphere (ϕ3.9 mm × 15 cm, 5μC18-100 Å, Waters) was used as a column. The temperature of the column was set at 40°C using an incubating water circulator. The column was balanced at a flow rate of 1 ml/min with 30% acetonitrile solution in 0.1% trifluoroacetic acid (TFA). Subsequently, 50 μl of Fr. 4 was injected, and acetonitrile in 0.1% TFA was eluted from 30% to 50% by a 25-minute linear gradient. Each peak of bilirubin-related compounds was traced at a wavelength of 436 nm.

Measurement of Reduced Glutahione (GSH)

After the serially resected liver was snap-frozen with liquid nitrogen, 0.5 g of liver was placed on a glass homogenizer, and 10 ml of 5% metaphosphoric acid solution was added. It was homogenized for 150 seconds. The homogenate was centrifuged at 6,000 × g at 4°C for 10 minutes. The supernatant was filtrated using a Morcat II (Nihon Millipore Industries, Co., Ltd.). The solution obtained by filtration was used as a specimen for HPLC. HPLC conditions were as follows; A TSKgel ODS-80-TM (Tosoh, Japan)(ϕ 4.6 mm × 15 cm) was used as a column. KH_2PO_4 (50 mM) and 1-heptanoic sodium sulfonate (13mM)/H_2O-contained 3% MeOH (pH = 3.0)(phosphoric acid) were used in the migration phase. The flow rate was 1.0 ml/min. The temperature was 30°C. Detection was at UV 210 nm at a range (AUFS) of 0.08. The injection amount was 10 μl.

Measurement of HO-1 mRNA Level in Liver

A cDNA (nucleotides 70-833) encoding a rat HO-1 (11) was prepared using polymerase chain reaction as described previously (2). Total RNA was extracted from the liver according to the method of Chomczynski and Sacchi (12). RNA (20 μg) was separated by electrophoresis on a 1% agarose gel containing 6.6%(w/v) formaldehyde, and immobilized on Hybond N+ membranes (Amersham) in 10 × SSC (1 × SSC is 150 mM NaCl and 150 mM sodium citrate). The membranes were baked at 80°C for 2h, and then hybridized with a probe (2×10^6 cpm/ml ^{32}P-labeled cDNAs) specific for HO-1 mRNA at 42°C overnight in a solution containing 50% (w/v) formamide, 5 × Denhardt's solution, 0.1% (w/v) sodium dodecyl sulfate, 5 × SSC, 50 mM sodium phosphate, and 0.05% (w/v) denatured salmon sperm DNA. The blots were washed twice with 0.1 × SSC containing 0.1% (w/v) sodium dodecyl sulfate at 55°C for 15 min. The washed blots were autoradiographed, after which the radioactivity on the band was measured with a Bioimage Analyzer System (BAS; Fuji Photographic Film Co. Ltd., Kanagawa, Japan). A cDNA of rat apolipoprotein-E (apo-E) (13) was kindly provided by Dr. J. M. Taylor, and used as a probe for hybridization.

Measurement of HO Activity

The HO activity in the liver measured in microsomal fractions of the liver by the method described by Kutty and Maines (14). The production of bilirubin was measured by an absorbance at 450 nm. The molecular extinction coefficient for the bilirubin used was 45 mM^{-1} cm^{-1}. The microsomal protein level was determined by the method described by Lowry et al. (15). The enzyme level producing 1 nmol of bilirubin per an hour under this condition was regarded as 1 unit.

RESULTS AND DISCUSSION

The concentration of biopyrrin in urine increased more than 20-fold 4 hours after reperfusion in the treatment group compared to that in the control group (simple laparotomy), and then decreased to the level observed before reperfusion (Fig. 1). Thereafter, the concentration was slightly increased again about 3-fold 12.5 hours after reperfusion. In the control group, the concentration of biopyrrin was increased 8 hours after treatment, and reached to about 5-fold 15 hours after

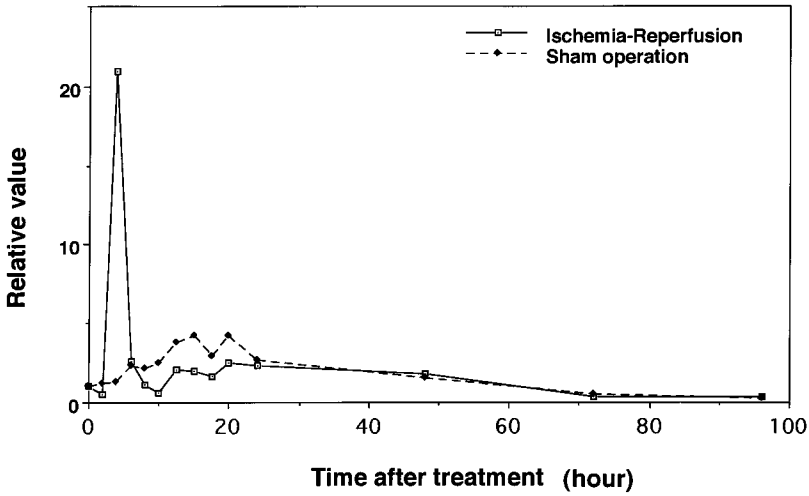

FIG. 1. Relative amounts of biopyrrins in rat urine treated by ischemia–reperfusion and sham operation. The value 1.0 denotes the baseline amount of biopyrrins in urine collected before ischemia reperfusion.

treatment. The levels of both groups were decreased to the concentrations observed before laparotomy about 4 days after treatment.

After the fractions (Fr. 1–Fr. 8) separated from urine using Sep-Pak C18 ENV were redissolved in 2 ml of purified water, the biopyrrin level in each fraction was determined by ELISA using mAb 24G7 in both the treatment group and the control group. A large amount of substances reactive to the antibody were detected in Frs. 4 and 5. Fr. 4, which showed a large amount of biotripyrrin-a and -b, was used as the HPLC specimen among fractions with a relatively large amount of reactive substances detected by ELISA. As shown in Fig. 2c, the biotripyrrin-a and -b levels were increased to 2.7-fold compared to those before laparotomy in the control group. On the other hand, the levels were increased to 7.2-fold in the treatment group with ischemia-reperfusion (Fig. 2d). Several unidentified bilirubin-related substances were also detected.

Fig. 3 shows the serial changes in the reduced glutathione level presented as the relative value. In the treatment group, the reduced glutathione level was decreased rapidly, and reached to the lowest level 10 hours after the reperfusion. Then, the level was recovered to the normal level at 48 hours after the reperfusion. In the control group, the reduced glutathione level was similarly decreased. However, it recovered to the normal level earlier than in the treatment group.

The HO-1 mRNA level in liver was increased 2 hours after the treatment, and reached a maximum value, 6-fold of the control group, 4 hours after the treatment. However, there was no significant increase in the control group after laparotomy (Fig. 4). There were no significant changes in the apo-E mRNA level after the treatment.

HO activity was increased 4 hours after the treatment, and then increased further to about 3-fold of the pre-treatment level by 6 hours after the treatment. High activity of HO remained until 24 hours after the reperfusion (Fig. 5). In the control group, the HO activity was increased 1.3-fold 6 hours after the laparotomy. However, it decreased to the pre-treatment levels by 24 hours after the treatment.

Ischemia-reperfusion promotes peroxidation of lipids in the membrane by producing reactive oxygen species, resulting in membrane disorders. In addition to changes in the membrane structure, damaged membrane proteins enhance the fragility or permeability of the membrane and inactivate membrane-binding proteins. Consumption of α-tocopherol or reduced glutathione, an antioxidant *in vivo* with preventive effects on membrane disorders, inhibits peroxidation of lipids (16,17). Our experiments also demonstrated reductions in reduced glutathione level immediately after the treat-

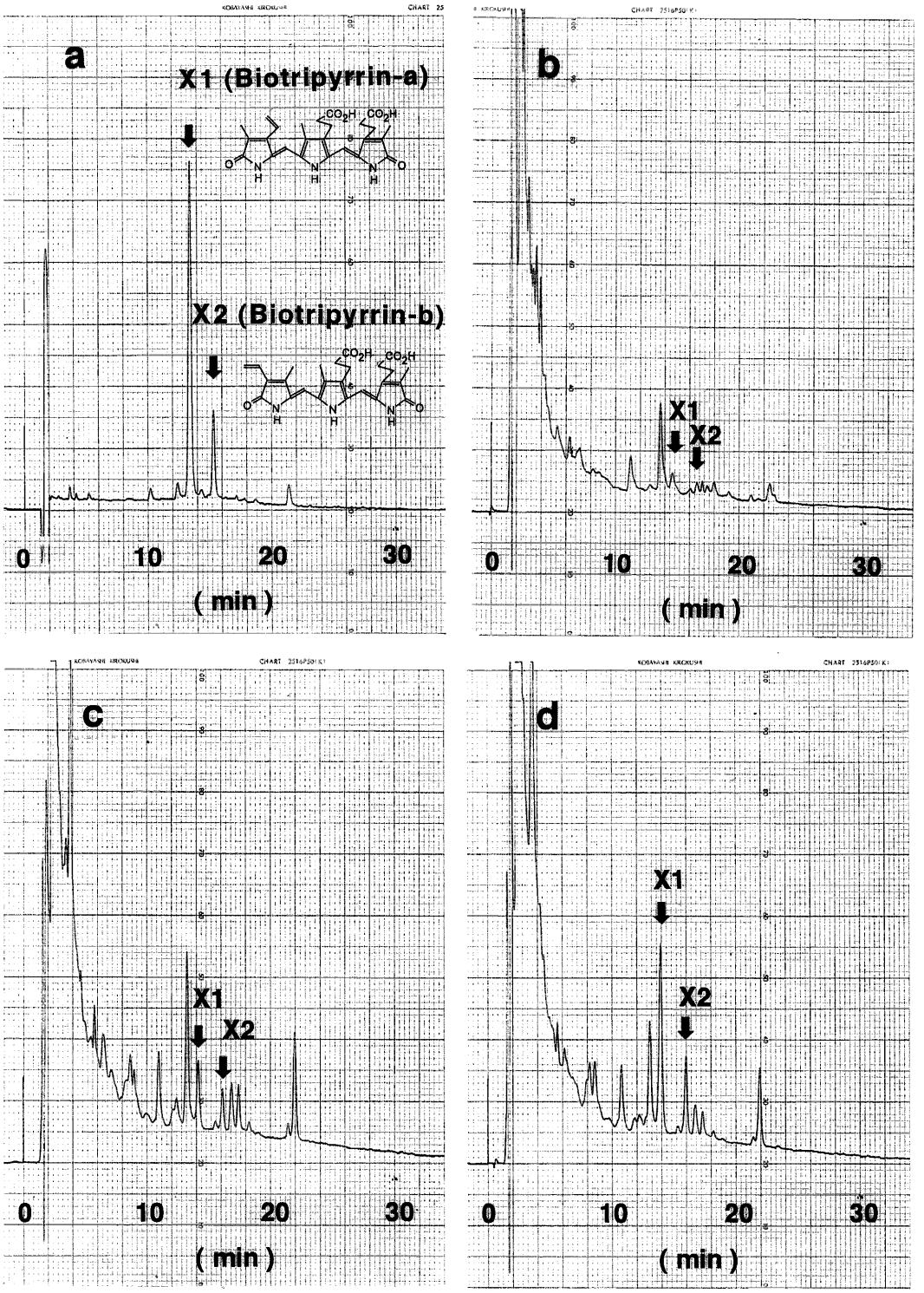

FIG. 2. HPLC elution profile of Fr. 4 samples separated by Sep-Pak C18 ENV of human urine (a) and urine (b, c, and d) from 1 to 10h after the treatment in rats. X1 & X2 denote biotripyrrin-a and -b, respectively. a: Healthy human b: Preoperation c: Sham operation d: Ischemia reperfusion

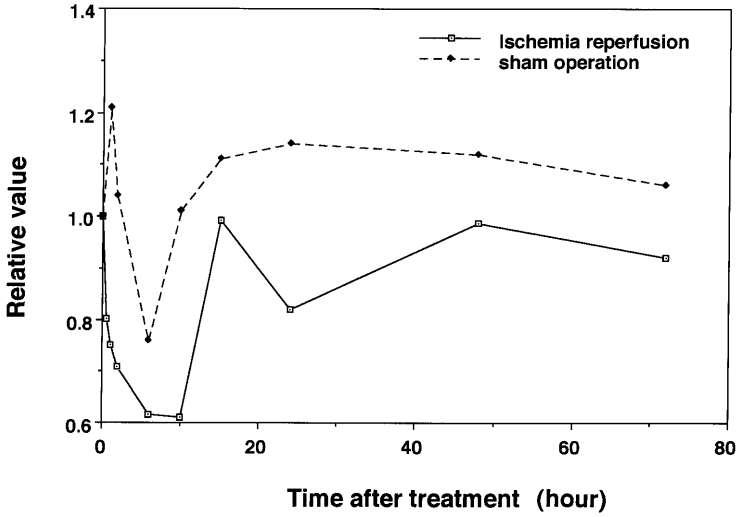

FIG. 3. Relative amounts of GSH in rat liver. The amount of GSH in liver before ischemia–reperfusion was presented as 1.0. Each value was the average of duplicate samples.

ment, suggesting production of reactive oxygen species (Fig. 3). According to the report by Stocker et al. (18,19), physiological concentration of bilirubin inhibited peroxidation of lipids in a homogeneous linoleic acid solution *in vitro.* The antioxidant effect of bilirubin was significantly lower than that of α-tocopherol under 20% oxygen. When the oxygen level was decreased from 20% to 2%, near the physiological intracellular oxygen level, the antioxidant effect of bilirubin exceeded that of α-tocopherol. Bilirubin also acts as a scavenger against reactive oxygen species via its own oxidation *in vivo,* and is excreted in the urine as various types of oxidative metabolites and biopyrrins. Some studies indicated that serum and urinary levels of bilirubin oxidative metabolites, biopyrrins, were increased immediately after surgery in patients undergoing laparotomy (8). Fur-

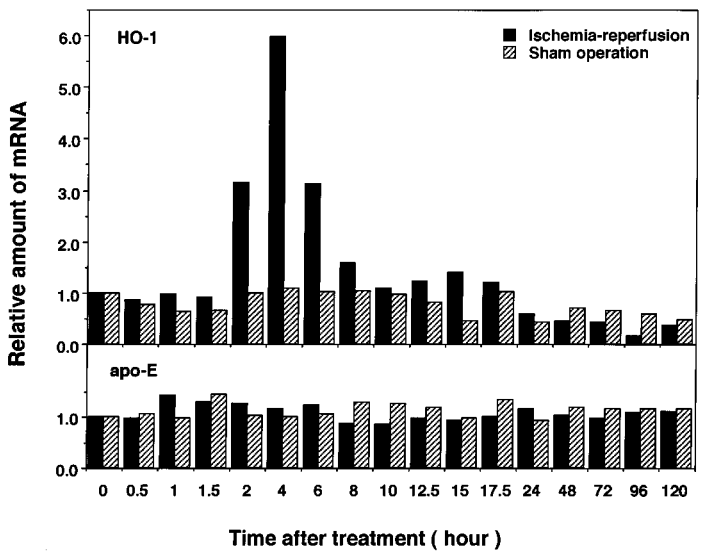

FIG. 4. Relative levels of HO-1 mRNA in rat liver. HO-1 mRNA level in liver before ischemia reperfusion was presented as 1.0.

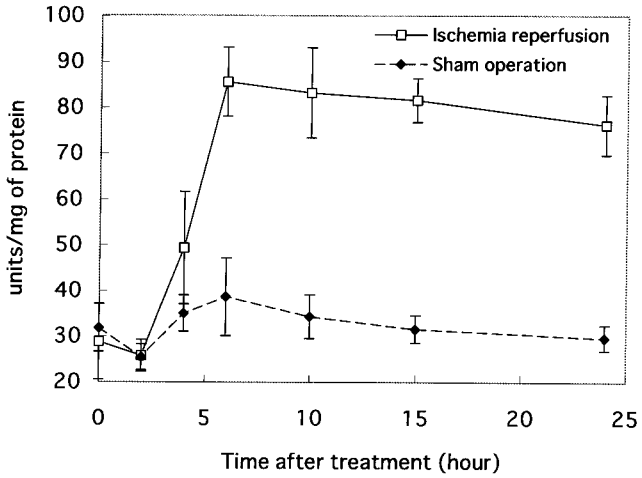

FIG. 5. Time course of heme oxygenase activity in liver microsomes after the treatment. The means and SE are reported. Data were analyzed by an ANOVA.

thermore, intraperitoneal administration of endotoxin (1 mg/kg body weight) into rats increased the urinary biopyrrin level about 3.5-fold, with a peak observed 10 hours after administration (2). The treatment with endotoxin is regarded as one of the oxidative stress, and may produce reactive oxygen species *in vivo.* (2,17). In our experiments, the urinary excretion of biopyrrin was also increased to more than 20-fold 4 hours after reperfusion compared to that in the control group (Fig. 1). Bilirubin may also be involved in scavenging reactive oxygen species. In our experimental models, we used the method to completely block the portal circulation to liver, which has been commonly used since Pringle used to prevent intraoperative hemorrhage in liver trauma in 1908. Complete liver ischemia at the hepatic portal region for 15 minutes caused congestion in the intestinal tract a few minutes after ischemia, resulting in bacterial translocation due to portal pooling. Therefore, we had to consider the possibility that endogenous endotoxemia develops in approximately 50%. However, the urinary biopyrrin level was increased more than 5.7 times compared to that in the endotoxin-treated models described above. This may be because the oxidative stress caused by ischemia-reperfusion was stronger than that induced by endotoxin *in vivo.* Furthermore, analysis by HPLC (1) showed that the peak in urinary biopyrrin level was consistent with those in novel classes of biopyrrins isolated from human urine and identified as biotripyrrin-a (X_1) and biotripyrrin-b (X_2) (Fig. 2)(1,2).

Heme oxygenase (HO), a rate-limiting enzyme of bilirubin biosynthesis, is composed of two isozymes, HO-1 (inducible) and HO-2 (constitutive). HO activity is high in the brain, spleen, liver and testis. HO-1 activity is enhanced by oxidative stress, heavy metals or hormones, but, it is thought that HO-2 activity is not enhanced by these factors. HO is an enzyme that decomposes intracellular heme into biliverdin via oxygenation. Biliverdin is then reduced by biliverdin reductase (20,21), resulting in the production of bilirubin which has been demonstrated to have antioxidant activity (2). Carbon monoxide (CO) is also produced during biliverdin production. The physiological effects of an intrinsic relaxing factor, CO, on blood flow disorders have been demonstrated (22). As shown in Fig. 4, the HO-1 mRNA level was increased 2 hours after the treatment, and reached a peak of about 6-fold at four hours after the treatment. HO has been demonstrated to be induced in liver even after a 15-minute ischemia. The HO-1 mRNA level began to rise at 2.5 hours after administration of endotoxin in rats, and reached a peak 5 hours after administration (2).

Following the increase in HO-1 mRNA, the HO activity was also increased 4 hours after the

treatment (Fig. 5), suggesting enhanced bilirubin production. In the control group, no changes in the HO-1 mRNA level were observed. These findings suggest that bilirubin constitutively existed and that newly produced bilirubin acts as a scavenger of reactive oxygen species against oxidative stress.

On immunohistochemical staining using an anti-HO-1 antibody, there were no hepatocytes showing staining before the treatment. However, stained hepatocytes were frequent along the line between the adjacent terminal portal venules 6 hours after reperfusion. Therefore, HO-1 antigen appeared to be expressed in hepatocytes after ischemia-reperfusion.

In conclusion, bilirubin acts as a scavenger via its own oxidation to scavenge reactive oxygen species produced by ischemia-reperfusion of the liver. Bilirubin oxidative metabolites are excreted in the urine in the form of biopyrrin. Induction of HO-1 mRNA and HO protein by ischemia-reperfusion may enhance the production of bilirubin, and has a protective effect against oxidative stress.

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