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Effect of Trolox on Altered Vasoregulatory Gene Expression in Hepatic Ischemia/Reperfusion

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This study was designed to investigate the effect of Trolox, a hydrophilic analogue of vitamin E, on the alteration of vasoregulatory gene expression during hepatic ischemia and reperfusion (I/R). Rats were subjected to 60 min of hepatic ischemia *in vivo*. The rats were treated intravenously with Trolox (2.5 mg/kg) or the vehicle as a control 5 min before reperfusion. Liver samples were obtained 5 h after reperfusion for a RT-PCR analysis on the mRNA for the genes of interest. These mRNA peptides are endothelin-1 (ET-1), potent vasoconstrictor peptide, its receptor ET_A and ET_B, vasodilator endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1), tumor necrosis factor-α (TNF-α) and cyclooxygenase-2 (COX-2). It was seen that serum alanine aminotransferase and lipid peroxidation levels were markedly increased after I/R and Trolox significantly suppressed this increase. In contrast, the glutathione concentration decreased in the I/R group, and this decrease was inhibited by Trolox. ET-1 mRNA expression was increased by I/R, an increase which was prevented by Trolox. The mRNA levels for ET_A receptor was significantly decreased, whereas ET_B receptor transcript increased in the I/R group. The increase in ET_A was prevented by Trolox. The mRNA levels for iNOS and HO-1 significantly increased in the I/R group and Trolox attenuated this increase. There were no significant differences in eNOS mRNA expression among any of the experimental groups. The mRNA levels for COX-2 and TNF-α significantly increased in I/R group and Trolox also attenuated this increase. Our findings suggest that I/R induces an imbalanced hepatic vasoregulatory gene expression and Trolox ameliorates this change through its free radical scavenging activity.

Key words: Trolox, Ischemia/Reperfusion, Lipid peroxidation, Vasoregulatory gene expression

INTRODUCTION

Hepatic ischemia/reperfusion (I/R) is a common problem encountered in many clinical conditions such as liver transplantation, hepatic failure after the shock of liver surgery, trauma and cancer. There is substantial experimental evidence to suggest that the integrity of the microcirculation during reperfusion is a critical determinant for the survival of the various tissues after ischemia (Nevalainen *et al.*, 1986), however, the mechanism responsible for microvascular failure during reperfusion remains unclear.

Liver microcirculation is normally maintained under the fine balance of vasoconstrictors and vasodilators such as endothelin-1 (ET-1), nitric oxide (NO) and carbon monoxide (CO) (Pannen *et al.*, 1996). In the stressed liver suffering from endotoxemic shock or hepatic I/R, this fine balance is disrupted, resulting in a dysregulation of the hepatic microcirculation (Sonin *et al.*, 1999).

It is well accepted that a major proportion of I/R injury occurs during the period of reperfusion when reactive oxygen species (ROS) are generated. ROS can cause direct cellular damage and/or act as second messengers in the activation of cellular responses that control the fate of cells and inflammation (Fan *et al.*, 1999). Nowadays, the generation of ROS during I/R may play a pivotal role in mediating tissue injury and the microvasculature is the primary target of the radicals (Muller *et al.*, 1995).

α-Tocopherol is considered as the most effective lipid soluble, chain-breaking antioxidant that protects cell membranes from oxidative damage. Trolox is a water-soluble analogue of α-tocopherol. Previous studies suggested

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that the α-tocopherol requires several days of pretreatment to exhibit the antioxidative benefits, while Trolox, due to its enhanced water solubility, may function more rapidly during acute oxidative stress (Wu *et al.*, 1991). However, the precise mechanism of the *in vivo* antioxidant effect of Trolox remains unclear.

This study was designed to investigate the effect of Trolox on vasoregulatory gene expression during I/R.

MATERIALS AND METHODS

Chemicals

Trolox was supplied by the Aldrich Chemical Co. (Gillingham, UK). Isopropanol, diethylpyrocarbonate (DEPC) and ethidium bromide (EtBr) was purchased from the Sigma Chemicals Co. (St. Louis, MO, USA). dNTP, oligo $(dT)_{12-18}$ primer and SuperScript™ II RNase H⁻ Reverse Transcriptase was supplied from Invitrogen Tech-Line™ (Carlsbad, CA, USA). All other chemicals used were of reagent grades and were locally and commercially available.

Animals

Male Sprague-Dawley rats weighing 270-300 g were obtained from Jeil animal breeding company of Korea and were acclimatized to laboratory conditions at Sungkyunkwan University for at least one week. The rats were kept in a temperature and humidity controlled room (25±1°C, 55±5%, respectively) with 12 h light-dark cycle and they were allowed to drink tap water *ad libitum*.

Hepatic ischemic procedure

After an overnight fasting, the rats were anesthetized with sodium pentobarbital (40 mg/kg). A midline incision was made to the abdomen, and the left parts of the portal vein and hepatic artery were clamped to induce complete ischemia of the median and left lateral lobes. The right lobes remained perfused to prevent intestinal congestion. At the end of 60 min of ischemia, the clip around the left branches of the portal vein was removed and the branch to the right lobes was ligated. Hayashi *et al.* (1986) has shown that following ischemia to the left lobes, the flow increased in the right robes due to increased vascular resistance in the postischemic lobes. In addition, they have also suggested the importance of the blood supply following ischemia if any therapeutic agent is to be effective. Control animals were prepared in the same manner except that the clip was not placed in the left and median lobes, but blood flow to the right lobes of the liver was occluded. After 5 h of reperfusion, a blood sample was obtained from the abdominal aorta. The left and median lobes of the liver were isolated and immediately used for preparation of RNA. They were at -75°C for later analysis.

Administration of Trolox

Trolox was dissolved in phosphate buffered saline (PBS, pH 7.4), and it was administered by intravenous injection at a dose of 2.5 mg/kg of body weight, 5 min before reperfusion. In the vehicle-treated rats, PBS was injected in the same volume and manner as Trolox. Four experimental groups were studied: (a) vehicle-treated control, (b) Trolox-treated control, (c) vehicle-treated ischemic and (d) Trolox-treated ischemic. Because there were no differences found in any of the parameter between Trolox- and vehicle-treated rats in the control group, the results of group (a) and (b) were pooled, and were both referred to as controls.

Serum alanine aminotransferase (ALT) activity

ALT activities were determined by spectrophotometric procedures by Sigma diagnostics INFINITY™ kits 52-UV.

Lipid peroxidation

Lipid peroxidation was estimated by the levels of thiobarbituric acid reactive substances using the method of Buege and Aust (1978). One volume of liver homogenate was mixed with 2 volumes of 0.25 N HCl solution containing 15% (w/v) trichloroacetic acid and 0.375% (w/v) thiobarbituric acid. The mixture was heated for 30 min in boiling water. After cooling at room temperature, the precipitate was removed by centrifugation at 1,000×g for 10 min. The absorbance of the clear supernatant was determined at 535 nm and the concentration of malondialdehyde (MDA), the end product of lipid peroxidation, was calculated using 1.56×10^5 $M^{-1}cm^{-1}$ as the molar extinction coefficient.

Hepatic glutathione concentration

Total free glutathione and reduced glutathione (GSH) were determined in an acid extract by the method of Brehe and Burch (1978). Glutathione disulfide (GSSG) was estimated by the reduction of the GSH from the total glutathione concentration.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from approximately 100 mg of liver tissue that was obtained from left lobe. In brief, the liver tissue was homogenized with 1 mL of TRIZOL® reagent (Invitrogen Tech-Line™, USA). After exrtraction with chloroform, the total RNA was precipitated from the aqueous phase by addition of isopropanol, and then it was washed with 75% ethanol. It was then dissolved in DEPC-treated deionized water and stored at -75°C. Reverse transcription of the total RNA was performed to synthesize the first strand cDNA using oligo$(dT)_{12-18}$ primer and SuperScript™ II RNase H⁻ Reverse Transcrip-

tase. The reverse transcriptase reaction was stopped by incubation at 70°C for 10 min. The reaction products (cDNAs) were immediately stored at -20°C until a PCR analysis was performed. PCR was carried out in each 20 μL volume reaction samples using gene specific primers (Table I). PCR was performed according to the following protocol: 2 μL of 2.5 mM dNTP, 2 μL of 10X PCR buffer, 10 pmol of each primer for the appropriate target sequence, 14.4 μL of DEPC-treated deionized water and 0.5 U/reaction Ex *Taq*® DNA polymerase. PCR was done in a GeneAmp 2700 thermocycler (Perkin-Elmer, Norwalk, USA). The PCR for ET-1, ET_A, ET_B, HO-1 and iNOS was performed with 30 cycles (95°C, 45 s; 65°C, 45 s; 72°C, 1 min) with an initial incubation at 95°C for 5 min and a final extension at 72°C for 7 min. The reaction conditions consisted of 38 cycles, 24 cycles and 26 cycles for COX-2, TNF-α and β-actin were kept at 95°C for a 30 second denaturation, 54°C for a 30 second annealing and 72°C for a 1 min extension, respectively.

To ensure that equal amounts of cDNA from the control and experimental samples were used in PCR, the aliquots of the RT products for PCR were used with the primers for the housekeeping gene, β-actin. Following RT-PCR, the amplified products were resolved by electrophoresis in 1.5% agarose gel and then stained with EtBr. The intensity of each PCR product was semi-quantitatively evaluated using a digital camera (DC120, Eastman Kodak, CT, USA) and a densitometric scanning analysis program (1D Main, Advanced American Biotechnology, CA, USA).

Statistical analysis

All the data are presented as means±SEM. A one-way analysis of variance (ANOVA), followed by a Dunnetts *t*-test was used to determine the statistical significance of the differences between experimental groups. The results were considered significant for a $P<0.05$.

RESULTS

Serum ALT activity and lipid peroxidation

The serum level of ALT in the control rats was 445±148 U/L 5 h after reperfusion. In the ischemic rats, the serum ALT levels increased to 5067±705 U/L 5 h after reperfusion. This increase was significantly suppressed by Trolox. In the control animals, the liver MDA level was approximately 0.95 nmol/mg protein/min. 5 h after reperfusion, the MDA level increased to 1.82±0.09 nmol/mg protein/min. This elevation was attenuated by Trolox (Table II).

Hepatic glutathione

The hepatic concentrations of GSH and GSSG in the controls were 3.25±0.02 and 0.19±0.01 μmol/g liver,

Table II. Effect of Trolox on ALT activity and lipid peroxidation in rat liver after ischemia/reperfusion

Groups	ALT (U/L)	MDA (nmol/mg protein/min)
Control	445.0 ± 148.4	0.95 ± 0.06
Ischemia/reperfusion	5067.4 ± 704.7**	1.82 ± 0.09**
Trolox + Ischemia/reperfusion	1131.5 ± 174.0**,++	1.17 ± 0.04++

Each value is the mean ± SEM of 8 animals per group
Significant difference (**P < 0.01) from controls
Significant difference (++P < 0.01) from ischemia/reperfusion

Table I. PCR primers utilized in the experiment

Gene	Primer sequences (5' → 3')	Product length (bp)
ET-1	sense : TCTTCTCTCTGCTGTTTGTGGCTT anti-sense : TCTTTTACGCCTTTCTGCATGGTA	407
ET_A	sense : AGTGCTAATCTAAGCAGCCAC anti-sense : CAGGAAGCCACTGCTCTGTAC	491
ET_B	sense : AGCTGGTGCCCTTCATACAGAAGGC anti-sense : TGCACACCTTTCCGCAAGCACG	919
iNOS	sense : TTCTTTGCTTCTGTGCTTAATGCG anti-sense : GTTGTTGCTGAACTTCCAATCGT	1061
eNOS	sense : TGGGCAGCATCACCTACGATA anti-sense : GGAACCACTCCTTTTGATCGAGTTAT	202
HO-1	sense : AAGGAGTTTCACATCCTTGCA anti-sense : ATGTTGAGCAGGAAGGCGGTC	568
COX-2	sense : CTGCATGTGGCTGATGTCATC anti-sense : AGGACCCGTCATCTCCAGGGTAATC	474
TNF-α	sense : CAGAGCAAGAGAGGCATCCT anti-sense : GGCAGCTCATAGCTCCTTCTC	346
β-Actin	sense : TTGTAACCAACTGGGACGATATGG anti-sense : GATCTTGATCTTCATGGTGCTAG	764

respectively. At 5 h after reperfusion, the concentration of GSH significantly decreased to 2.77±0.03 μmol/g liver. This decrease was attenuated by the Trolox treatment. In contrast, the concentration of GSSG increased 5 h after reperfusion. Treatment with Trolox also attenuated this increase in GSSG. The ratio of GSH to GSSG (an indicator of the hepatocellular redox state) markedly declined during reperfusion. The decrease in the ratio of GSH to GSSG was clearly attenuated by the Trolox treatment (Table III).

Vasoconstrictor gene expression

At 5 h after reperfusion, marked changes were observed in the levels of the mRNA of genes encoding for vasoconstrictors. Changes in expression of vasoconstrictor genes ET-1 and its receptors (ET_A and ET_B) during I/R are shown in Fig. 1. The level of ET-1 mRNA significantly increased in the I/R group. This increase was significantly attenuated by Trolox. In contrast, ET_A mRNA expression markedly decreased 5 h after reperfusion; Trolox pretreatment significantly prevented this decrease. The level of ET_B receptor mRNA increased in I/R group, but Trolox pretreatment did not affect the ET_B receptor mRNA expression during I/R.

Vasodilator gene expression

There were no significant differences in eNOS mRNA expression among any of the experimental groups. There was a low level of iNOS mRNA in the control group. However, the level of iNOS mRNA in the I/R group significantly increased; a Trolox pretreatment prevented this increase. The level of HO-1 mRNA significantly increased in the I/R group as compared to the control group. This increase was markedly reduced by Trolox (Fig. 2).

COX-2 and TNF-α gene expression

There was a low level of COX-2 mRNA in control group and the I/R upregulated the transcription of this gene. This increase was significantly prevented by the Trolox treatment. Similar to the COX-2 gene expression, TNF-α mRNA expression significantly increased after reperfusion. This increase was not prevented by Trolox (Fig. 3).

Table III. Effect of Trolox on hepatic concentrations of GSH, GSSG and GSH/GSSG ratio in rat liver after ischemia/reperfusion

Groups	GSH (μmol/g liver)	GSSG (μmol/g liver)	GSH/GSSG ratio
Control	3.25 ± 0.02	0.19 ± 0.01	17.21 ± 0.89
Ischemia/reperfusion	2.77 ± 0.03*	0.35 ± 0.03**	7.85 ± 0.83**
Trolox+Ischemia/reperfusion	3.21 ± 0.26++	0.22 ± 0.03++	14.92 ± 1.02++

Each value is the mean ± SEM of 8 animals per group.
Significant difference (*P < 0.05, **P < 0.01) from controls
Significant difference (++P < 0.01) from ischemia/reperfusion

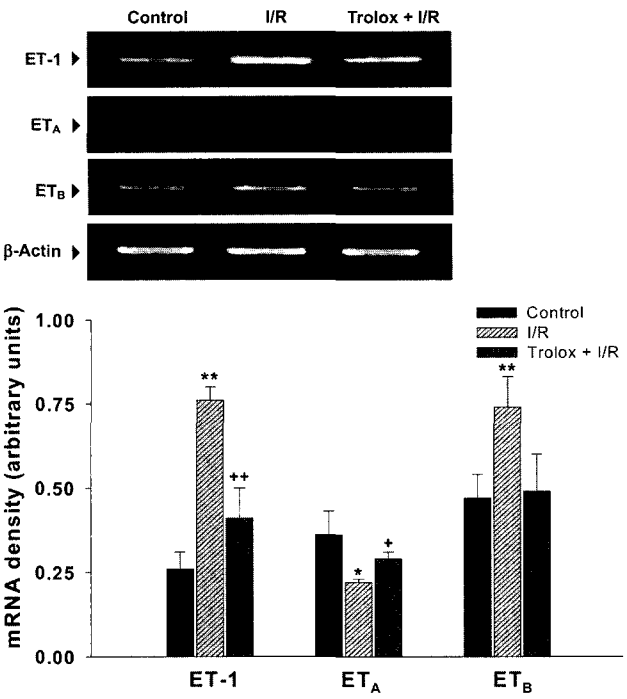

Fig. 1. The effect of Trolox on the mRNA levels for vasoconstrictor genes after ischemia and subsequent reperfusion in rats. Each value is a mean ± SEM for 8 rats per group. **Significant difference from the control (P<0.01). +,++Significant difference from ischemia/reperfusion (I/R) (P<0.05, P<0.01).

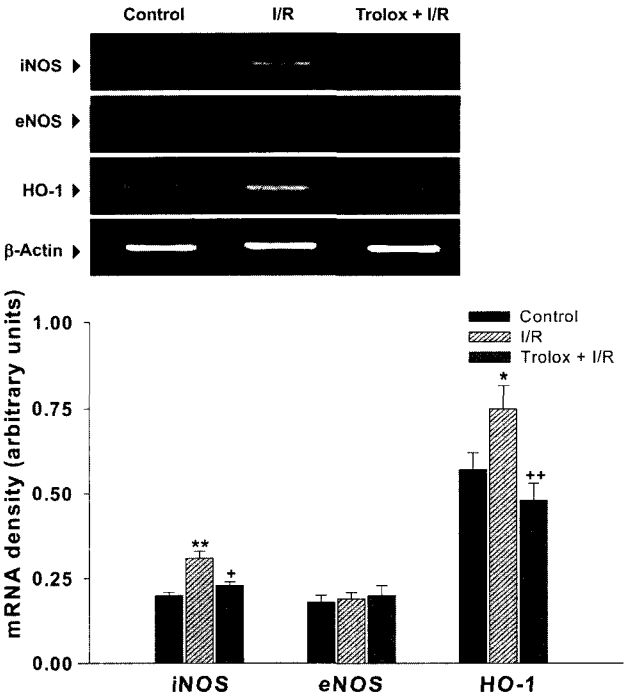

Fig. 2. The effect of Trolox on the mRNA levels for vasodilator genes after ischemia and subsequent reperfusion in rats. Each value is a mean ± SEM for 8 rats per group. *,**Significant difference from the control (P<0.05, P<0.01). +,++ Significant difference from the ischemia/reperfusion (I/R) (P<0.05, P<0.01).

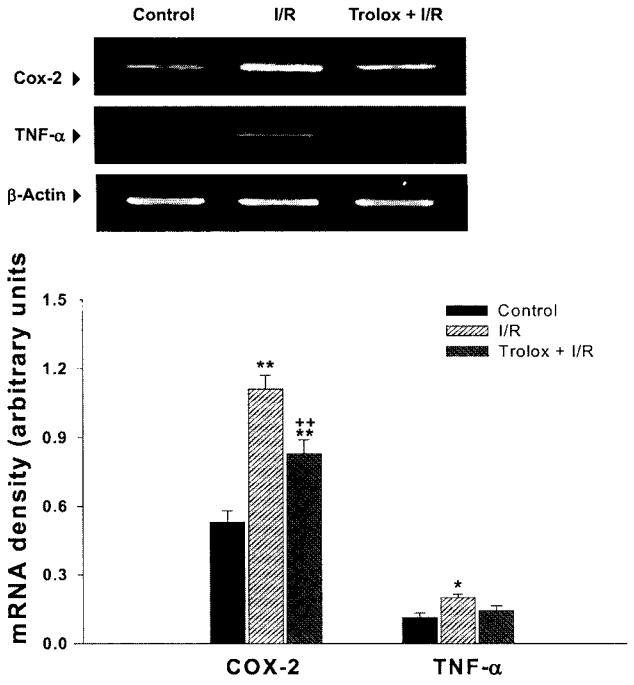

Fig. 3. The effect of Trolox on the mRNA levels for COX-2 and TNF-α after ischemia and subsequent reperfusion in rats. Each value is a mean ± SEM for 8 rats per group. *,**Significant difference from control ($P<0.05$, $P<0.01$). ++Significant difference from ischemia/reperfusion (I/R) ($P<0.01$).

DISCUSSION

Lipid peroxidation is related to a series of pathologic states, *e.g.* liver necrosis and ischemic brain damage (Bromont *et al.,* 1989), ischemic liver damage (Lee *et al.,* 2000) and ischemic heart disease (Petty *et al.,* 1991). Lipid peroxidation also induces structural and functional injury to the membranes of cell organelles. ROS-induced lipid peroxidation plays an important role in liver damage caused by I/R (Atalla *et al.,* 1985), and also it is involved in drug-induced lipid peroxidation in hepatic injury. It has been shown that the levels of lipid peroxidation products are lowered when GSH is added to the organ storage fluid (Bryan *et al.,* 1994). GSH plays an important role as a free radical scavenger. In addition, CCl_4 is converted to CCl_3· which causes lipid peroxidation in hepatocytes; lipid peroxide levels are also increased in chronic alcoholic patients. These symptoms are reduced by the antioxidants promethazine and α-tocopherol (Kawase *et al.,* 1989). These findings are in agreement with our data in which Trolox pretreatment prevented any increases in hepatic lipid peroxidation during I/R.

In ischemic rats, hepatic GSH significantly decreased 5 h after reperfusion. In contrast, lipid peroxidation significantly increased after reperfusion. Interestingly, ALT was also markedly increased after reperfusion. Thus, our results provide strong evidence that the ROS produced in post-ischemic livers causes direct cell damage through thiol oxidation and the subsequent lipid peroxidation. Moreover, treatment with Trolox attenuated a decrease in hepatic GSH content and lipid peroxidation. This suggests that it increases the hepatic pool of GSH and reduces the oxidative stress.

An accumulating body of evidence suggests that failure of the hepatic microcirculation is a major component of reperfusion injury in the liver. Pannen *et al.* (1998) showed that the physical prevention of a microvascular shutdown, using a flow-controlled reperfusion mode, largely prevented parenchymal cell necrosis in isolated perfused rat livers after I/R, *i.e.,* the degree of microcirculatory failure determined the extent of lethal hepatocyte injury (Chun *et al.,* 1994). However, the vasoactive mediators involved in the regulation following hepatic ischemia have yet to be identified.

The liver vascular response to the stress depends largely on the level of vasoactive mediators, ET-1, its receptors A and B, vasodilators nitric oxide (NO) and carbon monoxide (CO) (Clemens *et al.,* 1997). ET-1 is one of the most potent endogenous vasoconstrictive substances in the body (Prasad *et al.,* 1991), and the neutralization of ET-1, by a monoclonal antibody, or the inhibition of $ET_{A/B}$ receptors (Jin *et al.,* 1997) protected livers from a warm ischemia. Endothelin synthesis increases after the reperfusion of ischemic tissue (Watanabe *et al.,* 1991), and the blockade of endothelin receptors prevents organ damage following reperfusion (Brunner and Opie, 1998; Krause *et al.,* 1997). The mechanisms responsible for the increased endothelin synthesis following an ischemia-reperfusion are unknown, but the local release of ROS after tissue reperfusion (Ferrari, 1994) and the ability of ROS to induce other bioactive metabolites (Baud *et al.,* 1992; Lopez *et al.,* 1998) suggest that ROS may increase the synthesis of endothelin.

In the present study, ET-1 mRNA expression significantly increased after reperfusion; this increase was attenuated by Trolox. This result suggests that the ROS produced during I/R upregulates ET-1 mRNA expression.

Along with the increase of the ET-1 mRNA level, we observed approximately a 2-fold increase in the ET_B mRNA level in I/R liver, which is in consistent with previous reports of a higher ET_B receptor proportion during I/R (Yokoyama *et al.,* 2002). Trolox did not influence the expression of ET_B mRNA. This suggests that ROS are not essential in regulating the ET_B receptor gene expression during I/R. On the other hand, the level of ET_A mRNA transcription decreased after reperfusion, and this was prevented by the Trolox treatment. The increase in ET_B receptor expression, coupled with the decrease in the expression of ET_A receptors, would predict a predominant

effect on the presinusoidal constrictor response where ET_B receptor effects have been reported to be mediated (Zhang *et al.*, 1994). Thus, the increase in the available ET_B receptors would produce an increase in the presinusoidal constrictor response to ET-1, which acts on ET_B as well as ET_A receptors.

To examine the counterbalancing force to the pressor endothelin, we also determined the gene expression of iNOS and HO-1. NO and CO are strong vasodilators and most likely act in concert. Under normal conditions, NO is produced by eNOS in the sinusoidal endothelial cells, but an inflammatory response induces iNOS in multiple cell types in the liver including Kupffer cells, hepatocytes and hepatic stellate cells (Clemens *et al.*, 1994). In our study, we found a marked increased of iNOS mRNA in the I/R liver. Trolox treatment attenuated this increase. CO produced by heme oxygenase (HO) plays a pivotal role for regulation of hepatic vascular resistance in both the healthly and diseased states (Suematsu *et al.*, 1996). Two isoforms of HO have been characterized that contribute to catalytic activity and distinct genes encode for them both. Whereas the isoform HO-2 is constitutely expressed (Bauer *et al.*, 1998; Goda *et al.*, 1998), the isoform HO-1 is highly inducible (Rensing *et al.*, 1999). Our data show that HO-1 mRNA is highly induced in postischemic rats. These results are consistent with previous findings that the inhibition of HO-1 under I/R conditions results in significant increase in vascular resistance (Rensing *et al.*, 2002). Thus, ROS-dependent up-regulation of HO-1 and iNOS mRNA expression that may lead to the production of vasodilators, this appears to be a substantial part of the liver response to the stress.

COX-2, which is the inducible isoform of cyclooxygenase, is primarily responsible for the synthesis of prostaglandins during stressful conditions. Hormones, cytokines, and ROS rapidly induces the COX-2 gene, which is generally absent in resting cells. In the present study, we demonstrated that COX-2 mRNA was remarkably induced by the I/R, which coincides with the results of lipid peroxidation. This increase was prevented by Trolox. During hepatic I/R, activated Kupffer cells release a variety of proinflammatory cytokines including TNF-α, which is a key mediator in the pathogenesis of I/R injury (Colletti *et al.*, 1990). The generation of TNF-α in response to I/R propagates the hepatic microvascular dysfunction (Horie *et al.*, 1997). In this study, similar to COX-2, all ischemic animals demonstrated a marked increase in TNF-α mRNA expression as compared with the control animals. This increase was not prevented by Trolox treatment. This suggests that factors other than the ROS may also be responsible for the upregulation of TNF-α transcription.

The changes in mRNA expression that are reported here, if balanced in terms of constrictors-dilators expression, serve to protect against the injury, and can be considered as a preconditioning for future stress exposure. However, an imbalanced production of vasoactive mediators will result in the priming of the vasculature and a greater damage in at the time of the second stress.

Our findings suggest that ischemia and reperfusion induces an imbalance of the hepaic vasoregulatory gene expression and Trolox clearly ameliorates this change through its free radical scavenging activity.

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