Effects of Trolox on the activity and gene expression of cytochrome *P*450 in hepatic ischemia/reperfusion

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1 The aim of this study was to investigate the effect of Trolox on hepatic microsomal cytochrome *P*450 (CYP) activity and gene expression during ischemia and reperfusion (I/R).

2 Rats were subjected to 60 min of hepatic ischemia, and 5 h (acute phase) and 24 h (subacute phase) of reperfusion. Rats were treated intravenously with Trolox (2.5 mg kg^{-1}) or vehicle, 5 min before reperfusion.

3 The serum alanine aminotransferase level and lipid peroxidation were increased as a result of I/R. These increases were attenuated by Trolox. Reduced glutathione concentration decreased in I/R group, and this decrease was inhibited by Trolox.

4 Both total hepatic CYP content and NADPH-cytochrome *P*450 reductase activity decreased after I/R, which were restored by Trolox.

5 CYP1A1 activity and its protein level decreased 24 h after reperfusion; decreases which were prevented by Trolox. Both the activity and mRNA expression of CYP1A2 decreased 24 h after reperfusion. The decrease in *CYP1A2* mRNA was prevented by Trolox. CYP2B1 activity and mRNA expression decreased 5 h after reperfusion. The decrease in CYP2B1 activity was prevented by Trolox. In contrast, the CYP2E1 activity and its protein level increased 5 h after reperfusion and this increase was prevented by Trolox.

6 The expression of *TNF-α* and *iNOS* mRNAs increased after I/R. Trolox inhibited increase in *iNOS* mRNA expression.

7 Trolox ameliorates hepatic drug-metabolizing dysfunction, as indicated by abnormalities in CYP isoforms during I/R, and this protection is likely due to the scavenging of reactive oxygen species.
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Introduction

It is well accepted that a major proportion of ischemia and reperfusion (I/R) injury occurs during the period of reperfusion, when reactive oxygen species (ROS) are generated. During the acute phase of reperfusion, ROS can cause direct cellular damage through protein oxidation and degradation, lipid peroxidation, and DNA damage (Pardini, 1995). During the subacute phase of injury, cytokines produced by the initial acute phase activation of pro-inflammatory signal transduction cascades lead to the recruitment of neutrophils which amplify the redox burden in damaged tissue (Kurokawa *et al.*, 1996).

Among the stress-regulated genes are the cytochrome *P*450 (CYP) monooxygenase enzymes. These CYP enzymes are largely responsible for the metabolism and elimination of drugs, and form a large but closely related superfamily of distinct gene products with diverse substrate specificities (Nelson *et al.*, 1996). They are subject to regulation by a diverse array of xenobiotic chemicals, as well as by physiological factors, thus modifying the clinical and toxic effects of chemical agents in individuals (Jones *et al.*, 1989). Indeed, I/R decreases the activities of ethylmorphine demethylation and pentoxyresorufin dealkylation exerted by the hepatic microsomes of 3-methylcholanthrene-treated rats, but not those exerted by phenobarbital-treated rats, indicating that I/R may degrade CYP molecule form specifically (Lindstrom *et al.*, 1990). However, there is limited information available on the effect of I/R on individual CYP isoforms.

α-Tocopherol is considered as the most effective lipid-soluble antioxidant, protecting cell membranes from oxidative damage. Recently, α-tocopherol has been demonstrated to activate CYP gene expression *via* the pregnane X receptor, a nuclear receptor regulating a variety of drug-metabolizing enzymes (Landes *et al.*, 2003). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble analogue of the free radical scavenger α-tocopherol. A previous study suggested that α-tocopherol requires several days of pretreatment to exhibit antioxidative benefits, while Trolox, due to its enhanced water solubility, may function

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more rapidly during acute oxidative stress (Wu *et al.*, 1991). However, the precise mechanism of the *in vivo* antioxidant effect of Trolox remains unclear.

Therefore, this study was designed to investigate the effects that Trolox has on post-ischemic injury, particularly on the profile of CYP isozyme activity and gene expression.

Methods

Hepatic ischemia procedure

Male Sprague-Dawley rats (260–300 g) were fasted overnight, but allowed access to water. All animals were treated humanely under Sungkyunkwan University Animal Care Committee Guidelines. Under sodium pentobarbital anesthesia (40 mg kg^{-1}, i.p.), rats were laparotomized and liver ischemia was induced by clamping the pedicles of the left and median lobes for 60 min. At the end of ischemia, the clip around the left branches of the portal vein was removed and the branch to the right lobes was ligated. Control animals were prepared in the same manner except that the clip was not placed on the left and median lobes, but blood flow to the right lobes of the liver was occluded. At 5 and 24 h of reperfusion, blood was obtained from the abdominal aorta. The left and median lobes of the liver were isolated, immediately used for the preparation of microsome or RNA and stored at $-75°$C for later analysis.

Administration of Trolox

Trolox, dissolved in phosphate-buffered saline (pH 7.4, vehicle), was administered by intravenous injection at a dose of 2.5 mg kg^{-1} of body weight, 5 min before reperfusion. Four treatment groups were studied: (a) vehicle-treated control, (b) Trolox-treated control, (c) vehicle-treated ischemic, and (d) Trolox-treated ischemic. As 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 referred to as controls.

Isolation of hepatic microsomal fraction

The excised liver was minced, then homogenized with a Teflon® pestle homogenizer in 4 volumes of ice-cold 1.15 M KCl for 1 g of the liver, and centrifuged at $9,000 \times g$ for 60 min. The supernatant was collected and centrifuged at $105,000 \times g$ for 60 min. Microsomal precipitates were resuspended with 4 volumes of 0.1 M phosphate buffer, pH 7.4, for 1 g of the liver microsome and stored at $-75°$C until assayed.

Analytical procedures

Serum alanine aminotransferase was determined by standard spectrophotometric procedures using Sigma diagnostics IN-FINITY™ kit 52-UV (Sigma Chemical Co., St Louis, MO, U.S.A.). Lipid peroxide was assayed by the method of Buege & Aust (1978), and 1,1,3,3-tetraethoxypropane (malondialdehyde (MDA) tetraethyl acetal) was used as the standard. Total glutathione was determined in liver homogenates after precipitation with 1% picric acid and using yeast-glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and

NADPH, at 340 nm. Glutathione disulfide (GSSG) was determined by the same method in the presence of 2-vinylpyridine and reduced glutathione (GSH) calculated from the difference between total glutathione and GSSG (Anderson, 1985). CYP content was calculated by using the molar extinction coefficient of 91 mM^{-1}cm^{-1} for the absorbance difference between 450 and 480 nm measured with a differential spectrophotometer (Omura & Sato, 1964). The activity of NADPH-cytochrome *P*450 reductase was indirectly determined by its NADPH-cytochrome *c* reductase activity (Vermillion & Coon, 1978). Catalytic activities of CYP1A1, 1A2, and 2B1 in liver microsomal fraction were measured as 7-ethoxyresorufin *O*-deethylase (EROD), methoxyresorufin *O*-demethylase (MROD), and pentoxyresorufin *O*-dealkylase (PROD) activities, respectively, by the method of Burke *et al.* (1985). Microsomal CYP2E1 activity was determined by measuring 4-hydroxylation of aniline to *p*-aminophenol (Schenkman *et al.*, 1967). Protein content was estimated by the dye binding assay of Bradford (1976).

Total RNA extraction and reverse transcription–polymerase chain reaction (RT–PCR)

Isolation of total RNA was carried out according to the method described by Chomczynski & Sacchi (1987). Reverse transcription of total RNA was performed to synthesize the first-strand cDNA using oligo(dT)$_{12–18}$ primer and Super-Script™ II RNase H⁻ Reverse Transcriptase (Invitrogen Tech-Line™, Carlsbad, CA, U.S.A.). PCR reaction was performed with a diluted cDNA sample and amplified in each 20 μl reaction volume. The final reaction concentrations were: primers, 10 pmol; dNTP mix, 250 μM; 10 × PCR buffer; Ex *Taq* DNA polymerase, 0.5 U per reaction. The gene-specific primers are listed in Table 1. All PCR reactions had an initial denaturation step at 94°C for 5 min, and a final extension at 72°C for 5 min using GeneAmp 2700 thermocycler (Applied Biosystems, Foster city, CA, U.S.A.). Each PCR amplification cycling condition was as follows: 94°C 30 s, 57°C 30 s, 72°C 60 s, 30 cycles for *CYP1A1*; 94°C 30 s, 60°C 30 s, 72°C 60 s, 23 cycles for *CYP2E1*; 94°C 30 s, 62°C 30 s, 72°C 60 s, 22 cycles for *CYP1A2* and 23 cycles for *CYP2B1*; 94°C 45 s, 65°C, 45 s; 72°C, 60 s, 32 cycles for *iNOS*; 94°C 30 s, 54°C 30 s, 72°C 60 s, 26 cycles and 25 cycles for *TNF-α* and *β-actin*, respectively. Following RT–PCR, 10 μl samples of amplified products were resolved by electrophoresis in 1.5% agarose gel, and stained with ethidium bromide. The intensity of each PCR product was semiquantitatively evaluated using a digital camera (DC120, Eastman Kodak, New Haven, CT, U.S.A.) and a densitometric scanning analysis program (1D Main, Advanced American Biotechnology, Fullerton, CA, U.S.A.).

Western blot immunoassay

Microsomal proteins (10 μg per well) were separated by 10% SDS–PAGE and were transferred to nitrocellulose membrane using semi-dry transfer process. Bands were immunologically detected using polyclonal antibodies against rat CYP1A1, 2B1, and 2E1 (Gentest, Woburn, MA, U.S.A.) and visualized with an alkaline phosphatase-conjugated monoclonal rabbit anti-goat IgG secondary antibody using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as the substrate. The

Table 1 PCR primers used in the study

Gene (Accession number)	Primer sequences (5′ → 3′)	Product length (bp)
CYP1A1 (X00469)	sense : CTGGTTCTGGATACCCAGCTG anti-sense : CCTAGGGTTGGTTACCAGG	331
CYP1A2 (X01031)	sense : CAGTCACAACAGCCATCTTC anti-sense : CCACTGCTTCTCATCATGGT	302
CYP2B1 (XM_342078)	sense : TTGTTTGGTGCTGGGACAGAG anti-sense : GGCTAGGCCCTCTCCTGCACA	443
CYP2E1 (M20131)	sense : AAACTTCATGAAGAAATTGAC anti-sense : TCTCCAACACACACACGCTTCC	311
TNF-α (X66539)	sense : GTAGCCCACGTCGTAGCAAA anti-sense : CCCTTCTCCAGCTGGAAGAC	346
iNOS (D44591)	sense : TTCTTTGCTTCTGTGCTTAATGCG anti-sense : GTTGTTGCTGAACTTCCAATCGT	1061
β-actin (BC063166)	sense : TTGTAACCAACTGGGACGATATGG anti-sense : GATCTTGATCTTCATGGTGCTAG	764

Table 2 Effect of Trolox on ALT activity and lipid peroxidation in rat liver after ischemia and reperfusion

	ALT (i.u. l^{-1})		*MDA* (nmol mg^{-1} protein)	
Groups	5 h	24 h	5 h	24 h
Control	445.0 ± 148.4	68.5 ± 20.3	0.95 ± 0.06	1.12 ± 0.07
Ischemia/reperfusion	5067.4 ± 704.7**	623.9 ± 126.0**	1.82 ± 0.09**	1.61 ± 0.10**
Trolox + ischemia/reperfusion	1131.5 ± 174.0**,++	231.9 ± 30.1**,+	1.17 ± 0.04++	1.08 ± 0.03++

Each value is the mean ± s.e.m. of 7–10 animals per group. Significantly different (**$P < 0.01$) from controls. Significantly different (+ $P < 0.05$, ++ $P < 0.01$) from ischemia and reperfusion.

intensity of the immunoreactive bands was determined by densitometric analysis program.

Statistical analysis

Overall significance was tested by two-way ANOVA. Differences between groups at specific time points were considered significant at $P < 0.05$ with the appropriate Bonferronic correction made for multiple comparisons. All results are presented as means ± s.e.m.

Results

Serum alanine aminotransferase (ALT) and lipid peroxidation

The serum level of ALT in the control rats was 445 ± 148 i.u. l^{-1} 5 h after reperfusion and 69 ± 20 i.u. l^{-1} 24 h after reperfusion. In the ischemic group, the serum ALT level increased to 11.4-fold 5 h after reperfusion and 9.1-fold of control 24 h after reperfusion ($P < 0.01$), respectively. This increase was significantly suppressed by Trolox. In the control animals, the liver MDA level remained constant at approximately 1.03 nmol mg^{-1} protein throughout the experiment. At 5 and 24 h after reperfusion, the MDA level increased to 1.9-

and 1.4-fold of control ($P < 0.01$), respectively. This elevation was attenuated by Trolox (Table 2).

Hepatic glutathione

The hepatic concentrations of GSH in controls were 4.06 ± 0.34 and 5.68 ± 0.14 µmol g^{-1} liver 5 and 24 h after reperfusion, respectively. GSH concentration significantly decreased 5 and 24 h after reperfusion. This decrease was attenuated by Trolox. Hepatic GSSG concentration was unchanged among any of the experimental groups. 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 attenuated by Trolox (Table 3).

Total hepatic CYP content and NADPH-cytochrome P450 reductase activity

In the vehicle-treated ischemic group, the hepatic microsomal CYP content was found to significantly decrease after 5 h of reperfusion ($P < 0.01$). This decrease persisted at least until 24 h of reperfusion. The decrease in CYP content was attenuated by Trolox. Similar to CYP content, the hepatic microsomal NADPH-cytochrome *P*450 reductase activity significantly decreased both 5 and 24 h after reperfusion. This decrease was prevented by Trolox (Table 4).

Table 3 Effect of Trolox on concentrations of GSH, GSSG and GSH/GSSG ratio in rat liver after ischemia and reperfusion

Groups	GSH (μmol g^{-1} liver)		GSSG (μmol g^{-1} liver)		GSH/GSSG ratio	
	5 h	24 h	5 h	24 h	5 h	24 h
Control	4.06 ± 0.34	5.68 ± 0.14	0.26 ± 0.03	0.26 ± 0.01	15.64 ± 0.75	22.35 ± 1.27
Ischemia/reperfusion	3.02 ± 0.12*	4.32 ± 0.29*	0.32 ± 0.03	0.34 ± 0.05	9.44 ± 1.10**	13.90 ± 2.60**
Trolox + ischemia/reperfusion	3.82 ± 0.23[+]	6.40 ± 0.54[+]	0.27 ± 0.05	0.30 ± 0.02	14.15 ± 1.47[+]	21.36 ± 1.54[+]

Each value is the mean ± s.e.m. of 7–10 animals per group. Significantly different (*$P<0.05$, **$P<0.01$) from controls. Significantly different ([+]$P<0.05$) from ischemia and reperfusion.

Table 4 Effect of Trolox on total hepatic cytochrome *P*450 content and NADPH-cytochrome *P*450 reductase activity after ischemia and reperfusion

Groups	Cytochrome P450 content (nmol min^{-1} mg^{-1} protein)		NADPH-cytochrome P450 reductase activity (nmol min^{-1} mg^{-1} protein)	
	5 h	24 h	5 h	24 h
Control	0.41 ± 0.03	0.39 ± 0.03	86.0 ± 5.4	88.1 ± 2.9
Ischemia/reperfusion	0.26 ± 0.03**	0.23 ± 0.03**	69.3 ± 2.0**	66.0 ± 3.7**
Trolox + ischemia/reperfusion	0.36 ± 0.02[+]	0.29 ± 0.04*	80.6 ± 1.6[++]	75.7 ± 2.9*

Each value is the mean ± s.e.m. of 7–10 animals per group. Significantly different (*$P<0.05$, **$P<0.01$) from controls. Significantly different ([+]$P<0.05$, [++]$P<0.01$) from ischemia and reperfusion.

Table 5 Effect of Trolox on hepatic microsomal cytochrome *P*450 isozyme activities after ischemia and reperfusion

Groups	Ethoxyresorufin O-deethylase (pmol min^{-1} mg^{-1} protein)		Methoxyresorufin O-demethylase (pmol min^{-1} mg^{-1} protein)		Penthoxyresorufin O-dealkylase (pmol min^{-1} mg^{-1} protein)		Aniline p-hydroxylase (nmol min^{-1} mg^{-1} protein)	
	5 h	24 h	5 h	24 h	5 h	24 h	5 h	24 h
Control	50.9 ± 4.2	51.3 ± 6.6	24.8 ± 1.4	16.4 ± 0.7	31.2 ± 2.6	44.3 ± 2.3	0.23 ± 0.02	0.16 ± 0.01
Ischemia/reperfusion	34.2 ± 4.4	30.2 ± 2.5*	27.0 ± 2.0	7.2 ± 0.7**	15.4 ± 2.4**	35.9 ± 3.0	0.78 ± 0.09**	0.24 ± 0.02*
Trolox + ischemia/reperfusion	43.2 ± 3.0	46.7 ± 7.6[+]	26.4 ± 2.1	8.3 ± 0.4**	23.7 ± 1.4[+]	41.9 ± 4.2	0.31 ± 0.05[++]	0.17 ± 0.01[+]

Each value is the mean ± s.e.m. of 7–10 animals per group. Significantly different (*$P<0.05$, **$P<0.01$) from controls. Significantly different ([+]$P<0.05$, [++]$P<0.01$) from ischemia and reperfusion.

Hepatic microsomal CYP isozyme activities

The results of CYP isozyme activities are summarized in Table 5. No significant effects on CYP1A1 activity were observed in the vehicle-treated ischemic group 5 h after reperfusion. At 24 h of reperfusion, however, CYP1A1 activity was reduced in the vehicle-treated ischemic group to levels about 59% of those seen in microsomes from control ($P<0.05$). This decrease was ameliorated by Trolox. Although there were no changes until 5 h after reperfusion, the activity of CYP1A2 in the ischemic rats significantly decreased 24 h after reperfusion. Trolox had little effect on the decrease in CYP1A2 activity. CYP2B1 activity significantly decreased 5 h after reperfusion, a decrease which was attenuated by Trolox. CYP2B1 activity was unchanged among any of the experimental groups 24 h after reperfusion. In contrast to CYP1A1, 1A2, and 2B1, the CYP2E1 activity markedly increased 3.4 times (5 h after reperfusion) and 1.5 times (24 h after reperfusion) the control values in I/R rats, respectively. This increase was prevented by Trolox.

CYP isozyme protein expression

The amount of CYP1A1 protein in microsome significantly decreased both 5 and 24 h after reperfusion, a decrease which was significantly attenuated by Trolox. The amount of CYP2B1 protein was unchanged among any of the experimental groups. The amount of CYP2E1 protein significantly increased 5 h after reperfusion. This increase in CYP2E1 protein was prevented by Trolox (Figure 1).

CYP isozyme mRNA expression

As shown in Figure 2, the level of *CYP1A1* mRNA significantly decreased 5 h after reperfusion. Trolox had little effect on the decrease in *CYP1A1* mRNA expression. The level of *CYP1A2* mRNA expression significantly decreased 24 h after reperfusion, a decrease which was attenuated by Trolox. As shown in Figure 3, the level of *CYP2B1* mRNA significantly decreased 5 h after reperfusion. Trolox did not affect the level of *CYP2B1* mRNA. The level of *CYP2E1*
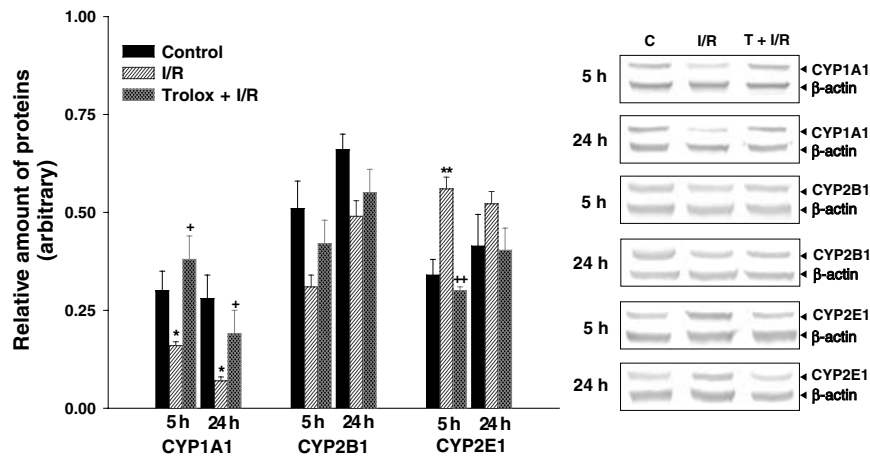
Figure 1 Effect of Trolox on CYP1A1, CYP 2B1 and 2E1 protein expression after hepatic ischemia and reperfusion. Values are means $\pm$ s.e.m. for 7–10 rats per group. *,**Significantly different ($P < 0.05$, $P < 0.01$) from controls. [+,++]Significantly different ($P < 0.05$, $P < 0.01$) from ischemia/reperfusion. C, control; I/R, ischemia/reperfusion; T, Trolox.
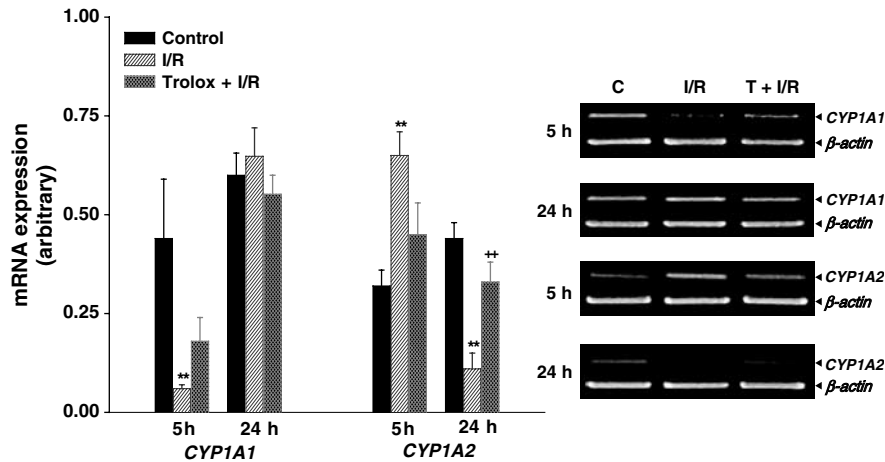

Figure 2 Effect of Trolox on *CYP1A1* and *CYP1A2* mRNA expression after hepatic ischemia and reperfusion. Values are means $\pm$ s.e.m. for 7–10 rats per group. **Significantly different ($P < 0.01$) from controls. [++]Significantly different ($P < 0.01$) from ischemia/reperfusion. C, control; I/R, ischemia/reperfusion; T, Trolox.
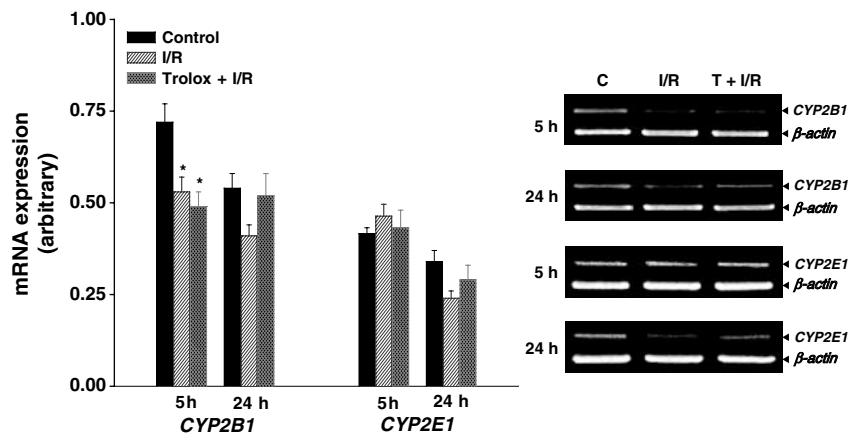

Figure 3 Effect of Trolox on *CYP2B1* and *CYP2E1* mRNA expression after hepatic ischemia and reperfusion. Values are means $\pm$ s.e.m. for 7–10 rats per group. *Significantly different ($P < 0.05$) from controls. C, control; I/R, ischemia/reperfusion; T, Trolox.
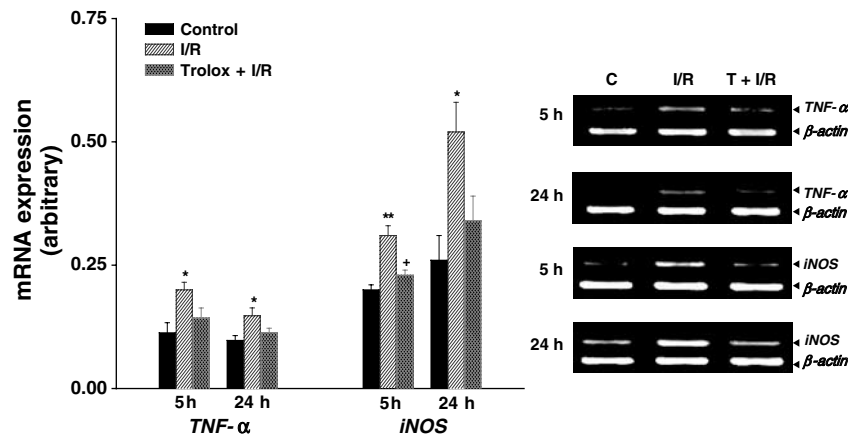
Figure 4 Effect of Trolox on *TNF*-α and *iNOS* mRNA expression after hepatic ischemia and reperfusion. Values are means ± s.e.m. for seven rats per group. *,**Significantly different ($P<0.05$, $P<0.01$) from controls. [+]Significantly different ($P<0.05$) from ischemia/reperfusion. C, control; I/R, ischemia/reperfusion; T, Trolox.

mRNA expression was unchanged among any of the experimental groups.

TNF-α *and iNOS mRNA expression*

TNF-α and iNOS have been demonstrated to act as mediators of tissue damage during I/R. To examine if the increased expression of *TNF*-α and *iNOS* mRNA after hepatic I/R is related to the production of ROS, we tested the effect of Trolox on their activation. As shown in Figure 4, there was a low level of *TNF*-α mRNA in the control group. The level of *TNF*-α mRNA expression significantly increased after reperfusion; an increase that was not prevented by Trolox. The level of *iNOS* mRNA in I/R group significantly increased; Trolox significantly inhibited this increase.

Discussion

There is now a substantial body of evidence supporting the notion that many of the pathophysiological events triggered by I/R injury are mediated through the production of ROS. I/R injury in the liver has been demonstrated to occur in a biphasic pattern, consisting of both acute- and subacute-phase responses. The acute phase, characterized by hepatocellular injury after 3–6 h of reperfusion, is associated with free radical generation with T-lymphocyte and Kupffer cell activation. Numerous studies suggest that the burst of ROS generated after reperfusion may contribute to the initiation of post-ischemic liver injury, and to the subsequent inflammatory infiltration (Zwacka *et al.*, 1997). The subacute-phase response following liver I/R is characterized by massive neutrophil infiltration, peaking after 18–24 h of reperfusion. Pro-inflammatory cytokines secreted by neutrophils during the subacute phase serve to perpetuate the organ damage and the generation of intracellular ROS in the damaged tissue through receptor-mediated pathways (Kurokawa *et al.*, 1996).

Trolox was reported to scavenge peroxyl radicals from artificial systems better than its parent compound. Wu *et al.* (1990) observed that Trolox protects human myocytes and hepatocytes against *in situ* generated oxyradicals. Further-

more, Trolox reduced hypoxia/reoxygenation-induced hepatic injury in the isolated perfused rat liver (Lee & Cho, 1997). However, few studies have rigorously determined whether Trolox exhibits antioxidant activity *in vivo* animal model of hepatic I/R.

In ischemic rats, ALT markedly increased after 5 h of reperfusion. After 24 h of reperfusion, the elevation in ALT activity declined, but still remained increased as compared with that in the control rats. Moreover, treatment with Trolox markedly attenuated ALT release after both 5 and 24 h of reperfusion. The present results demonstrate that treatment with Trolox improves hepatic function after ischemia and reperfusion. Accumulating evidences indicate that ROS play a major role in producing the microvascular and parenchymal cell damage associated with the reperfusion of ischemic tissues. ROS attack on biological membrane can lead to the oxidative destruction of the membrane polyunsaturated fatty acids by lipid peroxidation. It has been shown that the levels of lipid peroxidation products are lowered when GSH is added to organ storage fluids (Bryan *et al.*, 1994). GSH plays an important role as a free radical scavenger (Nakano *et al.*, 1995). In ischemic rats, hepatic GSH significantly decreased 5 and 24 h after reperfusion. In contrast, hepatic lipid peroxidation significantly increased after both 5 and 24 h of reperfusion. Thus, our results suggest that ROS, produced in post-ischemic livers, cause direct cell damage through thiol oxidation and subsequent lipid peroxidation. Moreover, treatment with Trolox attenuated a decrease in hepatic GSH content and lipid peroxidation, which suggest that it increases the hepatic pool of GSH and reduces oxidative stress.

Our previous study suggested that abnormalities in the microsomal drug-metabolizing function associated with lipid peroxidation occur following hepatic I/R *in vivo* (Lee *et al.*, 2000). In ischemic rats, CYP concentrations significantly decreased after both 5 and 24 h of reperfusion, which coincides with the results of lipid peroxidation. This decrease in CYP concentration was prevented by Trolox. Such a decrease in the total concentration of CYP suggests that the overall activity of CYP-dependent oxidations is likely to be similarly decreased and that this results from injury to the endoplasmic *via* disruption of the membrane lipid environment, as well as the

specific downregulation of specific CYP isoforms. CYP requires NADPH-cytochrome *P*450 reductase for electron transfer. After 5 and 24 h of reperfusion, the activity of NADPH-cytochrome *P*450 reductase decreased, as did that of CYP, and this decrease at 5 h of reperfusion was prevented by Trolox. This suggests that ROS may damage the CYP protein (heme protein), as well as the NADPH-cytochrome *P*450 reductase protein (non-heme protein) in the acute phase of reperfusion.

In the present study, both CYP1A1 and CYP1A2 activities significantly decreased in the late phase of reperfusion. Trolox attenuated the decrease in CYP1A1 activity, but not in CYP1A2 activity. Western blot analysis revealed that the levels of CYP1A1 protein significantly decreased after both 5 and 24 h of reperfusion; this decrease was prevented by Trolox. This suggests that ROS enhance the degradation of CYP1A1 protein and/or suppress CYP1A1 synthesis. Unfortunately, we could not identify any band equivalent to CYP1A2 enzyme size (59.3 kDa; data not shown).

Isozymes belonging to the CYP2B subfamily have been studied extensively in many species. In the rat, CYP2B1/2B2 is known as the major phenobarbital-inducible CYP isozyme (Guengerich *et al.*, 1982). In the present study, the activity of CYP2B1 decreased after 5 h of reperfusion. The decrease in the activity of CYP2B1 occurred earlier than the decrease in the activities of CYP1A1 and CYP1A2. This decrease in the activity of CYP2B1 was prevented by the Trolox. However, there was no appreciable decrease in the level of the CYP2B1 protein. Thus, these results indicate that the decrease in CYP2B1 activity is ROS-dependent, but is not explained by translational regulation. We may infer that ROS mediate their effects through a post-translational mechanism in which the enzyme protein is not active but is still recognized by the antibodies. There is evidence that ROS may have reduced the activity of selected isoforms of the CYP indirectly by inducing the phosphorylation of the isoforms. Effectively, activation of kinase can phosphorylate CYP, resulting in its inactivation (Rhee, 1999). CYP2E1 is significant for its adaptive response to high blood ethanol level, with a corresponding acceleration of ethanol metabolism, and is inducible by small organic molecules and pathophysiological states (Hong *et al.*, 1987). However, the 80% suppression of CYP2E1 mRNA caused by the injection of endotoxin has also been reported (Morgan, 1993). The CYP2E1 has been shown to be loosely coupled and produces ROS in high amounts relative to other CYP isoforms (Ekstrom & Ingelman-Sundberg, 1989), as CYP2E1 is rapidly reduced even in the absence of substrate (Guengerich & Johnson, 1997). In contrast to other CYP isozymes, the activity of CYP2E1 markedly increased, with a concomitant increase in its protein level, after 5 h of reperfusion, and this increase was prevented by Trolox. These results suggest that ROS is involved in the I/R-induced upregulation of CYP2E1 at the translational level.

It has been reported that the intracellular production of ROS is implicated in the activation of signal transduction cascades and in the regulation of gene expression. Changes in the cellular redox-state or the formation of ROS have been shown to activate redox-sensitive transcription factors, such as nuclear factor (NF)-*k*B and activator protein (AP)-1 in various cell lines (Palmer & Paulson, 1997). In the present study, both *CYP1A1* and *CYP2B1* mRNA expression were significantly downregulated during I/R, but Trolox did not affect the downregulation of CYP1A1 or CYP2B1 gene expression. This suggests that factors other (produced indirectly by cytokines generated during I/R) than ROS may also be responsible for the downregulation of *CYP1A1* and *CYP2B1* mRNA expression. *In vivo* and *in vitro* studies have shown that the cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α can mimic the downregulation of CYP gene products seen during inflammation. Cytokines also induce inducible nitric oxide synthase (iNOS) and result in the production of NO in many cell types. Several studies have suggested that NO mediate the suppression of hepatic CYP expression and activity (Carlson & Billings, 1996). Contrary evidence existed that NO is not required for the suppression of some CYP during inflammation (Sewer & Morgan, 1998). In the present study, we observed that levels of *iNOS* and *TNF*-α mRNA expression significantly increased during I/R. However, Trolox attenuated the increase in *iNOS* mRNA expression but did not affect the increase in *TNF*-α mRNA expression. These data indicate that the extent of iNOS expression is redox modulated and the inhibition of *iNOS* mRNA expression is responsible for the reduction of I/R-induced hepatic injury. The mRNA expression of *CYP1A2* decreased, with a concomitant decrease in its activity, after 24 h of reperfusion and this decrease was prevented by Trolox. This result suggests that I/R downregulates *CYP1A2* mRNA expression through a ROS-dependent mechanism.

Even though the full complex regulatory mechanisms of these inconsistent alterations in drug-metabolizing systems have not been identified, the individual CYP isozymes seem to be differentially affected by I/R injury, and the production of ROS during I/R may damage these CYP isoforms. These changes would be expected to variably affect the intrinsic hepatic clearance of certain drugs in patients with severe hepatic I/R injury. Furthermore, Trolox differently regulates not only the expression of each form of CYP, among the various CYP subfamilies, but also that of each isozyme within the same subfamily. Our data indicate that Trolox improves ischemia-induced hepatic drug-metabolizing dysfunction, which prevents a microsomal oxidant stress and lipid peroxidation.

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