Arch Pharm Res Vol 25, No 6, 940-945, 2002

Archives of Pharmacal Research
http://apr.psk.or.kr

Trolox C Ameliorates Hepatic Drug Metabolizing Dysfunction After Ischemia/Reperfusion

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(Received September 17, 2002)

The present study was done to determine the effect of trolox C, a hydrophilic analogue of vitamin E, on hepatic injury, especially the alteration in cytochrome P-450 (CYP)-dependent drug metabolism during ischemia and reperfusion (I/R). Rats were subjected to 60 min of hepatic ischemia and 5 h of reperfusion. Rats were treated intravenously with trolox C (2.5 mg/kg) or vehicle (PBS, pH 7.4), 5 min before reperfusion. Serum alanine aminotransferase and lipid peroxidation levels were markedly increased after I/R. This increase was significantly suppressed by trolox C. Cytochrome P-450 content was decreased after I/R but was restored by trolox C. There were no significant differences in ethoxyresorufin *O*-dealkylase (CYP 1A1) and methoxyresorufin *O*-dealkylase (CYP 1A2) activities among any of the experimental groups. Pentoxyresorufin *O*-dealkylase (CYP 2B1) activity was decreased and aniline *p*-hydroxylase (CYP 2E1) activity was increased after I/R. Both these changes were prevented by trolox C. Our findings suggest that trolox C reduces hepatocellular damage as indicated by abnormalities in microsomal drug-metabolizing function during I/R, and that this protection is, in part, caused by decreased lipid peroxidation.

Key words: Trolox C, Ischemia/reperfusion, Lipid peroxidation, Cytochrome P-450 isozyme activities

INTRODUCTION

Hepatic ischemia/reperfusion (I/R) is a common problem encountered in many clinical conditions, such as liver transplantation, hepatic failure after shock and liver surgery, trauma and cancer. Although much experimental work has been performed on the nature of this injury, the specific sequence of events that leads to the damage and eventual death of ischemic cells, despite reperfusion, is still not clear.

It is well accepted that a major part of I/R injury occurs during the period of reperfusion when reactive oxygen metabolites (ROM) are generated. ROM are generated early in reperfusion and are capable of interacting with a range of biomolecules leading to lipid peroxidation of cell membranes, increased membrane permeability and, ultimately, cell death via direct parenchymal injury and microvascular impairment (Jaeschke *et al.*, 1991). It is reported that exogenously added free radical scavengers alter favorably the post-ischemic deterioration of cell function (Evans *et al.*, 1997).

In patients with chronic liver disease, the elimination of drugs metabolized by the liver is often impaired (Huet and Villenueve, 1983). Indeed, our previous studies suggested that abnormalities in microsomal drug-metabolizing function and in hepatic secretory function associated with lipid peroxidation occur during hepatic I/R *in vivo*. Furthermore, this post-ischemic derangement in drug metabolism might be directly related to deficits in function of cytochrome P-450, an integral component of the hepatic microsomal oxidase (Lee *et al.*, 2000). However, a direct association between microsomal lipid peroxidation *in vivo* after I/R injury and changes in activities of cytochrome P-450 isozymes has not been established.

Trolox C, water-soluble analogue of vitamin E, was reported to be an excellent antioxidant *in vitro* (Doba *et al.*, 1985; Barkley *et al.*, 1985). In SDS micelles, trolox C was shown to scavenge peroxyl radicals eight times better than α-tocopherol (Castle and Perkins, 1986). We also observed that trolox C protected liver cells against

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hypoxia/reoxygenation injury in isolated perfused rat liver (Lee and Cho, 1997). However, the precise mechanism of the *in vivo* antioxidant effect of trolox C remains unclear.

Therefore, the purpose of this study was to investigate the effect of trolox C on post-ischemic injury, particularly on the deterioration of the microsomal drug metabolizing function.

MATERIALS AND METHODS

Chemicals

Trolox C was supplied by Aldrich Chemical Co. (Gillingham, U.K.). Ethoxyresorufin, methoxyresorufin, pentoxyresorufin, aniline, NADPH, HEPES and thiobarbituric acid were purchased from Sigma Chemicals Co. (St. Louis, MO). All other chemicals used in this study were of reagent grade and were locally and commercially available.

Animals

Male Sprague-Dawley rats weighing 260-300 g were obtained from Jeil animal breeding company of Korea and were acclimatized to laboratory conditions at Sungkyunkwan University for at least one week. Rats were kept in a temperature and humidity controlled room (25 ± 1°C, 55 ± 5%, respectively) with a 12 h light-dark cycle and were fasted for 18 h before the experiment and allowed to drink tap water *ad libitum*.

Hepatic ischemic procedure

Rats were anesthetized by the intraperitoneal injection of pentobarbital sodium (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 hepatic 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. Control animals were prepared in a similar 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 h of reperfusion, a blood sample was obtained from the abdominal aorta. The left and median lobes of the liver were then removed for the assay of protein, lipid peroxide and microsomal drug-metabolizing enzyme activities.

Administration of trolox C

Trolox C, dissolved in phosphate buffered saline (PBS, pH 7.4), 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 C. Four experimental groups were studied: (a) vehicle-treated control, (b) trolox C-treated control, (c) vehicle-treated ischemic, and (d) trolox C-treated ischemic.

Isolation of hepatic microsomal fraction

Liver samples were removed and placed in ice-cold 0.9% NaCl solution. They were then weighed, minced and homogenized with a teflon pestle homogenizer in 4 volumes of homogenizing buffer containing 1.15% KCl and 50 mM Tris HCl (pH 7.4). The whole homogenate was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was collected and centrifuged at 105,000 g for 60 min at 4°C. Microsomal precipitates were resuspended in 10 volumes of storage buffer containing 15% KCl, 10 mM HEPES and 1 mM EDTA (pH 7.6), for 1 g of the liver microsome and stored at -70°C until assayed. The content of microsomal protein was determined using the Bio-Rad protein assay reagent with bovine serum albumin as a standard.

Analytical procedures

Serum alanine aminotransferase (ALT) activity was determined by standard spectrophotometric procedure using a Sigma Kit 52-UV (Sigma Chemical Co., St. Louis, MO). Lipid peroxidation in the liver was estimated by the assay of levels of thiobarbituric acid reactants using the method of Buege and Aust (1978). Cytochrome P-450 content was calculated by using a molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ for the absorbance difference between 450 and 490 nm in a differential spectrophotometer (Omura and Sato, 1964). NADPH-cytochrome P-450 reductase was determined by its NADPH-cytochrome *c* reductase activity (Vermillion and Coon, 1978). Aniline *p*-hydroxylase activity (CYP 2E1) was determined by measuring the formation of *p*-aminophenol. Activities of CYP 1A1, 1A2, and 2B1 in liver microsomal fraction were measured as ethoxy-, methoxy-, and pentoxy-resorufin *O*-dealkylase activities, respectively by the methods of Pohl and Fouts (1980) and Burke *et al.* (1985). The reaction mixture contained 100 mM Tris-HCl buffer, pH 7.5, 25 mM $MgCl_2$, 5 μM substrates (ethoxy-, methoxy-, or pentoxy-resorufin) and microsome. The reaction was initiated by the addition of 1 mM NADPH and incubated at 37°C for 10 min. After incubation, reactions were terminated by adding methanol and the mixtures were centrifuged at 2,000 g for 10 min. Fluorescence of resorufin in the supernatant was measured at excitation and emission wavelengths of 550 and 580 nm, respectively.

Statistics

All data were expressed as means ± SEM. Overall significance was tested by one-way analysis of variance followed by Dunnetts *t*-test. The significance level was set at $p<0.05$.

RESULTS

Serum ALT

The serum ALT level in the vehicle-treated control rats and trolox C-treated control rats was 445 ± 148 U/L and 360 ± 137 U/L, respectively. After 60 min of ischemia without reperfusion, no changes were observed in serum ALT level in ischemic rats compared with the pre-ischemic values (data not shown). However, when the blood flow to the ischemic lobe of the liver was restored, the serum ALT activity increased to 5068 ± 704 U/L in the vehicle-treated ischemic rats. This increase in ALT activity was significantly suppressed by the administration of trolox C (Fig. 1).

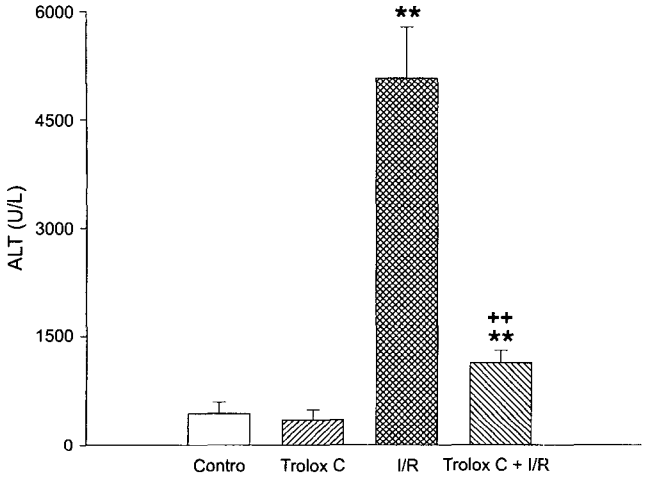

Fig. 1. Effect of trolox C on the serum alanine aminotransferase (ALT) activity after ischemia/reperfusion (I/R) of rat liver. ** = Significantly different ($p<0.01$) from controls. ++ = Significantly different ($p<0.01$) from I/R. Values are means ± SEM for 7-11 rats per group.

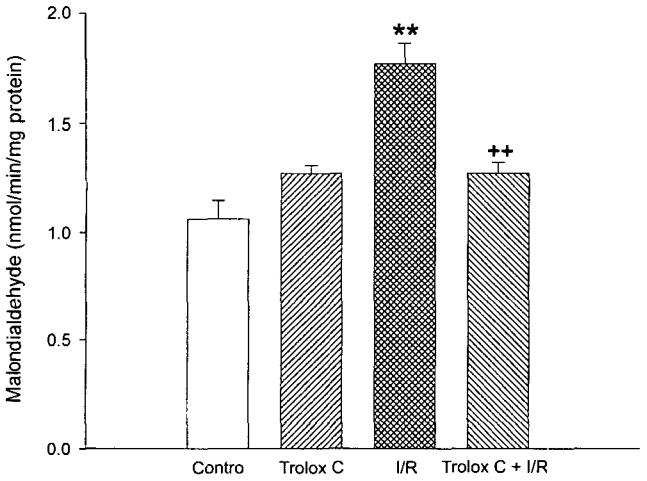

Fig. 2. Effect of trolox C on hepatic microsomal lipid peroxidation after ischemia/reperfusion (I/R). ** = Significantly different ($p<0.01$) from controls. ++ = Significantly different ($p<0.01$) from I/R. Values are means ± SEM for 7-11 rats per group.

Lipid peroxidation

The results of malondialdehyde (MDA) determination are presented in Fig. 2. In the vehicle-treated control rats and trolox C-treated control rats, the level of MDA in liver microsomes remained at approximately 1.06 ± 0.19 nmol/mg protein and 1.25 ± 0.04 nmol/mg protein, respectively. On the other hand, in the vehicle-treated ischemic rats, the MDA level increased to 1.77 ± 0.10 nmol/mg protein after reperfusion. This elevation in MDA was attenuated by trolox C administration (Fig. 2).

Cytochrome P-450 content

As shown in Fig. 3, the hepatic microsomal cytochrome P-450 content in the vehicle-treated control rats was 0.39

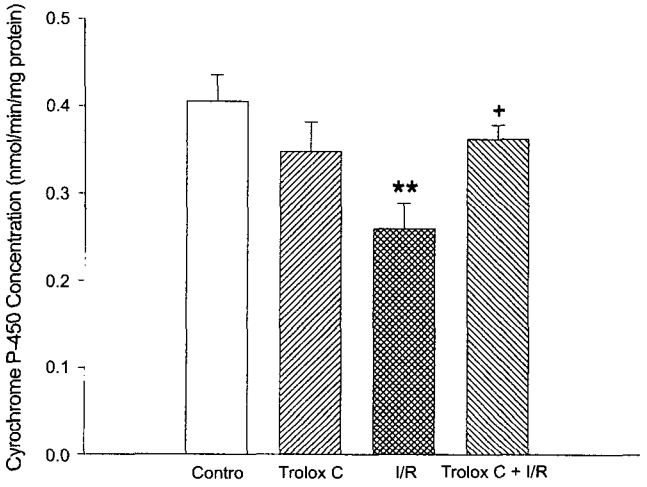

Fig. 3. Effect of trolox C on hepatic microsomal cytochrome P-450 concentration after ischemia/reperfusion (I/R). ** = Significantly different ($p<0.01$) from controls. * = Significantly different ($p<0.05$) from I/R. Values are means ± SEM for 7-11 rats per group.

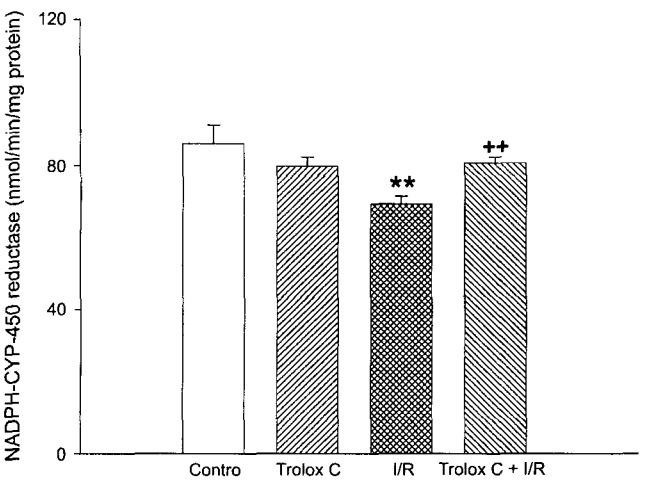

Fig. 4. Effect of trolox C on hepatic microsomal NADPH-cytochrome P-450 reductase activity after ischemia/reperfusion (I/R). ** = Significantly different ($p<0.01$) from controls. ++ = Significantly different ($p<0.01$) from I/R. Values are means ± SEM for 7-11 rats per group.

± 0.03 nmol/mg protein, with no changes being observed in trolox C-treated control rats compared with vehicle-treated control rats. However, cytochrome P-450 content in the vehicle-treated ischemic rats was found to significantly decrease after reperfusion; a decrease which was significantly attenuated by trolox C.

NADPH-cytochrome P-450 reductase activity

The results for NADPH-cytochrome P-450 reductase are displayed in Fig. 4. Similar to cytochrome P-450 content, the NADPH-cytochrome P-450 reductase activity significantly decreased after I/R. This decrease was significantly suppressed by trolox C.

Drug-metabolizing enzyme activity

As shown in Table I, ethoxyresorufin *O*-deethylase (CYP 1A1) activity in the control rats was 50.9 ± 4.2 pmol/min/mg protein. There were no significant differences in CYP 1A1 activity among any of the experimental groups. Similar to CYP 1A1, methoxyresorufin *O*-demethylase (CYP 1A2) activity was unchanged among all experimental groups. The pentoxyresorufin *O*-dealkylase (CYP 2B1) activity, 31.2 ± 2.6 pmol/min/mg protein in the control rats, was reduced in the trolox C-treated control rats and further reduced in the vehicle-treated ischemic rats. The decrease in CYP 2B1 after I/R was attenuated by trolox C. In contrast, aniline hydroxylase (CYP 2E1) activity was significantly increased after I/R (from 0.23 ± 0.02 to 0.78 ± 0.10 nmol/mg protein, $p<0.01$); an increase which was prevented by trolox C.

DISCUSSION

Lipid peroxidation is related to a series of pathologic states e.g. liver necrosis, ischemic brain damage (Bromant *et al.*, 1989), ischemic liver damage (Omar *et al.*, 1989) and ischemic heart disease (Petty *et al.*, 1990). The consequences of lipid peroxidation may be manifested as alterations in membrane integrity or membrane-associated functions in subcellular organelles. Reactive oxygen species-induced lipid peroxidation plays an important role in the extent of liver damage resulting from I/R. Our previous study reported that α-tocopherol pretreatment substantially attenuated increase in hepatic lipid peroxidation during I/R and that this correlated with improvement in some indicators of liver injury and cytochrome P-450 activity (Lee and Clemens, 1992). However, the pathophysiological role of oxygen free radicals in ischemic liver injury has yet to be determined.

α-Tocopherol is well known as a strong natural antioxidant. One of the major functions of α-tocopherol is to inhibit lipid peroxidation (Marubayasch *et al.*, 1988). However, α-tocopherol is extremely lipophilic and taken up by cells reactively slowly, *i.e.* within days or weeks (Ingold *et al.*, 1987). Therefore, it is not an ideal therapeutic antioxidant, especially in an emergency setting.

Trolox C, a hydrophilic analogue of α-tocopherol, was reported to scavenge peroxyl radicals from artificial system better than its parent compound. Wu *et al.* (1990) observed that trolox C protects human myocytes and hepatocytes against *in situ* generated oxyradicals. Furthermore, trolox C reduced hypoxia/reoxygenation-induced hepatic injury in isolated perfused rat liver (Lee and Cho, 1997). However, few studies cited have rigorously determined whether trolox C has antioxidant activity *in vivo* animal model of hepatic I/R.

In the vehicle-treated ischemic rats, significant increases in ALT and lipid peroxidation levels appeared after I/R. This shows that a temporal association exists between increased lipid peroxidation and hepatocyte injury. Moreover, administration with trolox C prevented lipid peroxidation and markedly attenuated ALT released by I/R. These results are similar to those of our previous work; pretreatment with α-tocopherol significantly reduced hepatocellular damage after 60 min ischemia and subsequent 5 h reperfusion. Although temporally associated, it is not clear that microsomal lipid peroxidation and hepatocyte necrosis are causally linked. The works of Jaeschke and Farhood (1991) and Jaeschke (1995) provide evidence that most early oxidative stress during reperfusion of the liver occurs extracellularly, mediated by Kupffer cells, whereas hepatocyte necrosis is associated with later neutrophil infiltration. Thus our findings may be indicative of hepatocyte oxidative stress that produces functional impairment of drug metabolism without direct contribution to hepatocyte necrosis.

After I/R, membrane-associated functions such as cytochrome P-450 activities may be more directly influenced by lipid peroxidation than other aspects of liver injury. Alterations in the cytochrome P-450 drug-metabolizing enzyme system during I/R in the liver are closely related to lipid peroxidation, and pretreatment with α-tocopherol reduced hepatocellular damage (Lee and Clemens, 1992). This study provides convincing evidence that trolox C has an antioxidant effect on the degradation of cytochrome P-450 induced by I/R. In vehicle-treated ischemic rats, cytochrome P-450 concentrations significantly decreased after I/R, and this decrease was inhibited by trolox C treatment.

Such a decrease in total content of cytochrome P-450 suggests that the overall activity of the cytochrome P-450-dependent oxidases would be similarly decreased. It seems likely that loss of cytochrome P-450 is a result of injury to the endoplasmic reticulum via disruption of the membrane lipid environment. In our present study, ethoxyresorufin *O*-deethylase (CYP 1A1) and methoxyresorufin *O*-demethylase (CYP 1A2) activities among all experi-

Table I. Effect of trolox C on cytochrome P-450 isozyme activities after ischemia and subsequent reperfusion (I/R)

Group	Ethoxyresorufin O-deethylase (pmol/min/mg protein)	Methoxyresorufin O-demethylase (pmol/min/mg protein)	Pentoxyresorufin O-dealkylase (pmol/min/mg protein)	Aniline p-hydroxylase (nmol/min/mg protein)
Control	50.9 ± 4.2	27.0 ± 2.0	31.2 ± 2.6	0.23 ± 0.02
Trolox C	41.3 ± 4.1	27.5 ± 1.8	23.2 ± 2.5*	0.28 ± 0.03
I/R	34.2 ± 4.4	27.0 ± 2.0	15.4 ± 2.4**	0.78 ± 0.10**
Trolox C + I/R	43.2 ± 3.5	26.4 ± 2.1	23.7 ± 1.4+	0.32 ± 0.05+*

*,** = significantly different ($p<0.05$, $p<0.01$) from controls. +,+* = Significantly different ($p<0.05$, $p<0.01$) from I/R. Values are means ± SEM for 7-11 rats per group.

mental groups were unchanged. The activity of pentoxyresorufin *O*-dealkylase (CYP 2B1) was decreased, whereas the activity of aniline *p*-hydroxylase (CYP 2E1) was increased after I/R. This phenomenon was alleviated by treatment with trolox C. Even though the mechanisms of these inconsistent alterations in drug metabolizing systems have not been identified, the individual cytochrome P-450 isozymes seem to be differentially affected by I/R injury. These changes would be expected to variably affect the intrinsic hepatic clearance of particular drugs in patients with severe liver I/R injury. Our data indicate that trolox C improved drug metabolism during reperfusion in ischemic liver by protecting against the functional damage of cellular and subcellular membranes that occurred during lipid peroxidation.

In conclusion, our findings suggest that administration of trolox C reduces hepatocellular damage caused by I/R and that this protection is, in part, caused by decreased lipid peroxidation.

ACKNOWLEDGEMENTS

This work was supported by grant R04-2001-000-00011-0 from the Basic Research Program of the Korea Science and Engineering Foundation.

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