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α-Tocopherol Transfer Protein Expression in Rat Liver Exposed to Hyperoxia

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α-Tochopherol transfer protein (αTTP), a 32 kDa protein exclusively expressed in liver cytosol, has a high binding affinity for α-tochopherol. The factors that regulate the expression of hepatic αTTP are not clearly understood. In this study, we investigated whether or not exposure to hyperoxia (>95% O_2 for 48 h) could alter the expression of hepatic αTTP. We also examined the association between the expression of antioxidant enzymes (hepatic glutathione peroxidase (GPX) and Mn-superoxide dismutase (Mn-SOD)) and the expression of hepatic αTTP. The levels of thiobarbituric acid-reactive substances (TBARS) in both plasma and liver were significantly higher after rats were exposed to hyperoxia for 48 h when compared with the levels in control rats. Northern blotting showed a decrease in the expression of αTTP messenger RNA (mRNA) after hyperoxia, although the αTTP protein level remained constant. Expression of Mn-SOD mRNA and protein, as well as the expression of GPX mRNA, were stable after hyperoxia. These findings indicate that mRNA for hepatic αTTP, rather than Mn-SOD or GPX, may be highly responsive to oxidative stress.

Keywords: α-Tocopherol; α-Tocopherol transfer protein; Hyperoxia; Mn-superoxide dismutase; Glutathione peroxidase

INTRODUCTION

α-Tocopherol is the most biologically active form of vitamin E, a fat-soluble antioxidant that prevents lipid peroxidation of cell membranes.[1] α-Tocopherol transfer protein (αTTP) is a 32 kDa protein exclusively expressed in liver cytosol which has a high binding affinity for α-tocopherol.[2,3] Arita *et al.* cloned the αTTP gene in rats and humans, and demonstrated that it was the causative gene for familial isolated vitamin E deficiency.[4,5] In normal individuals, αTTP protein has been shown to regulate the plasma level of α-tocopherol.[6]

Hepatic expression of αTTP protein and messenger RNA (mRNA) are decreased in vitamin E-replete rats, while expression of the mRNA is up-regulated in vitamin E-depleted rats.[7] In addition, the expression of αTTP protein and mRNA in the liver is decreased in rats fed a low protein diet. However, it has also been reported that hepatic expression of αTTP protein and mRNA was similar in vitamin E-replete or -depleted rats.[8] Furthermore, re-feeding of an α- or a δ-tocopherol-containing diet to vitamin E-depleted rats induces the hepatic expression of αTTP mRNA.[9]

It is known that the hepatic expression of cytochrome P 450,[10] hemopexin,[11] and inducible nitric oxide synthase (iNOS) was induced in rats exposed to hyperoxia ($\geqq$ 95% O_2 for 48 h), while hepatic glutathione levels were reduced.[12] The hepatic level of thiobarbituric acid-reactive substances (TBARS) was also increased in rats exposed to hyperoxia.[23]

Our previous study showed that the αTTP expression was up-regulated in rats with streptozotocin (STZ)-induced diabetes.[14] However, the mechanism involved is unknown because there are various biochemical changes in rats injected with

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STZ. The levels of malondialdehyde (MDA) and carbonyls in the liver, kidney, and pancreas were significantly increased in STZ-treated rats compared with control rats,[15] which might be related to the complications of diabetes mellitus. An increase of total superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase activity in these tissues also suggests the occurrence of adaptation to oxidative stress.[16] In the present study, we investigated whether or not exposure to hyperoxia induced the up-regulation of hepatic αTTP expression in rats in order to examine the relationship between oxidative stress and αTTP.

MATERIALS AND METHODS

Animals

Ten adult male *Wistar* rats which were seven months old weighing about 500 g each were divided into two groups of five rats (hyperoxia and normal control). Rats in the hyperoxia group were exposed to >95% oxygen for 48 h in a chamber with continuous monitoring of the oxygen concentration. Rats in the normal control group were kept in exposed to room air. In both groups, rats were allowed free access to food and drinking water during this experiment. The animals were sacrificed just after completion of their exposure to hyperoxia.

Western Blot Analysis

Rats were anesthetized by inhalation of ethyl ether and their livers were perfused with 0.9% sodium chloride via the portal vein. The livers were homogenized in 2.3 volumes of buffer (025 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). The cytosolic fraction was prepared from the homogenate by ultracentrifugation at 100,000*g* for 60 min and its protein content was determined by the method of Lowry. Cytosolic protein (40 μg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Then Western blotting was performed as described previously.[4] The blot was developed by soaking in a buffer (0.1 M $NaHCO_3$ and 1 mM $MgCl_2$, pH 9.8) containing 0.3 mg/ml nitroblue tetrazolium and 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate.

Northern Blot Analysis

Total RNA was isolated from each liver according to the method of Chomczynski and Sacchi.[17] mRNA was then extracted from the total RNA using a rapid method for purification of poly $(A)^+$-mRNA by oligo (dT)-Latex (Takara Co., Ltd, Shiga, Japan.).[18] The mRNA (2 μg) was run on 1.0% agarose gel, transferred to a nitrocellulose membrane, and cross-linked to the membrane by ultraviolet (UV) irradiation. Northern blot analysis was performed as described previously.[4] The αTTP, Mn-superoxide dismutase (Mn-SOD), and GPX cDNA probes were prepared as described previously[4,19] and were radiolabeled by the random primer labeling method.[4] β-actin was used as a control for equal loading of mRNA. Filters were subsequently exposed to a Fuji imaging plate (Fuji Photo Film Co., Ltd, Tokyo, Japan) and the radioactivity of the signals was quantified using a Fujix BAS-2000 Bio-imaging Analyzer (Fuji Photo Film Co., Ltd).

Assay of α-tocopherol in Plasma and Liver

Plasma was separated from heparinized whole blood by centrifugation at 800*g* for 15 min. Plasma and liver homogenates (0.5 ml) were assayed for tocopherol using high performance liquid chromatography (HPLC) with electrochemical detection, as described previously.[20]

Assay of Lipid Peroxide

Plasma TBARS was determined by the method of Yagi,[21] while hepatic TBARS was measured by the method of Uchiyama and Mihara with some modifications.[22]

Assay of Plasma Lipids and Alanine Transaminase (ALT)

Total lipids (the total of cholesterol, triglycerides and phospholipid) and ALT activity were determined by enzymatic assays using commercial kits (Wako Co., Ltd, Osaka, Japan).

Histological Examination of the Liver

Livers were fixed in 10% neutral formalin and routinely processed for light microscopy. Paraffin-embedded sections were stained with hematoxylin and eosin before observation.

TABLE I Plasma total lipids, ALT, α-tocopherol and TBARS levels and liver α-tocopherol and TBARS levels in 48 h hyperoxic group and control group

	Hyperoxia	Control
(Plasma)		
Total lipids (mg/dl)	366.5 ± 31.1	301.4 ± 21.2
ALT (IU/L)	28.7 ± 1.9	34.9 ± 7.4
α-Tocopherol (nmol/ml)	15.1 ± 0.5	12.5 ± 1.2
TBARS (nmol/ml) (liver)	122.6 ± 38.2*	28.8 ± 9.0
α-Tocopherol (nmol/g·protein)	289.8 ± 31.8	307.5 ± 23.2
TBARS (nmol/mg·protein)	5.6 ± 1.1*	1.4 ± 0.7

Values represent the mean ± SE. Comparison between groups was made by Mann–Whitney *U*-test. Asterisk indicates significant difference from control subjects ($p < 0.05$).

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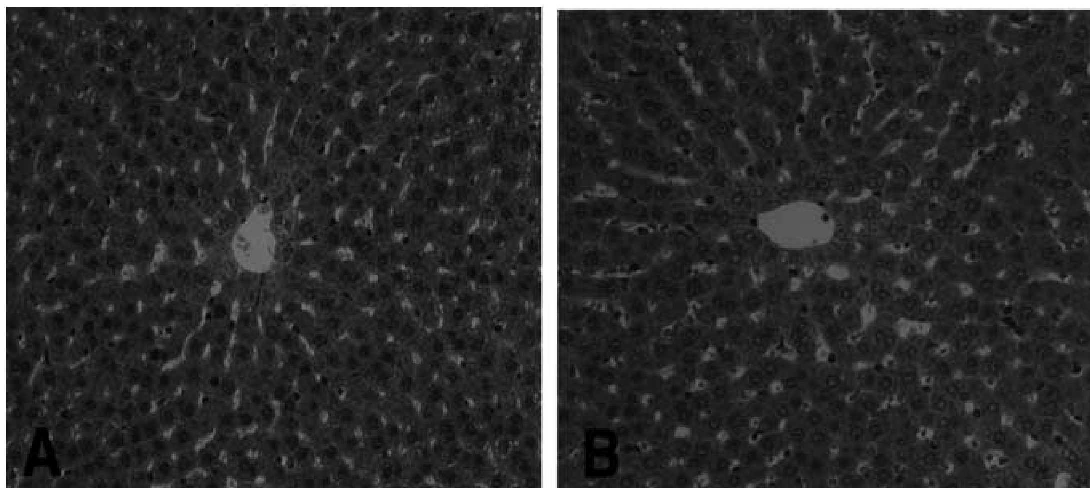

FIGURE 1 Histological findings of the liver. (A) Control rat. (B) Hyperoxic rat. (H&E; original magnification × 30).

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Statistical Analysis

All results were expressed as the mean ± SE. Differences between groups were assessed by the Mann–Whitney *U*-test and a *p* value less than 0.05 was considered to be significant.

RESULTS

Table I shows the levels of α-tocopherol and TBARS in plasma and liver as well as the total lipid and ALT levels in the plasma of hyperoxic and control rats. Plasma levels of total lipids, ALT, and α-tocopherol levels, as well as the hepatic α-tocopherol level, were not different between the control and hyperoxic groups. However, the TBARS levels in both plasma and liver from the hyperoxic group were significantly higher than in the control group.

Histological examination of the livers did not show any abnormalities in either group (Fig. 1).

To investigate the effect of hyperoxia on the expression of hepatic αTTP and antioxidant-related proteins, we evaluated the expression of αTTP and Mn-SOD mRNA and protein as well as the expression of GPX mRNA in rats exposed to hyperoxia (>95% O_2) for 3, 6, 24, or 48 h, followed by room air for the subsequent 72 h. There were five rats in each group. Typical results of Western and Northern blotting are shown in Figs. 2 and 3. Western blotting showed that the hepatic expression of αTTP and Mn-SOD protein did not change over time during hyperoxia (Fig. 2). When the changes of mRNA expression were analyzed by Northern blotting, hepatic αTTP mRNA showed a gradual decrease during hyperoxia up to 48 h and then was restored to the control level during subsequent exposure to room air for 72 h. In contrast, the expression of GPX and Mn-SOD mRNA showed no changes during the experiment (Fig. 3).

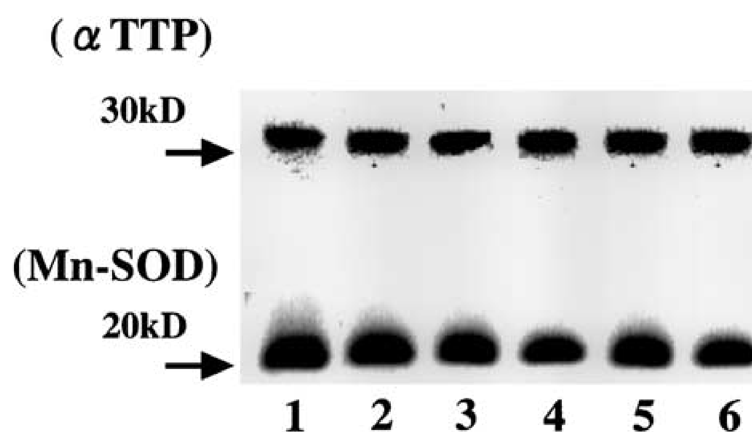

FIGURE 2 Western blot analysis of αTTP and Mn-SOD protein expression in the liver in each group of rats at different times. Lane 1: control; hyperoxia for 3 (lane 2), 6 (lane 3), 24 (lane 4), or 48 h(lane 5), and 21% oxygen (room air) for 72 h after hyperoxia for 48 h (lane 6).

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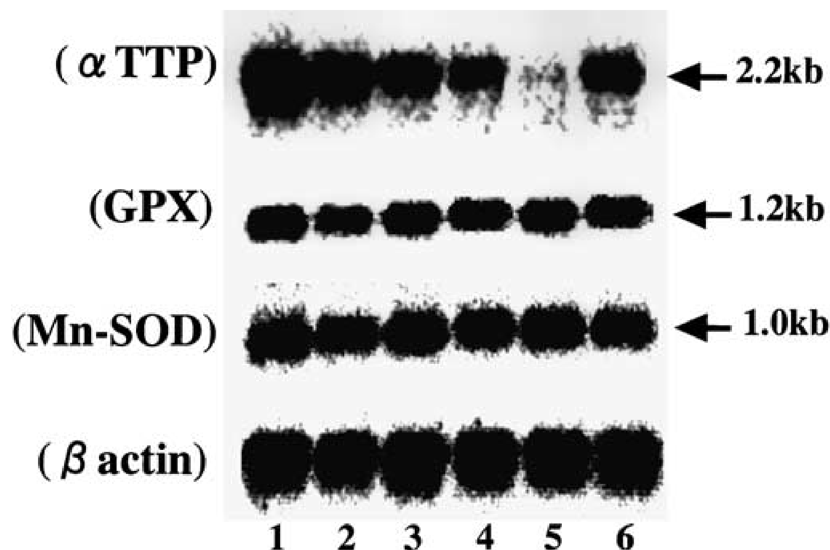

FIGURE 3 Northern blot analysis of αTTP, GPX, and Mn-SOD mRNA expression in the liver in each group of rats at different times. Lane 1: control; hyperoxia for 3 (lane 2), 6 (lane 3), 24 (lane 4), or 48 h (lane 5), and 21% oxygen (room air) for 72 h after hyperoxia for 48 h (lane 6). β-actin was used as a control to ensure equal loading of mRNA.

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DISCUSSION

It is well known that the plasma concentration of α-tocopherol is several times greater than that of γ-tocopherol despite a higher γ-tocopherol level in the diet.[23] This preferential increase of α-tocopherol is thought to be caused by αTTP in the liver. The secretion of α-tocopherol by cells expressing αTTP was found to be more efficient than by similar cells lacking αTTP in an experiment using the hepatoma cell line McARH7777.[24] However, the factors that regulate the expression of αTTP have not been clarified and the relationship between oxidative stress and αTTP expression has not been examined in detail. Recently, Arai *et al.* demonstrated that the placenta is protected from oxidative stress by vitamin E during development and that uterine αTTP may play an important role in governing the plasma α-tocopherol level, as well as hepatic αTTP, because pregnant αTTP knockout mice showed a marked reduction of labyrinthine trophoblasts and their embryos died at mid-gestation, while administration of α-tocopherol or a synthetic antioxidant dietary supplement prevented placental failure and allowed full-term pregnancy.[25] We have previously investigated factors affecting the levels of αTTP protein and mRNA, and we found that the vitamin E nutritional status can influence αTTP expression. We also found that rats with streptozotocin (STZ)-induced diabetes showed an increase of both αTTP protein and mRNA. These facts suggested that oxidative stress in STZ-induced diabetic rats might stimulate signal transduction and increase the expression of αTTP to elevate the circulating level of α-tocopherol. Therefore, we investigated another animal model of oxidative stress in the present study. Exposure to hyperoxia was previously used to investigate oxidative brain injury in rats.[26]

In the present study, both plasma and liver TBARS levels were significantly increased in rats exposed to hyperoxia for 48 h when compared to control rats, suggesting that peroxidation occurred in the hyperoxic animals. This finding was consistent with the report of Schweich *et al.*[13] Rats exposed to hyperoxia for 48 h showed normal biochemical parameters and hepatic histology, but rats exposed for 72 h developed severe liver damage leading to death (data not shown). There have been several reports[10–13,26] regarding the effect of oxidative stress in rats exposed to hyperoxia for 48 h. Therefore, we performed the hyperoxic experiment for 48 h.

We found that the αTTP protein level was not significantly altered, although the mRNA level gradually decreased in rats exposed to hyperoxia for up to 48 h and was restored to the pre-exposure level at three days after the cessation of hyperoxia. This findings suggested the possible stability of αTTP protein. The discrepancy between the expression of αTTP protein and mRNA may be due to slow hepatic turnover of the protein, which means that the protein level was not affected by mRNA degradation after 48 h of hyperoxia. Likewise, plasma and liver α-tocopherol levels did not differ between control and rats exposed to hyperoxia for 48 h because αTTP protein expression was constant during the experiment.

The discrepancy between the expression of αTTP in rats exposed to hyperoxia and rats injected with STZ was probably due to the difference in the type of stress, and the increase of αTTP expression in rats injected with STZ may be due to effects other than

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oxidative stress such as hyperglycemia or hyperlipidemia.

Frank reported an increase of pulmonaly SOD, glutathione reductase (GR), glutathine peroxidase (GPX) activity, and glutathione (GSH) in neonatal rats during oxygen challenge, suggesting that this may be the basis for their increased tolerance of hyperoxic lung injury when compared with adults.[27] We found no change of hepatic αTTP and Mn-SOD protein levels in rats exposed to hyperoxia for 48 h. In contrast, there was a time-dependent decrease of αTTP mRNA, although there were no change of GPX and Mn-SOD mRNA. The lack of any change in Mn-SOD and GPX during hyperoxia was consistent with Frank's report, except that we examined liver enzymes. Our findings suggested that the αTTP mRNA was more sensitive to hyperoxia than Mn-SOD or GPX mRNA. However, we currently have no evidence that the down-regulation of αTTP mRNA is induced by oxidative signal transduction.

In conclusion, we speculate that mRNA for antioxidant enzymes shows stability in the presence of oxidative stress, while αTTP mRNA expression is highly sensitive to oxidative stress, although the mechanism by which oxidative stress influences hepatic expression of αTTP mRNA is still unclear. Oxidative stress may be one of the factors that regulates αTTP expression, but further studies are needed to understand the physiological significance of these findings.

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