Hepatoprotective Effect of Fe-TPEN on Carbon Tetrachloride Induced Liver Injury in Rats

Jeon-Ok MOON,^{*,a} Su-Kyung PARK,^a and Tetsuo NAGANO^b

College of Pharmacy, Pusan National University,^a Keumjeung-ku, Pusan 609-735, Korea and Faculty of Pharmaceutical Sciences,^b The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Received October 13, 1997; accepted December 25, 1997

Fe(II)-tetrakis-N,N,N',N'-(2-pyridylmethyl) ethylenediamine (Fe-TPEN) catalyzes the dismutation of superoxide, and blocks the toxic effect of paraquat on *Escherichia coli* growth and survival. We examined antioxidative effects of Fe-TPEN on lipid peroxidation and *t*-butyl hydroperoxide induced cell damage. Fe-TPEN inhibited the FeSO₄/H₂O₂ induced lipid peroxidation in the rat liver homogenates with an IC₅₀ value of 30.2 μM, and protected Ac2F cell damage by *t*-butyl hydroperoxide in a dose-dependent manner (EC₅₀ value is 2.6 μM). Also, hepatoprotective effect of Fe-TPEN (5 mg/kg, i.p.) was investigated using CCl₄ induced liver injury in rats. This complex inhibited the elevation of serum alanine aminotransferase (AST) and aspartate aminotransferase (ALT) levels in CCl₄ induced liver injuries, and improved submassive necrosis and fatty degeneration of the hepatocytes. Fe-TPEN also prevented the loss of total and nonprotein SH contents, glutathione peroxidase and glutathione-S-transferase activity in cytosol of rat liver. Although the exact mechanism of action is not clear, antioxidative properties as well as attenuation of hepatocellular defense systems by Fe-TPEN seem to be important on its potent hepatoprotective effect in CCl₄-intoxicated rat.

Key words Fe-TPEN; hepatoprotective effect; antioxidative effect

Superoxide dismutase (SOD) catalyzes the conversion of superoxide to hydrogen peroxide and dioxygen.¹⁾ SOD provides a defense system against several diseases in which superoxide appears to play an important role,^{2,3)} including inflammation,^{4,5)} carcinogenesis,^{6,7)} and aging.^{8,9)} There have been reports of beneficial effects of SODs in clinical settings.¹⁰⁾ It is difficult, however, to employ SODs because they are readily cleared by the kidney and are unable to enter cells due to their high molecular weight.¹¹⁾ Therefore, useful SOD mimics that are capable of crossing cell membranes as well as stable, active, and nontoxic need to be developed. One attractive candidate is Fe(II)-tetrakis-N,N,N',N'-(2-pyridyl methyl) ethylenediamine (Fe-TPEN, Fig. 1). This metal complex catalyzed the dismutation of superoxide, and 0.8 μM Fe-TPEN showed activity equivalent to 1 unit of SOD in the xanthine oxidase-cytochrome *c* assay. This complex also blocked the toxic effect of paraquat on *Escherichia coli* growth and survival without causing induction of SOD.¹²⁾ We now report some of additional properties of Fe-TPEN that include inhibitory effect against the lipid peroxidation induced by Fenton reaction, antioxidative effect against Ac2F cell toxicity by *t*-butyl hydroperoxide (*t*-BHP) and hepatoprotective activity against CCl₄-induced liver injury in rats.

Fig. 1. Structure of Fe-TPEN

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals and Materials Thiobarbituric acid, glutamine, dimethylsulfoxide (DMSO), glutathione (GSH), NADPH, GSH reductase, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), *t*-BHP, tetrakis-N,N,N',N'-(2-pyridylmethyl) ethylenediamine (TPEN), 1,1,3,3-tetraethoxypropane and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Fetal bovine serum, Dulbecco's Modified Eagle Media (DMEM), trypsin-EDTA and antibiotic-antimycotic were purchased from Gibco Chemical Co. (Grand Island, U.S.A.). FeSO₄ was from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), and H₂O₂, CCl₄ and butylated hydroxytoluene (BHT) from Junsei Chemical Co., Ltd. Fe-TPEN was prepared as described in previous paper.¹²⁾

Animal Male Sprague-Dawley (SD) rats, 6 weeks of age, weighing 150—170 g were obtained from Dae Han Laboratory Animal Research Center Co., Ltd., Korea, and maintained under 12 h light/dark cycle in a temperature and humidity controlled room.

Inhibitory Effects on Lipid Peroxidation in Rat Liver Homogenate After rats had been fasted for 24 h, livers were perfused with an ice-cold 1.19% KCl solution *in situ*, then cut into thin pieces and homogenized in cold phosphate buffer (pH 7.4). The homogenate was centrifuged at 700×g for 10 min at 4 °C. The supernatant was then collected and used for the lipid peroxidation reaction. Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard.¹³⁾

Lipid peroxidation in rat liver homogenate induced by Fenton reaction composing of 0.1 mM FeSO₄, 3 mM H₂O₂, various concentrations of the tested substances and liver homogenate (7.5 mg protein/ml) was measured by the method of Buege and Aust with some modifications.¹⁴⁾ The reaction was started by the addition of FeSO₄ and H₂O₂ and then incu-

bated at 37 °C for 10 min. The reaction was stopped by mixing with 3 ml of a stock solution of 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, 0.125 M hydrochloric acid, and 0.6 mM BHT. The combination of reaction mixture and stock solution was heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1250×*g* for 20 min. The absorbance of the sample was determined at 535 nm and the malonaldehyde (MDA) concentration was calculated using 1,1,3,3-tetraethoxypropane as a standard. For control, water was used instead of the sample solution, and as the normal, water was used instead of FeSO₄ and H₂O₂. The percent inhibition of tested substances on the formation of MDA was calculated based on the amount of MDA formation of control after subtracting the normal.

Protective Activity of Fe-TPEN on Cell Damage Induced by *t*-BHP Ac2F, the normal liver cell of Donryu rat was obtained from Japan Cancer Research Resources Bank, Tokyo, Japan. The cells were routinely cultured in the complete medium composed of DMEM supplemented with 10% fetal bovine serum, 2% glutamine, penicillin (100 µg/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml) in a humidified atmosphere of 5% CO₂, 95% air. The cells were placed in 24-well plates at a cell density of 5.0×10⁴ cells *per* well. After 18–20 h cultivation, the complete medium of the plates was replaced by serum free medium (SFM) and the tested compounds were added to the cells. Thirty minutes later, the cells were exposed to 1 mM *t*-BHP for 2 h. After incubation, 100 µl of MTT solution (5 mg/ml in phosphate buffered saline) was added to each well and incubated for another 4 h. After the culture medium had been removed, 1 ml of a 1 : 1 DMSO and ethanol mixture was added and mixed to dissolve the MTT formazan. The plates were read on a microplate reader (Packard Instrument Co., U.S.A.) using a wavelength of 570 nm.¹⁵ The survival values, used to examine the protective effects of the compounds on cell damage by *t*-BHP, were expressed as a percentage of the absorbance of the normal cells.

CCl₄-Induced Liver Injury *in Vivo* Liver injury in rat was induced by i.p. injection of 25% (w/v) CCl₄ (in olive oil) at a dose of 0.6 g CCl₄/kg. Fe-TPEN was injected at a dose of 5 mg/kg, i.p. twice at 16 h and at 30 min before CCl₄ intoxication. TPEN was injected at a dose of 3 mg/kg, i.p. twice at 16 h and at 30 min before CCl₄ intoxication. After 24 h from CCl₄ injection, blood samples were collected and serum enzyme levels were measured to indicate the extent of liver damage.

Histopathological Examination A portion of the median lobe of the liver was removed 24 h after CCl₄ injection in rats. It was fixed in 10% neutralized formalin solution, embedded in paraffin, sectioned, and stained with hematoxylin–eosin (H.E.). Liver cell necrosis and fatty change were graded: normal (–), slight (+), moderate (++), severe (+++).

Enzyme Assay Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in rat were measured by the method of Reitman–Frankel using GOT and GPT assay kits (Eiken Chemical Co., Ltd., Tokyo, Japan).¹⁶

GSH peroxidase (GPx) was assayed essentially as described by Paglia and Valentine using H₂O₂ (0.5 mmol/l) following the disappearance of NADPH at 340 nm.¹⁷ Reaction

solution (1.0 ml) contained 100 mM sodium phosphate buffer (pH 7.0), 4 mM EDTA, 1 mM GSH, 0.15 mM NADPH, 1 unit of GSH reductase, 1 mM NaN₃ and about 20 µg of cytosolic protein.

GSH-S-transferase (GST) activity was determined by GSH conjugation of 1-chloro-2,4-dinitrobenzene (1 mmol/l) at 340 nm. Reaction solution (1.0 ml) contained 250 mM potassium phosphate buffer (pH 6.5), 1 mM CDNB, 1 mM GSH and cytosolic fraction (approximately 7 µg protein).¹⁷

Determination of Total SH and Nonprotein SH¹⁹ For total SH measurement, samples were homogenized in 100 mM sodium phosphate buffer (pH 7.4). Homogenate (100 µl) was mixed with 100 µl 0.01 M DTNB, 4 ml methanol and 1 ml 0.2 M Tris buffer (pH 8.2) and kept at 24 °C for 15 min. The sample was centrifuged for 30 min at 1250×*g*. An aliquot of the resulting supernatant was detected at 412 nm.

For the determination of nonprotein SH, homogenate was treated with trichloroacetic acid and centrifuged. The supernatant (100 µl) was mixed with 0.05 ml 0.01 M NaNO₂, and 0.45 ml 0.2 N H₂SO₄, and permitted to stand at 5 min. To sample was added 0.5% sulfamic acid ammonium solution (0.2 ml), 0.1 ml 1% HgCl₂, 0.9 ml 3.4% sulfanilamide and 1 ml 0.1% N-naphthyl ethylenediamine (in 0.4 N HCl). The mixture was kept for 5 min and detected at 540 nm. The standard used was 125 nM GSH solution.

Statistical Analysis All values were expressed as mean ± S.E. for *n* experiments. Student's *t*-test for unpaired observations between control and tested samples was carried out to identify statistical differences: a *p* value of 0.05 or less was considered statistically significant.

RESULT

Inhibitory Effects of Fe-TPEN on FeSO₄/H₂O₂ Induced Lipid Peroxidation in Rat Liver Homogenate Antioxidant activity of Fe-TPEN was estimated by examination of the inhibitory effect against FeSO₄/H₂O₂-induced lipid peroxidation in rat homogenate and the results are shown in Table 1. As a positive control, α-tocopherol was also tested. α-Tocopherol is a well-known chain-breaking antioxidant because it donates one hydrogen to a free radical and the radical is converted to a nonradical product. Fe-TPEN had a high inhibitory effect against FeSO₄/H₂O₂ induced lipid peroxida-

Table 1. Effects of Fe-TPEN on the Lipid Peroxidation of Rat Liver Homogenate Induced by FeSO₄ and H₂O₂

Substances	Concentration (µM)	Inhibition (%) ^a	IC ₅₀ (µM)
Fe-TPEN	10	16.3±6.7	30.2
	50	61.9±8.4	
	100	90.7±4.3	
α-Tocopherol	4	31.4±9.2	15.3
	20	61.6±1.5	
	100	65.5±4.9	

Results are expressed as mean ± S.E. (*n*=3). The reaction mixture (1.0 ml) was composed of the rat liver homogenate (7.5 mg prot.), 0.1 mM FeSO₄, 3 mM H₂O₂, and various concentrations of Fe-TPEN or α-tocopherol. After incubation at 37 °C for 10 min, the amount of MDA formation was measured by the method of Buege and Aust. ^a Inhibition (%) of MDA formation in Fe-TPEN or α-tocopherol was calculated based on the amount of MDA formation of the FeSO₄/H₂O₂ treated control (21.5 µM) after subtracting the normal (0.54 µM).

tion with an IC_{50} values of $30.2 \mu M$, which was slightly lower than that of α -tocopherol ($15.3 \mu M$).

The Protective Effect of Fe-TPEN on Cell Damage Induced by *t*-BHP *t*-BHP is a cytotoxic agent which is metabolized to the free radical such as *t*-butoxyl, *t*-butylperoxyl or methyl radical to interfere with cellular functions.²⁰⁾ The protective effect of Fe-TPEN on cell damage induced by *t*-BHP was examined (Table 2). In this study, by exposing of the cells to 1 mM *t*-BHP for 2 h, the survival of cells decreased to 45.6%. Fe-TPEN was found to protect the cell damage dose-dependently, and the EC_{50} was calculated as $2.6 \mu M$. Protective effect of α -tocopherol on the same system was examined as a positive control. Although α -tocopherol exhibited higher inhibitory activity on lipid peroxidation than Fe-TPEN, protective effect of Fe-TPEN on cell damage induced by *t*-BHP was more potent than that of α -tocopherol (EC_{50} $103.4 \mu M$).

Effect of Fe-TPEN on CCl_4 -Induced Liver Injury The hepatoprotective effect of Fe-TPEN on CCl_4 -induced liver in-

Table 2. Protective Activity of Fe-TPEN on Ac2F Cell Damage Induced by *t*-Butyl Hydroperoxide

Substances	Concentration (μM)	Survival (%)
Control		45.6 ± 0.9
Fe-TPEN	0.5	59.9 ± 2.2
	2.5	67.3 ± 0.9
	12.5	88.0 ± 1.5
α -Tocopherol	4	52.6 ± 1.1
	20	62.3 ± 0.9
	100	75.0 ± 2.3

Cells treated with Fe-TPEN or α -tocopherol for 30 min were exposed to 1 mM *t*-BHP for 2 h. Survivals estimated by MTT assay are expressed percentages for survival of the normal cells. Data are the mean $\pm$ S.E. ($n=4$).

Table 3. Effects of Fe-TPEN on AST, ALT and the Liver Weight to Body Weight % in CCl_4 -Intoxicated Rats^{a)}

	AST (Karmen) (U/l)	ALT (Karmen) (U/l)	% Liver weight to body weight
Control	56.6 ± 6.2	48.0 ± 2.8	3.76 ± 0.05
CCl_4	$573.2 \pm 143.2^{b)}$	$306.0 \pm 42.2^{b)}$	$4.56 \pm 0.07^{c)}$
Fe-TPEN + CCl_4	$179.7 \pm 37.3^{d)}$	$82.0 \pm 11.2^{d)}$	$4.19 \pm 0.08^{e)}$

a) Each value represents the mean $\pm$ S.E. ($n=6$). Fe-TPEN (5 mg/kg) was administered intraperitoneally, 16 h and 30 min before i.p. administration of CCl_4 (0.6 g/kg). Blood samples were collected 24 h after CCl_4 injection. b) $p < 0.01$, c) $p < 0.001$ vs. controls, d) $p < 0.01$, e) $p < 0.05$ vs. CCl_4 -treated controls, by Student's *t*-test.

Table 4. Effects of Fe-TPEN on CCl_4 -Induced Liver Injury by Histopathological Examination

	Number of rats							
	Necrosis				Fatty degeneration			
	-	+	++	+++	-	+	++	+++
CCl_4 treated control	0	0	3	3	0	0	3	3
Fe-TPEN + CCl_4	1	5	0	0	2	3	1	0

Liver injury in rat was induced by i.p. injection of 25% (w/v) CCl_4 (in olive oil) at a dose of 0.6 g CCl_4 /kg. Fe-TPEN was injected at a dose of 5 mg/kg, i.p. twice at 16 h and at 30 min before CCl_4 intoxication. Intensity of the histopathological findings; observed under a microscope and scored as normal (-), slight (+), moderate (++), severe (+++).

jury in rats is shown in Table 3. In normal rats, serum AST and ALT levels were 56.6 ± 5.8 and 48.0 ± 0.4 U/l, respectively. In CCl_4 -treated controls, serum AST and ALT levels were elevated to 573.2 ± 143.2 (1013% of control) and 306.0 ± 42.2 U/l (638% of control), respectively, after 24 h from CCl_4 administration. In contrast, in the Fe-TPEN-pretreated group, serum AST and ALT levels were lowered to 179.7 ± 37.3 (76% of protection) and 82.0 ± 11.2 U/l (88% of protection), respectively. TPEN alone could not prevent CCl_4 -induced liver damage of rat (Table 5).

By injection of CCl_4 , ratio of liver to body weight is significantly increased to $4.56 \pm 0.17\%$ (121% of the normal), while with the pretreatment of Fe-TPEN, the ratio is significantly reduced to $4.19 \pm 0.78\%$ (109% of the normal) showing a protection of 54%. The effect of Fe-TPEN on histopathological examination was also evaluated using this model and the results are shown in Table 4 and Fig. 2. Under

Table 5. Effects of TPEN on AST and ALT in CCl_4 -Intoxicated Rats^{a)}

	AST (Karmen) (U/l)	ALT (Karmen) (U/l)
Control	58.5 ± 3.8	15.0 ± 2.4
TPEN only	$74.8 \pm 3.5^{a)}$	16.3 ± 2.3
CCl_4	$568.5 \pm 131.9^{a)}$	$269.3 \pm 64.3^{b)}$
TPEN + CCl_4	649.0 ± 105.5	279.0 ± 85.5

a) Each value represents the mean $\pm$ S.E. ($n=6$). TPEN (3 mg/kg) was administered intraperitoneally, 16 h and 30 min before i.p. administration of CCl_4 (0.6 g/kg). Blood samples were collected 24 h after CCl_4 injection. a) $p < 0.01$, b) $p < 0.001$ vs. controls, by Student's *t*-test.

Fig. 2. Microscopic Appearance of Rat Livers after CCl_4 Treatment with or without Fe-TPEN (H. E. Stained, $\times 200$)

Rats were treated with 5 mg/kg of Fe-TPEN at 16 h and 30 min before CCl_4 administration. (A) CCl_4 -treated control; submassive hepatocellular necrosis is seen (B) Fe-TPEN and CCl_4 -treated group; The pathological alterations are reduced by pretreatment of Fe-TPEN with only slight centrilobular necrosis. C, central vein P, portal area.

Table 6. Effects of Fe-TPEN on the Contents of Total SH, and Nonprotein SH in Liver Homogenate and GST, and GPx in Liver Cytosol of CCl₄-Intoxicated Rats

	Total SH (nmol/mg prot.)	Nonprotein SH (nmol/mg prot.)	GST (unit/mg prot.)	GPx (unit/mg prot.)
Control	157.72±4.43	22.75±4.01	2.84±0.18	1.65±0.05
CCl ₄	147.04±3.37 ^{a)}	14.57±1.17 ^{a)}	2.46±0.06	1.29±0.03 ^{b)}
Fe-TPEN+CCl ₄	183.71±8.72 ^{c)}	43.60±10.62 ^{d)}	3.04±0.14 ^{c)}	1.58±0.06 ^{c)}

Values are mean±S.E. (n=6). Liver injury in rat was induced by i.p. injection of 25% (w/v) CCl₄ (in olive oil) at a dose of 0.6 g CCl₄/kg. Fe-TPEN was injected at a dose of 5 mg/kg, i.p. twice at 16 h and at 30 min before CCl₄ intoxication. a) $p<0.05$, b) $p<0.01$ vs. controls, c) $p<0.01$, d) $p<0.001$ vs. CCl₄-treated controls, by Student's *t*-test.

pathological conditions associated with CCl₄ injection, a well-defined hepatic lobular organization as characterized by an irregular branching and anastomosis of cells into plates and the morphology of parenchymal cell was disrupted. Submassive necrosis and fatty degeneration of the hepatocytes were observed. These pathological alterations were reduced by pretreatment using 5 mg/kg of Fe-TPEN, with only slight centrilobular necrosis or near-normal conditions.

The histopathological test, as well as serum parameters and ratio of liver to body weight, suggested that Fe-TPEN is a very strong hepatoprotective agent for CCl₄ induced liver injury.

Contents of Total, and Nonprotein SH in Rat Liver Homogenate and GST and GPx Activity in Cytosol of Rat Liver The effects of Fe-TPEN on the contents of total SH, and nonprotein SH in liver homogenate, and GST and GPx in liver cytosol in CCl₄-intoxicated rat were examined (Table 6). Contents of total SH and nonprotein SH group in liver homogenate of rat were significantly reduced by administration of CCl₄. However, these contents were significantly enhanced by Fe-TPEN pretreatment. In the case of nonprotein SH, the content was almost twice that of the normal. GST activity in the cytosol decreased by the administration of CCl₄ was significantly increased by the pretreatment of Fe-TPEN. GPx activity in the cytosol of CCl₄-intoxicated rats was significantly reduced to 1.29±0.03 unit/mg protein (78% of the control), and was significantly protected to 96% with the pretreatment of Fe-TPEN.

DISCUSSION

Reactive oxygen species are generated by enzymatic and nonenzymatic mechanisms. Under normal physiological conditions, about 3% to 10% of molecular oxygen seems to be converted to reactive oxygen species. Approximately 90 nmol of reactive oxygen species is produced *per min per g* of rat liver.²¹⁾ Most but not all reactive oxygen species rapidly react with a variety of molecules and thereby interfere with cellular functions. Therefore, protection of oxidative stress linked to various pathological state has received increasing attention in order to minimize cellular damages and to preserve the functional integrity of cells.²²⁾

It was demonstrated that Fe-TPEN catalyzes the dismutation of superoxide and blocks the toxic effect of paraquat on *Escherichia coli* growth and survival.¹²⁾ In this study we investigated whether this iron complex can scavenge different types of reactive oxygen species including hydroxyl radical, peroxy radicals. We evaluated the antioxidative activity of Fe-TPEN on the lipid peroxidation of rat liver homogenate induced by Fenton reaction (Table 1), and found that Fe-

TPEN has a high inhibitory effect against FeSO₄/H₂O₂-induced lipid peroxidation with an IC₅₀ value of 30.2 μM. The protective effect of Fe-TPEN on cell damage induced by *t*-BHP was also examined. Low concentrations of Fe-TPEN protected the cell from *t*-BHP toxicity (Table 2).

We then, examined the hepatoprotective effect of Fe-TPEN using CCl₄-induced rat model. CCl₄ is a substrate for cytochrome P450. It is converted to a CCl₃ radical, which generates CCl₃OO· by reacting with molecular oxygen.^{23,24)} Since CCl₃OO· reacts with microsomal membranes and induces lipid peroxidation, membrane damage by free radical chain reaction has been postulated as the major cause of hepatocellular injury by this compound.^{25,26)} In CCl₄-induced injury, antioxidants are widely known to be able to protect against hepatocyte necrosis because they intercept the CCl₄-induced oxidative stress in hepatocytes by scavenging ·CCl₃ and lipid peroxy radicals.²⁷⁾ It is evident that Fe-TPEN has very strong hepatoprotective effect in CCl₄-intoxicated rat, because it hindered the elevation of serum AST and ALT levels, lowered ratio of liver to body weight (Table 3), and protected CCl₄-induced submassive necrosis and fatty degeneration of the hepatocytes of rat administered CCl₄ (Fig. 2 and Table 4). Therefore, there is the possibility that antioxidative properties of Fe-TPEN might be attributed to its hepatoprotective effect in CCl₄-intoxicated rats. In fact, TPEN alone (TPEN without Fe) could not affect cell damage induced by *t*-BHP and TPEN administration was not able to prevent CCl₄-induced liver damage of rat (Table 5).

In the body, intoxication and detoxication mechanisms are delicately balanced. When this balance is overwhelmed by the formation of toxic metabolites to an extent that exceeds the capacity of the detoxication system, resulting in hepatotoxicity. Thiols act as intracellular antioxidants by maintaining a low intracellular redox potential, by scavenging free radicals and through several enzymatic reactions. GSH is a naturally occurring major thiol and is synthesized in cytosol in its reduced form. Under oxidative stress, GSH is oxidized to GSSG which is actively secreted by hepatocytes, potentially leading to depletion of the intracellular GSH pool. GSH peroxidase is a preventive antioxidant since it catalyzes the conversion of hydroperoxides to stable nonradical products in which the generation of free radicals is inhibited. GST, in addition to conjugating hepatotoxic electrophiles catalyzes reduction of hydroperoxides.²⁸⁾

Alteration of several hepatocellular defense systems by Fe-TPEN was examined (Table 6). Total and nonprotein SH contents in rat liver homogenate and GPx and GST activities were depressed by the administration of CCl₄. Fe-TPEN prevented the CCl₄-induced loss of total and nonprotein SH contents and GSH-dependent enzyme activity (GST and GPx).

Table 7. Effects of Fe-TPEN on the Contents of Total SH and Nonprotein SH in Liver Homogenate, and GST and GPx in Liver Cytosol of Rats

	Total SH (nmol/mg prot.)	Nonprotein SH (nmol/mg prot.)	GST (unit/mg prot.)	GPx (unit/mg prot.)
Control	100.94±3.89	18.09±0.56	2.62±0.10	1.75±0.07
Fe-TPEN	125.84±6.03 ^{a)}	22.22±1.52 ^{b)}	2.76±0.07	1.47±0.07 ^{a)}

Values are mean±S.E. (n=6). Liver injury in rat was induced by i.p. injection of 25% (w/v) CCl₄ (in olive oil) at a dose of 0.6 g CCl₄/kg. Fe-TPEN was injected at a dose of 5 mg/kg, i.p. twice at 16 h and at 30 min before CCl₄ intoxication. a) $p<0.01$, b) $p<0.05$ vs. controls, by Student's *t*-test.

We examined direct effect of Fe-TPEN on the thiol pool in liver. We found that Fe-TPEN caused significant increases in protein-bound thiol and non-protein SH contents (Table 7).

In conclusion, Fe-TPEN showed significant inhibitory effect against the lipid peroxidation induced by Fenton reaction and protective effect on the cell toxicity by *t*-BHP, and have potent hepatoprotective effect against CCl₄ induced liver injury in rats. Although the exact mechanism of action is still not clear, antioxidative properties as well as attenuation of cellular contents of the thiol pool by Fe-TPEN seems to be important on its hepatoprotective effect in CCl₄-intoxicated rats.

REFERENCES

- McCord J. M., Fridovich I., *J. Biol. Chem.*, **244**, 6049—6055 (1969).
- Halliwell B., Gutteridge J. M. C., *Biochem. J.*, **219**, 1—14 (1984).
- Halliwell B., Gutteridge J. M. C., *Arch. Biochem. Biophys.*, **246**, 501—514 (1986).
- McCord J. M., *Science*, **185**, 529—531 (1974).
- Oyanagui Y., "Biological and Clinical Aspects of Superoxide and Superoxide Dismutase," Vol. 11B, ed. by Bannister W. H., Bannister J. V., Elsevier/North-Holland, New York, 1980, pp. 147—153.
- Oberley L. W., Bize I. B., Sahu S. K., Lauthauser S. W. H. C., Gruber H. E., *J. Natl. Cancer Inst.*, **61**, 375—379 (1978).
- Oberley L. W., Buettner G. R., *Cancer Res.*, **39**, 1141—1149 (1979).
- Fong K., McCay P. B., Poyer J. L., Keele B. B., Misra H., *J. Biol. Chem.*, **248**, 7792—7797 (1973).
- Pederson T. C., Aust S. D., *Biochem. Biophys. Res. Commun.*, **52**, 1071—1078 (1973).
- McCord J. M., Syokes S. H., Wong K., *Adv. Inflamm. Res.*, **1**, 273—278 (1979).
- Huber W., Saifer M. G. P., Williams L. D., "Biological and Clinical Aspects of Superoxide and Superoxide Dismutase," Vol. 11B, ed. by Bannister W. H., Bannister J. V., Elsevier/North-Holland, New York, 1980, pp. 395—407.
- Nagano T., Hirano T., Hirobe M., *J. Biol. Chem.*, **264**, 9243—9249 (1989).
- Lowry O. H., Rosebrough N. R., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- Buege J. A., Aust S. D., *Methods Enzymol.*, **12**, 302—310 (1978).
- Tim M., *J. Immunol. Method*, **15**, 5363—5370 (1983).
- Reitmans S., Frankel S., *Am. J. Clin. Pathol.*, **28**, 56—63 (1957).
- Paglia D. E., Valentine W. N., *J. Lab. Clin. Med.*, **70**, 158—169 (1967).
- Habig W. H., Pabst M. J., Jakoby W. B., *J. Biol. Chem.*, **249**, 7130—7139 (1974).
- Sedlak J., Lindsay R. H., *Anal. Chem.*, **25**, 192—205 (1968).
- Christopher H. K., Daniel F. C., Gray W. W., William A. P., *Free Radic. Biol. Med.*, **12**, 381—387 (1992).
- Chance B., Sies H., Boveris A., *Physiol. Rev.*, **59**, 527—605 (1979).
- Rice-Evans C. A., Diplock A. T., *Free Radic. Biol. Med.*, **15**, 77—96 (1993).
- Butler T. C., *J. Pharmacol. Exp. Ther.*, **134**, 311—319 (1961).
- Lai E. K., McCay P. B., Noguchi T., Fong K. L., *Biochem. Pharmacol.*, **28**, 2231—2235 (1979).
- Slater T. F., *Biochem. J.*, **222**, 1—15 (1984).
- Yasuda H., Izumi N., Shimada O., Kobayakawa T., Nakanishi T., *Toxicol. Appl. Pharmacol.*, **52**, 407—413 (1980).
- Castro J. A., Sasame H. A., Sussman H., Gillette J. R., *Life Sci.*, **7**, 129—136 (1968).
- Inoue M., "The Liver: Biology and Pathobiology," ed. by Arias I. M., Boyer J. L., Fausto N., Jakoby W. B., Schachter D. A., Shafritz D. A., Raven Press, Ltd., New York, 1994, pp. 443—459.