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Effects of Iron Overload on Hepatic and Renal Metallothionein Levels in Rats

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Metallothionein (MT) is one of the stress proteins induced by various heavy metals and oxidative stress. Although induction by Fe has not been well established, its overload is documented to cause oxidative stress in experimental animals. To investigate alteration in tissue MT levels that were possibly caused by Fe overload, male Wistar rats were fed a diet containing 3.5% Fe(II) fumarate (1.2% as Fe) for up to 21 days. Tissue Fe levels in liver and kidney increased in time–dependent manners up to 7.2 and 2-fold, respectively, by the end of the experiment. Interestingly, plasma Fe levels showed a maximum on day 3, then decreased to the control level. The hepatic MT levels showed a transient decrease on day 3, then turned to increase throughout the experiment period, with the final level higher than that in control animals that fed on a normal diet. MT levels in the kidney decreased to nearly 1/3 of the control on day 3, with the values unchanged thereafter. Although Zn levels in liver, plasma and kidney showed a transient but significant reduction on day 3 of the Fe feeding, they recovered to the control values in the later period. Thus, a time–dependent change in the hepatic MT levels was quite similar to that of the tissue Zn levels. The renal Zn levels could not account for the change in the tissue MT levels, at least in the latter period of the experiment. Hybridization analysis of the MT mRNA in the kidney of Fe-fed rats did not differ from control-diet rats, suggesting Fe feeding would not alter synthetic rates of the renal MT. The turnover rates of the renal MT estimated from a time–dependent alteration after its induction by $HgCl_2$ treatment seemed to be enhanced in Fe-fed rats. Thus, the Fe-induced decrease in the renal MT level could possibly be due to an enhanced turnover rate. Despite the reduced renal MT levels, the Fe-treated rats showed no sign of a renal failure indicated by a stable plasma creatinine levels and sustained increase of body weight, suggesting that the Fe-induced suppression of the renal MT levels would not be one of the toxic effects by this metal. Nevertheless, the $HgCl_2$ injection experiment revealed Fe-fed rats had a slightly higher susceptibility against $HgCl_2$ nephrotoxicity than the control-diet rats.

Key words —— iron overload, metallothionein, kidney, rats

INTRODUCTION

Metallothionein (MT) is one of the stress-induced proteins rich in cysteine residues. Not only various heavy metals, but also a wide variety of reagents, such as herbicides, cytokines and pro-oxidants, as well as physical stresses, such as X-ray, UV and fasting, can induce MT.[1] MT functions in the cytosolic compartment as a chelator of various heavy metal ions, and as a scavenger of reactive oxygen species both *in vivo* and *in vitro*. Iron is the most abundant essential metal on the earth and its toxicity is well documented.[2,3] Although Good and Vašák[4] demonstrated the existence of the iron-thiolate cluster, there has been no report on *in vivo* occurrence of Fe-thionein. However, induction of hepatic MT was documented by some investigators after intraperitoneal injections of Fe in rats and chickens.[5,6] Fleet *et al*.[5] showed interesting results that feeding on an Fe-enriched diet caused no effect on the hepatic MT level in chickens, despite a significant Fe accumulation in the liver. He suggested that parenteral injection of iron would cause significant stress and, as such, promote MT biosynthesis.

Iron loading has often been employed to enhance oxidative status in experimental animals.[6–8] Younes *et al*.[8] reported that rats fed on a diet containing 3.5% Fe(II) fumarate enhanced a susceptibility to xenobiotics-induced hepatic damage *via* selective accumulation of Fe in the hepatocytes. Previously, the authors suggested the enhanced production of

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hydroxyl radicals in the liver using the rat model of Younes *et al.*[8] that resulted in a stimulation of MeHg degradation in the liver.[9] We also found reduced inorganic Hg accumulation in Fe-fed rats, and speculated that suppressed tissue MT levels might be responsible for it.[9] In the present study, we examined the possibility that feeding on an Fe-enriched diet caused alterations in tissue MT levels.

MATERIALS AND METHODS

Fe(II) fumarate and $HgCl_2$ were purchased from Wako Pure Chemical Co. (Osaka, Japan). 2-Thiobarbituric acid (TBA) and L-buthionine-(S,R)-sulfoximine were obtained from Merck (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, U.S.A.), respectively. Male Wistar rats (9 weeks of age) were obtained from CLEA Japan (Osaka, Japan), and maintained at 23 ± 2°C and 55 ± 5% humidity. Mouse MT-I cDNA was kindly provided by Dr. A. Naganuma (Tohoku University, Japan).

Effect of Fe-Loading Period on Tissue Fe, Zn and MT Levels ——— The rats of 4 groups (5 rats in each group) were fed on a powdered diet (CE-2, CLEA Japan) containing 3.5% Fe(II) fumarate (1.2% as Fe) for 3, 7 and 21 days. Normal powdered diet (CE-2) was given to control animals for 21 days. At the end of each feeding period, blood was collected from the heart in a heparinized syringe under ether anesthesia, then rats were perfused with cold saline. Blood samples were centrifuged at 3,000 × *g* for 10 min to separate plasma. A portion of the liver (*ca.* 1 g) and one kidney (left side) were used in analysis of MT levels. Another portion of the liver (*ca.* 1 g) and another kidney (right side) were kept at –80°C for determination of the Fe and Zn contents. For MT analysis, the freshly obtained tissues were homogenized in ice-cold 1.15% KCl under N_2 atmosphere, and assayed for MT levels using the Cd^{2+}–Hg^{2+} saturation method according to Naganuma *et al.*[10] with a slight modification.[11] Briefly, the homogenate (1 ml) was treated successively with diethylmalate (5 μl) and 10 mM $CdCl_2$ (25 μl), and heated at 95°C for 5 min to precipitate high-molecular weight proteins. Following cooling and centrifugation, 0.5 ml of the supernatant was successively treated with 5 mM $HgCl_2$ (25 μl), 1 mM ovalbumin (225 μl), and 12.5% TCA (250 μl). After centrifugation, the supernatant was filtered through a membrane of 0.22-μm pore diameter (Ultrafree C3, Millipore). The total MT levels were determined by analyzing the Hg concentration in the final filtrate. Hg concentrations were determined by the oxygen combustion-gold amalgamation method[12] using an atomic absorption spectrophotometer MD-A (Nippon Instruments Co. Ltd., Osaka, Japan). MT levels were expressed as the amount of Hg bound to the thionein molecule. Fe and Zn levels in liver and kidney were determined by plasma emission analysis using a Spectraspan IIIB emission spectrometer (Spectrametrics Inc., Andover, MA, U.S.A.) after digestion in concentrated HNO_3. Plasma Fe and Zn levels were determined using the commercially available assay kits Fe C-Test Wako and Zn-Test Wako (Wako Pure Chemical Co.), respectively.

Since the feeding period of 7 days on the Fe-diet was considered to be long enough to cause Fe-induced alteration in rat tissue MT levels, at least in the kidney, these rats were used as a Fe-loaded model in the following experiments.

Hybridization Analysis of MT mRNA in the Kidney ——— Kidney samples for MT mRNA analysis (0.06–0.08 g of the cortical portions) were removed from the two dietary groups of rats. Total RNA was isolated by a modified acid guanidium-phenol extraction method[13] using an RNA isolation reagent ISOGEN (Nippongene, Tokyo, Japan) according to the protocol recommended by the manufacturer. Yield and purity of the RNA samples were determined by absorbance at 260 nm and absorbance ratio A_{260}/A_{280} (1.80–1.95). RNA samples (10 μg each) thus prepared were diluted in 0.2 ml of H_2O/20 × SSC/37% formaldehyde (2 : 1 : 1; 1 × SSC is 0.15 M NaCl, 15 mM sodium citrate), then incubated at 65°C for 15 min. The samples were loaded in a slot-blot apparatus (Nippon Bio-Rad Laboratories, Tokyo, Japan) onto a Clear Blot Membrane-N (Atto Co., Tokyo, Japan). MT mRNA on the membrane was analyzed by a solution hybridization procedure described by Hamilton *et al.*[14] using α-^{32}P-dCTP and mouse MT-I cDNA as a probe. The density of each slot was determined using a laser densitometer UltroScan XL (Pharmacia LKB Biotechnology, Uppsala, Sweden). The MT mRNA level was estimated by comparison with β-actin mRNA level.

Renal Dysfunction and MT Induction by $HgCl_2$ Injection ——— To examine a renal responsiveness against $HgCl_2$, two dietary groups (12 for each group) of rats were injected intravenously $HgCl_2$ at a dose level of 0, 0.5, or 1 mg/kg. Twenty-four hours after the injection, blood samples were collected from the heart under ether anesthesia. After centrifuging at 10000 × *g* for 1 min, the plasma samples obtained

Table 1. Effects of Fe Loading on Rat Tissue Fe and Zn Levels

Tissue	Day 0	Day 3	Day 7	Day 2
Liver				
Fe (μg/g)	140 ± 10	195 ± 15**	485 ± 90**	1010 ± 250**
Zn (μg/g)	28.4 ± 1.0	24.4 ± 0.3**	28.8 ± 4.0	34.0 ± 1.8**
Kidney				
Fe (μg/g)	27.0 ± 1.5	31.5 ± 3.0**	44.5 ± 8.8**	55.2 ± 4.8**
Zn (μg/g)	13.2 ± 0.5	11.2 ± 0.3**	12.6 ± 1.2	12.0 ± 0.80
Plasma				
Fe (μg/ml)	4.22 ± 0.88	9.60 ± 0.91**	6.84 ± 0.65**	3.54 ± 0.89
Zn (μg/ml)	143 ± 12	102 ± 10**	132 ± 6*	151 ± 7

Rats were fed on a powdered diet (CE-2, CLEA Japan) containing 3.5% Fe(II) fumarate (1.2% as Fe) for 3, 7 and 21 days, and tissue Fe and Zn levels were determined. Each value represents mean ± S.D. obtained from 5 rats. Significant differences from control rats were shown by * ($p < 0.05$) and ** ($p < 0.01$).

were subjected to HPLC analysis to determine creatinine levels. An aliquot of the plasma sample was eluted with 25 mM sodium phosphate buffer (pH 3.65, 2.5% acetonitrile) using a Waters SCX cation exchange column, and absorption at 215 nm was determined.

In another experiment, 12 rats in each dietary group were intravenously injected with 0.5 mg/kg of $HgCl_2$. Four rats in each group were sacrificed on days 1, 3 and 5 to excise a kidney. MT levels in the kidney samples were determined as above.

Effect of Vitamin E on Fe-Induced Alterations of Lipid Peroxidation and MT Levels —— Two groups of rats (5 in each group) were fed on an Fe-containing diet for 7 days. One group was orally administered α-tocopherol acetate (25 μl/kg/day) as an olive oil solution (10 μl/ml) during the 7-day Fe-loading period. The other group was given olive oil (2.5 ml/kg/day) as a control. Twenty-four hours after the final administration, rats were sacrificed and liver and kidney samples were assayed for MT levels as described above. TBA-reactive substances (TBA-RS) levels in each tissue were determined according to Ohkawa *et al.*[15] using tetraethoxypropane as an external standard.

Effect of Fe-Loading on Tissue Glutathione Level and its Half-Life —— Two groups of rats (5 for each group), the 7-day Fe-loading and normal diet groups, were sacrificed under ether anesthesia, then liver and kidney samples were immediately homogenized in ice-cold 5% perchloric acid containing 1 mM EDTA. Total glutathione levels in the samples thus obtained were determined according to an enzymic recycling method.[16]

RESULTS AND DISCUSSION

Feeding rats on 3.5% Fe(II) fumarate diet was reported as a procedure for effective Fe accumulation in the liver.[8] In the present study, the hepatic Fe levels increased up to 7.2-fold of the control group after 21 days (Table 1). The increase in the renal Fe levels was more moderate than in the liver levels. Interestingly, the plasma levels showed a maximum on day 3, and declined to control levels by the end of the experiment. Since the excess amount of Fe in the diet was expected to alter Zn absorption, tissue and plasma Zn levels were examined. Storey and Greger[17] demonstrated that an excess of Fe in the diet did not affect Zn absorption and its tissue levels. In the present study, however, Zn levels in the liver, kidney and plasma showed a transient decrease at the early phase of the experiment, although the levels recovered at the later phase (Table 1). The excess amount of Fe in the diet might transiently inhibit Zn absorption in the small intestine.

Induction of MT in the liver and kidney was reported in animals exposed to heavy metals such as Cd and Hg.[18,19] In contrast to these heavy metals, the effects of Fe on tissue MT levels are not so well established. A few investigators have reported that intraperitoneal injection of $FeCl_3$ caused increases of hepatic MT levels in experimental animals.[5,6] Fleet *et al.*[5] showed, however, that oral administration of Fe caused no effect on the hepatic MT levels in chickens, despite a significant accumulation of Fe in the liver. They suggested parenteral Fe injection might cause MT induction *via* stress-induced changes in adrenal cortical hormone secretion. However, the present study demonstrated that feeding on the Fe-enriched diet brought about significant alter-

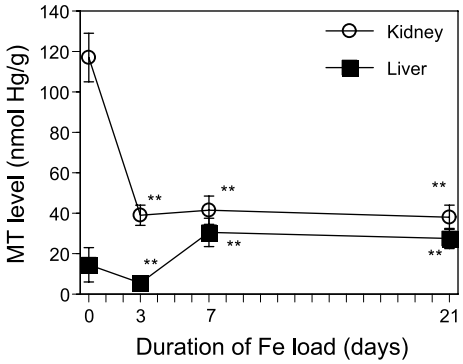

Fig. 1. Liver and Kidney Metallothionein Levels of Fe-Overload Rats

Rats were fed a powdered diet (CE-2, CLEA Japan) containing 3.5% Fe(II) fumarate (1.2% as Fe) for 3, 7 and 21 days, and MT levels in the liver and kidney were determined. Each value represents mean ± S.D. obtained from 5 rats. **Significantly different from day 0 control ($p < 0.01$).

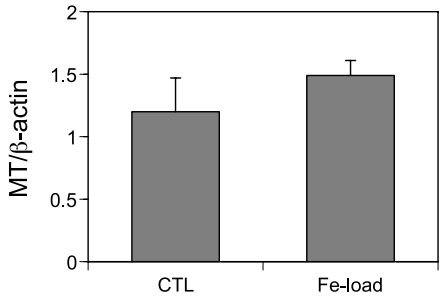

Fig. 2. Quantification of MT mRNA in the Kidney of Fe-Overload Rats

Total RNA prepared from the kidney of Fe-loaded rats for 7 days was loaded onto a membrane using a slot-blot apparatus, and hybridized with mouse MT-I cDNA. Each value represents mean ± S.D. obtained from three rats in relation to β-actin mRNA amounts.

ations of MT levels both in the liver and kidney with concomitant Fe accumulation. The model rats showed, following a transient decrease on day 3, a significant increase in the hepatic MT levels on day 7 and later (Fig. 1), by the oral Fe uptake. This result suggested that Fe accumulation in the liver, even when taken up orally, might be sufficient for an increase in the tissue MT, at least in rats. The time–dependent alteration in the hepatic MT levels was in fair agreement with that in the tissue Zn levels (Table 1), suggesting that the hepatic MT levels might, at least partly, be controlled by the tissue Zn levels, which were affected by the Fe-loading.

In contrast to the hepatic MT, the renal MT showed quite a different feature. The renal MT levels in the Fe-loaded rats decreased to 1/3 of the initial level within 3 days of Fe-feeding, and the lowered level was quite stable during the following period until day 21 (Fig. 1). Since Zn in this tissue had already recovered to the control level on day 7 (Table 1), the renal MT levels seemed to be controlled by factors other than tissue Zn levels in the Fe-fed rats. For the lowered renal MT level, suppressed synthesis and/or enhanced turnover rate were expected. To examine an effect of Fe loading on MT synthesis in the kidney, MT mRNA levels in rats fed on an Fe diet for 7 days were compared with control rats. Hybridization analysis of the renal MT mRNA revealed that Fe feeding caused a slight increase in the messenger levels (Fig. 2). This result indicated that MT biosynthesis in the kidney would never be lowered by the Fe overload. To obtain information on the turnover rate of the renal MT, rats of both dietary groups were intravenously injected with 0.5 mg/kg of $HgCl_2$ to induce MT in the kidney. The renal MT contents in both groups increased to a similar level at 24 hr after injection, despite the initial lower levels in the Fe-loaded group (Fig. 3). This suggested that, despite the depressed levels, the renal MT in Fe-fed rats still showed a normal response against $HgCl_2$ invasion. It should be noted, however, that the elevated MT levels in the Fe-loaded group began to decline on day 5 after $HgCl_2$ injection, when the levels in control rats were still at a plateau (Fig. 3). This result suggests that Fe feeding might enhance the turnover rate of the renal MT, and this might account for its reduced level. The enhanced turnover rate of the renal MT might compensate, at least partly, for the lowered level by accelerating the elimination of MT-bound xenobiotics.

MT functions to suppress the toxicity of heavy metals, such as Cd and Hg, by chelating them. In this context, the kidney of Fe-fed rats might show higher susceptibility to nephrotoxic heavy metals due to its lowered MT levels. To examine this possibility, a toxic dose of $HgCl_2$ (0.5 and 1 mg/kg) was injected into the rats, and plasma creatinine levels were examined 24 hr after the injection as a marker of renal dysfunction. Rats of both dietary groups showed a dose–dependent increase in plasma creatinine levels after $HgCl_2$ treatment (Fig. 4). The increasing rates were somewhat higher in the Fe-fed group than in the control. Twenty-four hours after $HgCl_2$ treatment of 1 mg/kg, the creatinine increased to 13.3 (4.3-fold of the non-treated group) and 16.7 μg/ml (6.9-fold of the non-treated group) in the control and Fe-fed rats, respectively. These results suggested that the Fe-fed rats showed slightly higher susceptibility against $HgCl_2$ nephrotoxic action than

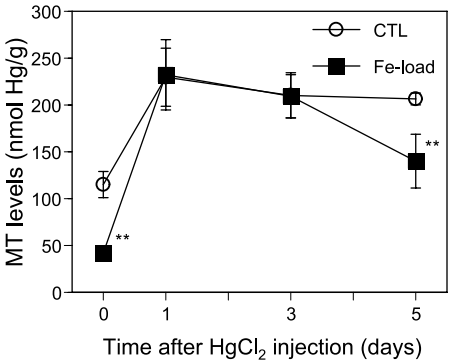

Fig. 3. Time–Depend Alterations of the Renal MT Levels in $HgCl_2$-Treated Rats

Control and Fe-loaded (7 days) rats were intravenously injected with 0.5 mg/kg of $HgCl_2$. On days 1, 3 and 5, MT levels in the kidney were determined. Each value represents mean ± S.D. obtained from 4 rats. **Significantly different from control rats ($p < 0.01$).

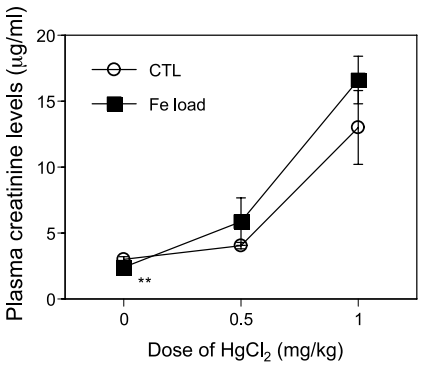

Fig. 4. Effect of $HgCl_2$ on Plasma Creatinine Levels in Fe-Loaded Rats

Twenty-four hours after intravenous $HgCl_2$ injection of 0.5 and 1.0 mg/kg to control and Fe-loaded (7 days) rats, creatinine levels in plasma were determined by HPLC analysis using a Waters SCX column. **Significantly different from control diet group ($p < 0.01$).

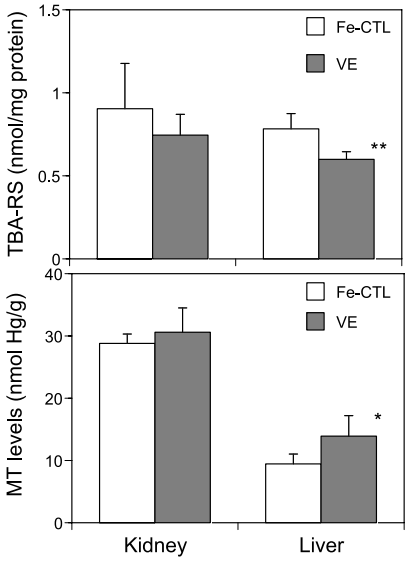

Fig. 5. Effects of α-Tocopherol on MT and TBA-RS Levels in the Liver and Kidney of Fe-Loaded Rats

Two groups of rats were fed on an Fe-containing diet for 7 days. One group was orally administered α-tocopherol acetate (25 μl/kg/day) as an olive oil solution (10 μl/ml) during the Fe-loading period. The other group was given olive oil (2.5 ml/kg/day) as a control. Twenty-four hours after the final administration, rats were sacrificed and excised tissue samples were assayed for MT and TBA-RS levels. Each value represents mean ± S.D. obtained from 5 rats. Significantly differences from control rats were shown by *($p < 0.05$) and **($p < 0.01$).

control rats, probably due to the suppressed renal MT level. Although the enhanced turnover rate might contribute to protection from $HgCl_2$ nephrotoxicity, it would not be sufficient to compensate for the lowered level.

Feeding rats on a 3.5% Fe(II) fumarate diet caused enhanced oxidative status in liver and kidney indicated by increased TBA-RS levels, as well as tissue Fe accumulations.[9] To examine a possible contribution of oxidative stress on Fe-induced alterations of tissue MT, rats were injected with α-tocopherol acetate during the Fe-feeding period to suppress the oxidative stress. The vitamin suppressed lipid peroxide levels, but increased MT levels in the liver (Fig. 5). However, the effect on the kidney was not as significant as on the liver. The effects on the liver might suggest that Fe-induced alteration of the tissue MT seemed be separate from oxidative stress. Sato and Sasaki[20] reported similar results that vitamin E treatment did not affect hepatic MT levels which were induced by paraquat or CCl_4 administration in rats, though their TBA-RS levels were reduced by the vitamin treatment. They suggested that enhanced lipid peroxidation was not necessary for induction of MT synthesis. Although MT is well known to be induced by oxidative stress, acceleration of lipid peroxidation seems to be not directly linked with MT induction in the liver of their animal models and the present Fe-fed rats. Oxidative status at the cytosolic environment in which MT functions may not necessarily depend on the membranous environment to which α-tocopherol would participate.

Glutathione, the most abundant nonprotein thiol in the cytosolic compartment, serves an important role in the protection against heavy metal toxicity and oxidative stress, as does MT.[21,22] Elevation of tissue glutathione levels in response to oxidative challenge was shown in various organisms.[23–25] In this context, the glutathione level was expected to increase in response to the reduced renal MT level

Table 2. Effects of Fe Loading on Tissue Glutathione Levels in Fe-Diet Fed Rats

Animal group	Fe-loaded	Control
Kidney (mM)	2.81 ± 0.25	2.53 ± 0.30
Liver (mM)	7.34 ± 0.29	8.26 ± 0.29

Each value represents mean ± S.D. obtained from 5 rats.

or enhanced oxidative stress in the liver of Fe-fed rats. To examine the possibility of modulation in the tissue glutathione status in response to altered MT levels and oxidative stress, its tissue levels were determined (Table 2). Contrary to expectation, Fe-loading caused no change in the level of the tripeptide either in the liver or kidney. Enhanced oxidative status and reduced MT levels as a result of Fe-overload would not be so hazardous as to disturb glutathione metabolism in either tissue.

Many reports using experimental animals are published on MT induction by various kinds of stimulation, such as heavy metals, oxidative stress and cytokines. However, finding that a decrease in its levels without appreciable adverse effects on the animals is very few. Despite a 1/3 MT level in the kidney, the Fe-loaded rats showed no sign of renal dysfunction as indicated by plasma creatinine levels. Furthermore, the body weight of the rat, following a transient and slight loss at the initial 24 hr, maintained about 90% of the control animal weight for 3 weeks (data not shown). These results suggested that the lowered renal MT caused by Fe feeding might be of a sufficient level to keep homeostasis in the kidney. Although the Zn-deficient rat model presented by Sato and Nagai[26] showed lower liver and kidney MT levels than the Zn-adequate rat, its body weight was about half that of the Zn-adequate rat after 4 weeks. The present Fe-loaded rat may be a unique model with a reduced MT level limited to the kidney.

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REFERENCES

1) Sato, M. and Bremner, I. (1993) Oxygen free radicals and metallothionein. *Free Radic. Biol. Med.*, **14**, 325–337.
2) Galleano, M. and Puntarulo, S. (1992) Hepatic chemiluminescence and lipid peroxidation in mild iron overload. *Toxicology*, **76**, 27–38.
3) Carthew, P., Dorman, B. M., Edwards, R. E., Francis, J. E. and Smith, A. G. (1993) A unique rodent model for both the cardiotoxic and hepatotoxic effects of prolonged iron overload. *Lab. Invest.*, **69**, 217–222.
4) Good, M. and Vašák, M. (1986) Iron(II)-substituted metallothionein: evidence for the existence of iron-thiolate clusters. *Biochemistry*, **25**, 8353–8356.
5) Fleet, J. C., Andrews, G. K. and McCormick, C. C. (1990) Iron-induced metallothionein in chick liver: a rapid, route-dependent effect independent of zinc status. *J. Nutr.*, **120**, 1214–1222.
6) Günther, T., Grossrau, R., Höllriegl, V. and Vormann, J. (1991) Effects of Fe, salicylate and Zn on metallothionein and lipid peroxidation *in vivo*. *J. Trace Elem. Electrolytes Health Dis.*, **5**, 95–100.
7) Bacon, B. R., Tavill, A. S., Brittenham, G. M., Park, C. H. and Recknagel, R. O. (1983) Hepatic lipid peroxidation in vivo in rats with chronic iron overload. *J. Clin. Invest.*, **71**, 429–439.
8) Younes, M., Eberhardt, I. and Lemoine, R. (1989) Effect of iron overload on spontaneous and xenobiotic-induced lipid peroxidation *in vivo*. *J. Appl. Toxicol.*, **9**, 103–108.
9) Yasutake, A., Adachi, T., Suda, I. and Hirayama, K. (1993) Effect of Fe-overload on the biotransformation of methylmercury in rat. *Jpn. J. Toxicol. Environ. Health*, **39**, 106–113.
10) Naganuma, A., Satoh, M. and Imura, N. (1987) Prevention of lethal and renal toxicity of *cis*-diamminedichloroplatinum(II) by induction of metallothionein synthesis without compromising its antitumore activity in mice. *Cancer Res.*, **47**, 983–987.
11) Yasutake, A., Nakano, A. and Hirayama, K. (1998) Induction by mercury compounds of brain metallothionein in rats: Hg^0 exposure induces long-lived brain metallothionein. *Arch. Toxicol.*, **72**, 187–191.
12) Ohkawa, T., Uenoyama, H., Tanida, K. and Ohmae, T. (1977) Ultra trace mercury analysis by dry thermal decomposition in alumina porcelain tube. *J. Hyg. Chem.*, **23**, 13–22.
13) Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA by acid guanidium thiocyanate-phenol-chloroform extracion. *Anal. Biochem.*, **162**, 156–159.
14) Hamilton, J. W., Bement, W. J., Sinclair, P. R., Sinclair, J. F. and Wetterhahn, K. E. (1988) Expression of 5-aminolaevulinate synthase and cytochrome P-450 mRNA in chicken embryo hepatocytes *in vivo* and in culture. *Biochem. J.*, **255**, 267–375.
15) Ohkawa, H., Ohishi, N. and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric

acid reaction. *Anal. Biochem.*, **95**, 351–358.
16) Tietze, F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.*, **27**, 502–522.
17) Storey, M. L. and Greger, J. L. (1987) Iron, zinc and copper interactions: chronic versus acute responses of rats. *J. Nutr.*, **117**, 1434–1442.
18) Cherian, M. G. and Goyar, R. A. (1978) Metallothioneins and their role in the metabolism and toxicity of metals. *Life Sci.*, **23**, 1–10.
19) Webb, M. and Cain, K. (1982) Function of metallothionein. *Biochem. Pharmacol.*, **31**, 137–142.
20) Sato, M. and Sasaki, M. (1991) Enhanced lipid peroxidation is not necessary for induction of metallothionein-I by oxidative stress. *Chem.-Biol. Interact.*, **78**, 143–154.
21) Meister, A. and Anderson, M. E. (1983) Other aspect of glutathione function. *Ann. Rev. Biochem.*, **52**, 711–760.
22) Brambilla, G., Carlo, P. and Finollo, R. (1992) Effect of ten thiocompounds on rat liver DNA damage induced by a small dose of N-nitrosodimethylamine. *Arch. Toxicol.*, **66**, 286–290.
23) Woods, J. S., Davis, H. A. and Baer, R. P. (1992) Enhancement of γ-glutamylcyteine synthetase and mRNA in rat kidney by methyl mercury. *Arch. Bipchem. Biophys.*, **296**, 350–353.
24) Yasutake, A. and Hirayama, K. (1994) Acute effects of methylmercury on hepatic and renal glutathione metabolism in mice. *Arch. Toxicol.*, **68**, 512–516.
25) Taniguchi, M. (1997) Effects of N-nitrosodimethylamine on glutathione levels during development of chick-embryo. *Chem.-Biol. Interact.*, **105**, 65–75.
26) Sato, M. and Nagai, Y. (1989) Effect of zinc deficiency on the accumulation of metallothionein and cadmium in rat liver and kidney. *Arch. Environ. Contam. Toxicol.*, **18**, 587–593.