J. Biochem. Mol. Biol. Vol. 28, No. 6 pp. 490~493 November 30, 1995

The Effect of Hydrogen Peroxide-Treated Metallothionein on the Hepatic Xanthine Oxidase Activity

Keun Huh*, Uk Seob Shin and Sang Il Lee[1]

Department of Pharmacology, College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea
[1]Department of Microbiology & Immunology, Tulane University Medical Center, New Orleans, Louisiana 70112-2699, USA

(Received July 6, 1995)

Abstract: We investigated the effect of hydrogen peroxide-treated metallothionein on the hepatic xanthine oxidase activity *in vitro*. When the metallothionein was preincubated with 1 mM of hydrogen peroxide, the activity of xanthine oxidase and type conversion were elevated dose-dependently by the addition of metallothionein into the reaction mixture. While increasing the treatment of hydrogen peroxide to the 50 μg of metallothionein, the xanthine oxidase activity and type conversion ratio were remarkably elevated dose dependently compared to the control. When cadmium ion was added to the reaction mixture, the increasing pattern of the enzyme activity was similar to the effect of hydrogen peroxide-treated metallothionein. DTT or penicillamine restored the increasing activity and type conversion of xanthine oxidase by the cadmium ion to the control level.

Key words: cadmium ion, hydrogen peroxide, metallothionein, xanthine oxidase.

Metallothionein is present in numerous animal tissues and is a group of low molecular weight proteins, with an unique amino acid composition, such as a large content (approximately 33%) of cysteine residue, which endows metallothionein with a very high affinity for heavy metals such as zinc, copper and cadmium (Hamer, 1986). Another unique property of metallothionein is its inducibility upon exposure to these heavy metals as well as in response to various exogenous xenobiotics and endogenous factors (Andrews, 1990). According to these chemical properties of metallothionein, much attention has been given to the physiological role of metallothionein in the detoxification of harmful heavy metals such as cadmium and mercury as well as to its involvement in the absorption, storage and transport of essential metals, but no definitive conclusion has been reached on metallothionein functions (Juan *et al.*, 1988).

Recently, it was reported that the primary determinant of metallothionein protection appeared to be metal release and subsequent uptake by the membranes (Thomas *et al.*, 1986). These results have important implications for the antioxidant role of metallothionein, a metalloprotein known to be induced by various prooxidant conditions. An intriguing possibility was that upon metal release, metals might act as a compensatory messenger of oxidative stress, stimulating some factor in the enhancer region of the metallothionein gene (Andrews, 1990). It was also reported that metallothionein was susceptible to intracellular degradation depending on the aging process, related to oxidative stress or chemical pretreatment, and that copper-metallothionein may have a prooxidative property, and tissues with high copper-metallothionein levels may be particulary susceptible to oxidative stress (Zacharias and Edmund, 1991). However, the prooxidant mechanism of metallothionein was not fully explained.

In this study, the mechanism of the prooxidative action of hydrogen peroxide-treated metallothionein was investigated with regard to the xanthine oxidase activity and type conversion of this enzyme.

Materials and Methods

Chemicals and Animals

Cadmium chloride, hydrogen peroxide, metallothionein (Cd, Zn-metallothionein), xanthine sodium salt, bovine serum albumin (BSA), nicotinamide adenine dinucleotide (NAD), dithiothreitol (DTT), penicillamine were obtained from Sigma Chemical Co. (St. Louis, USA). All other reagents were of the highest quality available. Male Sprague-Dawley rats (250~280 g) were purchased from the center of Life Science (Taegu, Korea). Rats

*To whom correspondence should be addressed.
Tel: 82-53-810-2814, Fax: 92-53-811-3871.

were maintained in the chambers at a temperature of 22± 2℃ and a 12 h/12 h (light/dark) cycle on the standard diet and water ad libitum.

Enzyme preparation

Male rats weighing 250~280 g were killed by exsanguination from the abdominal aorta under light ether anesthesia. The liver was exhaustively perfused with ice-cold saline through the portal vein until uniformly pale, and immediately removed and weighed. After being trimmed and minced, the pieces of liver were homogenized with 4 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) solution. The homogenate was centrifuged at 10,000×*g* for 20 min. The supernatant was further centrifuged at 105,000×*g* for 60 min. The resultant cytosolic fraction was used at the enzyme sources of xanthine dehydrogenase and xanthine oxidase assay.

Enzymatic assay of xanthine dehydrogenase

The xanthine dehydrogenase activity was assayed by measuring spectrophotometrically the amount of uric acid formed from xanthine sodium with NAD^+ as electron acceptor in the incubation mixture (Stirpe *et al.*, 1969).

Enzymatic assay of xanthine oxidase activity and type conversion

Xanthine oxidase activity was aerobically determined by measuring the rates of uric acid formation without NAD^+ from xanthine sodium as substrate in the reaction mixture (Stirpe *et al.*, 1969). The reaction mixture contains 0.1 M potassium phosphate buffer (pH 7.5), enzyme solution (2~4 mg protein) and 0.06 mM of the substrate in a final volume. The ratio of type conversion from xanthine dehydrogenase (type D) to xanthine oxidase (type O) is representated as type O/type D+O.

Protein determination

Protein content (Lowry *et al.*, 1951) was determined using bovine serum albumin as a standard.

Results and Discussion

Effect of hydrogen peroxide-treated metallothionein on the xanthine oxidase activity and type conversion

It is widely accepted that oxygen free radical production and its consequences, such as lipid peroxidation and protein damage, appear to play a major role in the pathogenesis of injuries after ischemia and particulary reperfusion (Comporti, 1985; Lunec, 1990; Hirafuji and Ogura, 1985). Oxygen free radicals can be produced by various sources such as oxidizing enzymes (Batteli *et al.*, 1972; Mackelvey *et al.*, 1988; Ross, 1989; Tubaro *et al.*, 1980). McCord (1983) demonstrated that xanthine dehydrogenase was converted to xanthine oxidase in pathophysiological conditions, and has hypothesized that the oxygen free radical production of xanthine oxidase is an important mechanism of cellular injury.

The changes in the xanthine oxidase activity and type conversion by varying concentrarions of metallothionein previously subjected to oxidative stress by hydrogen peroxide (1 mM) was shown in Table 1. Compared to the control, the activity of xanthine oxidase was elevated remarkably by increasing the amount of metallothionein treated with hydrogen peroxide. When 50 μg/ml of hydrogen peroxide-treated metallothionein

Table 1. The effect of hydrogen peroxide-treated metallothionein on the hepatic xanthine oxidase activity and type conversion *in vitro*

Dose of metallothionein (μg/ml)	Specific activity[a]		Type conversion ratio (%)
	Type O	Type D+O	
Vehicle	0.476	2.937	16.2
0	0.611	2.940	20.8
5	0.843	2.937	28.7
10	1.514	2.958	51.2
20	1.997	2.972	67.2
50	2.824	2.998	94.2
100	3.108	3.108	100.0

Each concentration of metallothionein (0~100 μg/ml) treated with H_2O_2 (1 mM) were added in reaction mixture. The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments.
[a]Uric acid nmol/mg protein/min.

Table 2. The effect of the treatment concentration of hydrogen peroxide to metallothionein on hepatic xanthine oxidase activity and type conversion *in vitro*

Dose of H_2O_2 (mM)	Specific activity[a]		Type conversion ratio (%)
	Type O	Type D+O	
Vehicle	0.476	2.937	16.2
0	0.527	2.976	17.7
0.1	0.793	2.992	26.5
0.5	1.271	2.994	42.5
1.0	2.824	2.998	94.2
2.0	2.998	2.998	100.0

Additional concentration of metallothionein in reaction mixture was 50 μg/ml. The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments.
[a]Uric acid nmol/mg protein/min.

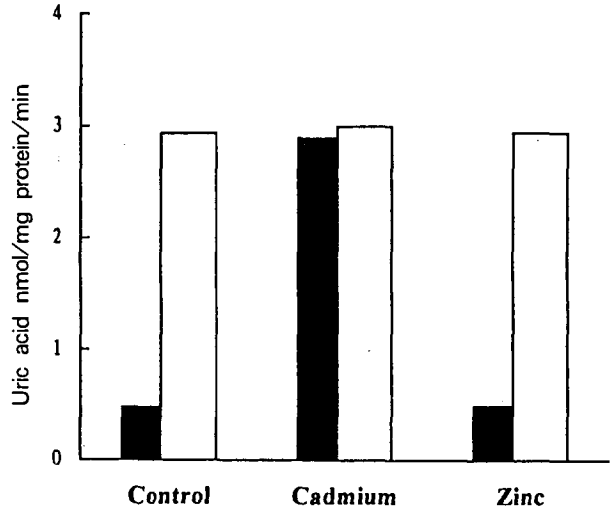

Fig. 1. The effect of cadmium or zinc on the hepatic xanthine oxidase activity *in vitro*. The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments. Cd^{+2} (3.35 μg/ml) or Zn^{+2} (0.25 μg/ml) was added in the reaction mixture which correspond with 50 μg/ml of metallothionein. ■ : xanthine oxidase (type O), □ : xanthine oxidase+xanthine dehydrogenase (type D+O).

was added to the reaction mixture, the activity was 2.824 nmol and this was four times higher than the control level. Type conversion from xanthine dehydrogenase to xanthine oxidase was potentiated in proportion to the concentration of hydrogen peroxide-treated metallothionein. Table 2 showed the change of xanthine oxidase activity and type conversion by 50 μg/ml of metallothionein treated with various concentrations of hydrogen peroxide. By increasing the concentration of hydrogen peroxide, xanthine oxidase activity and type conversion were increased compared with control. At more than 1mM concentration of hydrogen peroxide, xanthine dehydrogenase (type D) was completely converted into xanthine oxidase(type O).

These results suggested that the treatment with hydrogen peroxide may induce conformational changes in the chemical structure of metallothionein, along with cadmium or zinc ion released from metallothionein. So, we assume that these released free metal ions may facilitate the induction of xanthine oxidase activity and type conversion.

Effect of cadmium or zinc ion on the xanthine oxidase activity and type conversion

Metallothionein is generally recognized to possess antioxidant properties and is a metal binding protein that is synthesized in the liver and kidney and other major organs in response to certain metal ions such as zinc, cadmium and copper (Kögi and Vallee, 1961; Kögi *et al.*, 1974; Suntres and Lui, 1991). In these studies, we focused on zinc and cadmium ion-containing metallothionein to examine the role of hydrogen peroxide-treated metallothionein in the enhancement of xanthine oxidase activity and type conversion. When cadmium and zinc ion corresponded to 50 μg/ml of metallothionein in the reaction mixture, the change in xanthine oxidase activity occurred as shown in Fig. 1. In the control, the type O (xanthine oxidase) activity was 0.476 nmol. When cadmium ion was added to the reaction mixture, the activity was 2.900 nmol, which was more than 6 times that of the control level. When zinc ion was added, there was no change in the activity level. Total type (D+O) activity was not affected by cadmium or zinc ion in any case. When the incubating reaction mixture contained cadmium ion, the activity of xanthine oxidase and type conversion ratio occurred dose-dependently (see Table 3). By the addition of cadmium ion in doses from 0.34 μg/ml to 6.70 μg/ml (from 5 μg to 100 μg measured as metallothionein) in the assay mixture, the xanthine oxidase activity and type conversion were remarkably increased compared to the control level and its pattern was dose-dependent. These results indicated that the released cadmium ion from metallothionein might influence the xanthine oxidase activity and type conversion. It is postulated that xanthine dehydrogenase (type D) is very rapidly converted to the xanthine oxidase (type O) which dose produce the superoxide anion or hydroxyl radicals during pathophysiological conditions such as ischemia or hypoxia (McCord and Roy, 1982; Roy and McCord, 1982; McCord, 1983). Thus, we assume that the effect of cadmium ion on the xanthine oxidase activity and type conversion is very closely related to the progress of cadmium toxicity by oxygen species radicals-induced oxygen stress in the body.

Table 3. The effect of Cd^{+2} on the heaptic xanthine oxidase activity and type conversion *in vitro*

Dose of Cadmium (μg/ml)	Specific activity[a]		Type conversion ratio (%)
	Type O	Type D+O	
0	0.476	2.937	16.2
0.34	0.942	2.944	32.0
0.67	1.608	2.962	54.3
1.34	2.092	2.984	70.1
3.35	2.900	2.998	96.7
6.70	3.128	3.128	100.0

The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments.
[a]Uric acid nmol/mg protein/min.

Effect of DTT or penicillamine on Cd^{+2}-induced xanthine oxidase activity and type conversion

Table 4. The effect of dithiothreitol or penicillamine on the Cd^{+2} -induced xanthine oxidase activity *in vitro*

	Specific activity[a]	
	Type O	Type D+O
Control	0.476	2.937
CD	2.900	2.998
DT	0.506	2.944
PC	0.459	2.942
CDD	0.528	2.939
CDP	0.600	2.920

The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments. CD : cadmium ion (3.35 μg/ml of reaction mixture), DT : dithiothreitol (10 mM), PC : penicillamine (10 mM), CDD : cadmium + dithiothreitol, CDP : cadmium + penicillamine.
[a]Uric acid nmol/mg protein/min.

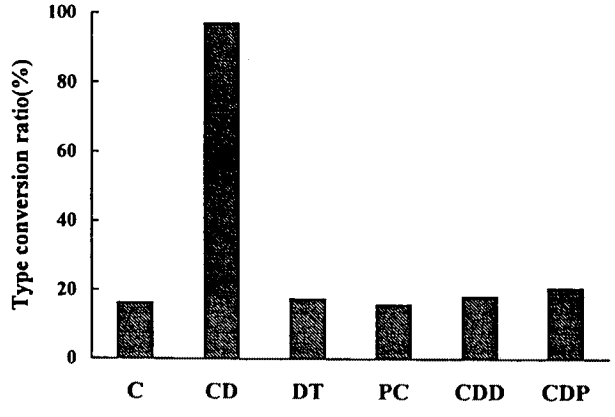

Fig. 2. The effect of dithiothreitol or penicillamine on the Cd^{+2} -induced type conversion of xanthine oxidase *in vitro*. The assay procedure was described in the experimental methods. Values are mean for 3 separate experiments. C : control, CD : Cd^{+2} (3.35 μg/ml), DT : dithiothreitol (10 mM), PC : penicillamine (10 mM), CDD : Cd^{+2} + dithiothreitol, CDP : Cd^{+2} + penicillamine.

We measured that the effect of DTT or penicillamine on the cadmium-induced activity and type conversion of xanthine oxidase. As shown in table 4 and Fig. 2, the addition of cadmium ion (3.35 μg/ml) markedly enhanced the xanthine oxidase activity and type conversion compared to the control. In contrast, when DTT (10 mM) or penicillamine (10 mM) was added into this reaction mixture, the resulting increment of the activity and type conversion of xanthine oxidase by the cadmium ion was reduced to the control level. The effect of DTT or penicillamine on the inducing activity and type conversion of xanthine oxidase by the hydrogen peroxide-treated metallothionein is similar to its effect on the induction due to cadmium ion (data not shown). These results indicated that penicillamine acts as a direct cadmium chelator in the xanthine oxidase activity and type conversion by cadmium ion.

Therefore, we conclude that the lability of metallothionein may lead to release of cadmium ion due to oxidative stress and releasing free cadmium ion from metallothionein may induce the activation of xanthine oxidase which seems to be related strongly to the free radicals generating process, and may have a prooxidant function.

Acknowledgement

This study was partially supported by research grants from the Academic Promotion Fund, Korea Science and Engineering Foundation(KOSEF 91-07-00-13).

References

Andrews, G. K. (1990) *Prog. Food Nutri. Sci.* **14**, 193.
Battelli, M. G., Della Corte, E. and Stirpe, F. (1972) *Biochem. J.* **126**, 747.
Comporti, M. (1985) *Lab. Invest.* **53**, 599.
Hamer, D. H. (1986) *Annu. Rev. Biochem.* **55**, 913.
Hirafuji, M. and Ogura, J. (1985) *Biochem. Pharmacol.* **34**, 933.
Juan, H., LLuis, C., Marta, B., Justine, S. G. and Antonio, A. (1988) *Am. J. Physiol.* **255**, E518.
Kögi, J. H. R., Himmelhoch, S. R., Whanger, P. D., Bethune, J. L. and Vallee, B. L. (1974) *J. Biol. Chem.* **249**, 3537.
Kögi, J. H. R. and Vallee, B. L. (1961) *J. Biol. Chem.* **236**, 2435.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
Lunec, J. (1990) *Annu. Clin. Biochem.* **27**, 173.
Mackelvey, T. C., Hollworth, M. E., Granger, D. N., Urikelander, T. D. and Jones, H. (1988) *Am. J. Physiol.* **254**, G753.
McCord, J. M. (1983) *Surgery* **94**, 412.
McCord, J. M. and Roy, R. S. (1982) *Can. J. Physiol. Pharmacol.* **60**, 1346.
Ross, D. (1989) *J. Pharm. Pharmacol.* **41**, 505.
Roy, R. S. and McCord, J. M. (1982) *Fedration Proc.* **41**, 767.
Stirpe, F. and Della Corte, E. (1969) *J. Biol. Chem.* **244**, 3855.
Suntres, Z. E. and Lui, E. M. K. (1991) *J. Pharmacol. Exp. Ther.* **258**, 797.
Thomas, J. P., Bachowski, G. J. and Girotti, A. W. (1986) *Biochim. Biophys. Acta.* **884**, 448.
Tubaro, E., Lotti, B., Croce, C., Caballo, G. and Borelli, G. (1980) *Biochem. Pharmacol.* **29**, 1939.
Zacharias, E. S. and Edmund, M. K. L. (1991) *J. Pharmacol. Exp. Ther.* **258**(3), 797.