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Protection against chronic cadmium toxicity by glycine

Zahir A. Shaikh *, Weifeng Tang

Department of Biomedical Sciences, University of Rhode Island, Kingston, RI 02881, USA

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Abstract

A Japanese drug containing glycine, glycyrrhizin, and cysteine (Stronger Neo-Minophagen C®) has been reported to protect against chronic cadmium (Cd) toxicity. The present study was conducted to evaluate which of the three constituents of this drug was the main antagonist for Cd toxicity and whether the mechanism of protection involved antioxidant action. Adult female Sprague–Dawley rats were injected sc with 5 μmol $CdCl_2$/kg per day, five times per week, for 15 weeks. Four groups of Cd-injected animals received co-treatments with either 10 mg glycyrrhizin/kg, 100 mg glycine/kg, 5 mg cysteine/kg, or with a mixture of all three compounds, five times per week, starting from week 7. An additional Cd-injected group was co-treated with vitamin E (100 mg/kg, five times per week, starting from week 7) as a positive control. Only those animals that received vitamin E, Minophagen mixture, or glycine were protected against Cd-induced hepatotoxicity as well as nephrotoxicity. All three co-treatments suppressed Cd-induced hepatic and renal lipid peroxidation. We conclude that the reported beneficial effects of Stronger Neo-Minophagen C® are due to glycine, which appears to protect against chronic Cd toxicity by reducing oxidative stress.

Keywords: Minophagen; Glycyrrhizin; Glycine; Cadmium; Nephrotoxicity; Hepatotoxicity; Lipid peroxidation; Glutathione.

1. Introduction

Chronic exposure to cadmium (Cd) can damage the renal proximal tubular epithelial cells and cause proximal tubular dysfunction manifested by low-molecular weight proteinuria, glucosuria, aminoaciduria, and phosphaturia (Piscator, 1986). Renal injury is believed to be caused by Cd-metallothionein that is originally produced in the liver, released into circulation, taken up by the renal proximal tubular epithelial cells, and degraded to liberate toxic Cd ions (Squibb and Fowler, 1984; Dudley et al., 1985; Chan et al., 1993). Chronic Cd administration is associated with increased lipid peroxidation in the liver and kidney and cotreatment with antioxidants, such as vitamin E and *N*-acetyl cysteine, greatly reduce the Cd-

* Corresponding author. Fax: +1-401-8745048; e-mail: zshaikh@uriacc.uri.edu.

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induced hepatotoxicity as well as nephrotoxicity (Shaikh et al., 1998).

Recently, Nomiyama and Nomiyama (1993) reported that co-treatment of Cd-injected rabbits with Stronger Neo-Minophagen C® (Minophagen) markedly reduced Cd-induced hepatic and renal injury. We also reported the beneficial effect of Minophagen in chronic Cd-induced nephrotoxicity in rats (Shaikh et al., 1998). Minophagen is a mixture of glycyrrhizin, glycine, and cysteine. Nomiyama and Nomiyama (1993) presumed that the active principal in Minophagen for this action was glycyrrhizin. These investigators also suggested that the primary action of this drug was in preventing hepatotoxicity, which resulted in reduction of Cd-metallothionein release from the liver and indirect lowering of nephrotoxicity.

Of the three components of Minophagen, cysteine is capable of forming a complex with Cd and this complex is more nephrotoxic than Cd alone (Maitani et al., 1986). Glycyrrhizin, on the other hand, has been broadly used as a medicine for over 2000 years (Van Rossum et al., 1998) and is apparently beneficial against chronic viral hepatitis (Chen and Chen, 1998), and against carbontetrachloride- and acetaminophen-induced hepatotoxicity (Kiso et al., 1984; Liu et al., 1994). However, Liu et al. (1994) found that glycyrrhizin offered no protection against acute Cd-induced hepatotoxicity in rats. The third component, glycine, has been shown to depress hypoxia-reperfusion injury in the liver and also prevent acute Cd toxicity (Deters et al., 1997, 1998). The purpose of the present study, therefore, was to investigate whether the protective effect of Minophagen in chronic Cd toxicity was due to glycine, and if so, whether it was accompanied with a reduction of Cd-induced oxidative stress.

2. Materials and methods

2.1. Chemicals

$CdCl_2$ was purchased from Alfa ÆSAR (Ward Hill, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Animal treatment and sample collection

Seven groups of six female Sprague–Dawley rats (Charles River, Wilmington, MA) each, weighing 200–250 g, were given unlimited access to rat chow and water. The animals were housed at 22°C with 12-h light and dark cycles. A control group of six animals received sc injections of 3 ml saline/kg body weight/day, five times per week, for 15 weeks. The remaining six groups received sc injections of 5 μmol $CdCl_2$/kg body weight per day, five times per week, for 15 weeks. Starting from weeks 7, four of these groups also received ip injections (3 ml/kg, five times per week) of one of the following solutions: 100 mg glycine/kg, 10 mg glycyrrhizin/kg, 5 mg cysteine/kg, or a mixture of all three components (Minophagen mixture). A fifth group was co-treated, starting from week 7, with 100 mg vitamin E/kg, five times per week, and served as a positive control for antioxidative protection against Cd toxicity. At periodic intervals the animals were held in plastic metabolic cages (Nalgene, Rochester, NY) for 24 h and urine was collected over ice to preserve the activity of lactate dehydrogenase (LDH). All animals were killed at the end of week 15 by exsanguination under ether anesthesia and serum, liver, and renal cortex were used for biochemical analyses.

2.3. Analytical methods

LDH activity in serum and urine was measured by the method of Hochella and Weinhouse (1965). Urinary creatinine was measured by the procedure of Heinegard and Tiderstrom (1973). Malondialdehyde (MDA), a product of lipid peroxidation, was measured by the thiobarbituric acid-reactive assay, as described by Sunderman et al. (1985). Tissue glutathione (GSH) levels were measured by a kinetic assay, according to the dithiobisnitrobenzoic acid recycling method of Anderson (1985).

2.4. Statistics

Statistical analysis was performed by one-way analysis of variance followed by Newman–Keuls test at $p < 0.05$.

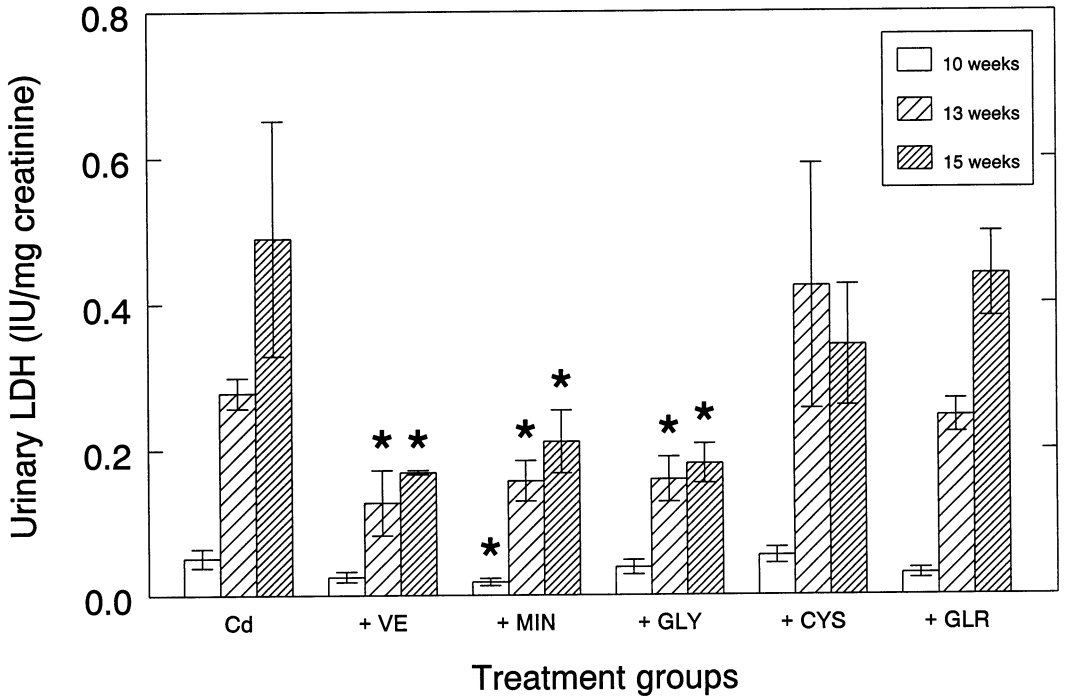

Fig. 1. Effect of vitamin E, Minophagen mixture, glycine, cysteine, and glycyrrhizin on Cd-induced nephrotoxicity. Urinary LDH activity was used as an indicator of nephrotoxicity. The control group received sc injections of 3 ml saline/kg per day, five times per week, for 15 weeks and an additional six groups received sc injections of 5 μmol $CdCl_2$/kg/day, five times per week for 15 weeks. From week 7 on, five of these groups were co-treated ip, 5 days/week, with one of the following: 100 mg/kg vitamin E, 3 ml/kg Minophagen mixture (100 mg glycine, 5 mg cysteine, and 10 mg glycyrrhizin/3 ml), or individual components of the mixture at the same dose levels. Urine samples were collected at the end of weeks 10, 13, and 15 for a period of 24 h. Data plotted are means $\pm$ S.E. of six animals per group. * Significantly lower than the Cd alone group at the same time point ($P < 0.05$).

3. Results

3.1. Protection against Cd-induced nephrotoxicity

The nephrotoxicity was evaluated by measuring urinary LDH activity (Fig. 1). In saline-injected controls the LDH levels were very low (average ~ 0.015 IU/mg creatinine). The LDH activity in urine increased rapidly after about 10 weeks of Cd administration and increased 33-fold by week 15. In comparison, in vitamin E, Minophagen mixture, and glycine co-treatment groups the LDH activities rose only about half as much as in the Cd alone group by week 13 and did not rise any further thereafter. On the other hand, rats co-treated with either cysteine or glycyrrhizin exhibited no protection from Cd-induced nephrotoxicity.

3.2. Protection against Cd-induced hepatotoxicity

Cd-induced hepatotoxicity was evaluated by monitoring serum LDH activity (Fig. 2). After 15 weeks of treatment with saline the serum LDH activity was 0.136 ± 0.013 IU/ml. Administration of Cd during the same period resulted in hepatotoxicity and caused a 14-fold increase in LDH activity. Vitamin E, Minophagen mixture, and glycine co-treatments were hepatoprotective. There were no significant differences in serum LDH activities between these groups and the LDH activities were roughly half of those of the

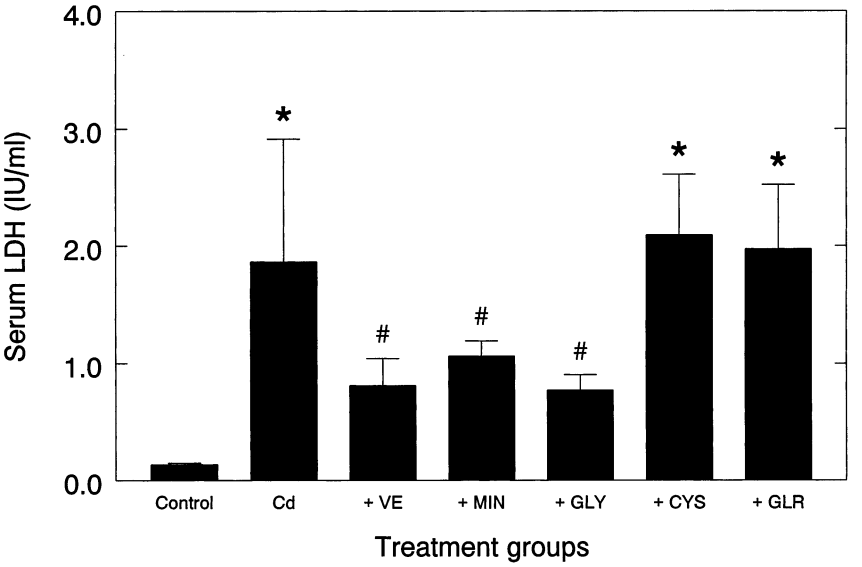

Fig. 2. Effect of vitamin E, Minophagen mixture, glycine, cysteine, and glycyrrhizin on Cd-induced hepatotoxicity. Serum LDH activity was measured as an index of hepatotoxicity at the end of week 15. See legend for Fig. 1 for experimental details. Data plotted are means $\pm$S.E. of six animals per group. * Significantly higher than the saline-injected control group ($P < 0.05$). # Significantly higher than the saline-injected control group but lower than the Cd alone group ($P < 0.05$).

Cd alone group. As with nephrotoxicity, cysteine and glycyrrhizin co-treatments had no beneficial effect on Cd-induced hepatotoxicity.

3.3. Oxidative stress as a mechanism of protection by glycine

From the above results it was evident that the protective effect of Minophagen against Cd toxicity was due to only one of its components, namely, glycine. Next, the possibility that glycine protected by depressing oxidative stress was evaluated and compared with vitamin E. Both hepatic and renal cortex GSH and MDA levels were measured. The hepatic GSH levels in the saline-treated group were 4.1 ± 0.2 μmol/g (Fig. 3A). Administration of Cd caused a 2-fold increase in hepatic GSH levels. Co-treatment with vitamin E, Minophagen mixture, or glycine did not significantly alter the Cd-induced elevation in hepatic GSH levels.

Even though Cd caused an elevation in GSH levels, it resulted in a 2.3-fold increase in MDA levels over the saline controls (0.135 ± 0.014 μmol/g) (Fig. 3B). All three co-treatments were effective in controlling the Cd-induced lipid peroxidation. Although the mean MDA level in the Minophagen mixture group was 1.5-fold higher than the saline controls, there was no significant difference between the vitamin E and glycine groups, or between the glycine and Minophagen mixture groups.

At week 15 the renal cortex GSH and MDA levels in the saline control group were 1.79 ± 0.17 and 0.136 ± 0.011 μmol/g, respectively (Fig. 3C and D). In the Cd-injected group the renal cortex GSH concentration increased 2.7-fold and the MDA concentration increased only 1.5-fold. As in the liver, co-administration of vitamin E or Minophagen mixture for 9 weeks had no effect on the Cd-induced GSH levels. In the glycine co-treatment group the GSH levels were about 20% lower than in the Cd alone group. As in the liver, all three co-treatments reduced the Cd-induced lipid peroxidation. Although the MDA values for the glycine group were significantly higher (25%) than the controls, there was no significant difference in the MDA levels between the vitamin E and Minophagen mixture groups or between the mixture and the glycine groups.

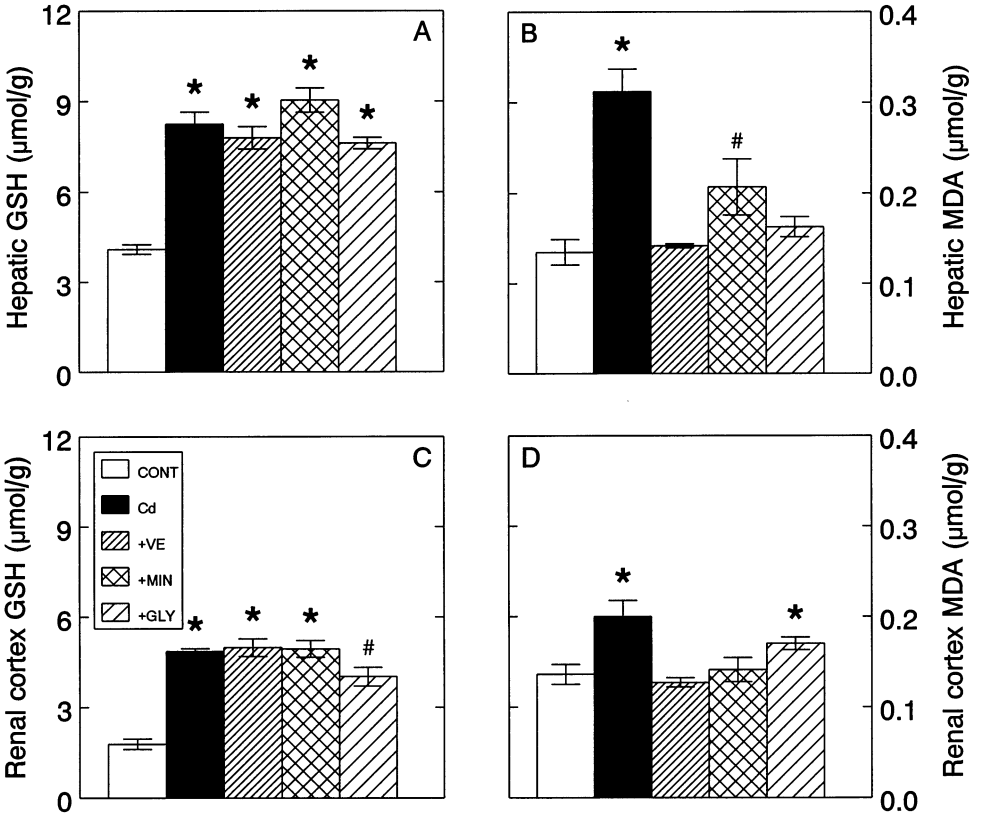

Fig. 3. Effect of vitamin E, Minophagen mixture, and glycine on hepatic and renal cortex GSH and MDA levels in Cd-injected rats. (A) Hepatic GSH, (B) hepatic MDA, (C) renal cortex GSH, and (D) renal cortex MDA levels were determined at the end of week 15. See legend for Fig. 1 for experimental details. Data plotted are means ± S.E. of six animals per group. * Significantly higher than the saline-injected control group ($P < 0.05$). # Significantly higher than the saline-injected control group but lower than the Cd alone group ($P < 0.05$).

3.4. Effect of co-treatments on hepatic and renal cortex Cd levels

The mean hepatic and renal Cd concentrations in the Cd alone group were about 600 and 250 µg/g, respectively, at the end of week 15. Neither vitamin E, nor Minophagen mixture or glycine had any significant effect on hepatic or renal cortex Cd accumulation (data not shown). This suggested that the co-treatments did not protect by lowering tissue Cd burden.

4. Discussion

The chronic Cd-induced nephrotoxicity model used in this study has been previously established in our laboratory (Shaikh and Hirayama, 1979; Tohyama and Shaikh, 1981; Tohyama et al., 1981). That Cd-induced nephrotoxicity involves necrosis of the proximal tubular epithelial cells has been reported by several investigators, including Goyer et al. (1989). The Cd-induced renal proximal tubular damage and dysfunction, as well as recovery from nephrotoxicity are commonly monitored by analyzing enzymuria, proteinuria, glucosuria, and aminoaciduria (Nomiyama and Nomiyama, 1984; Dudley et al., 1985; Kido et al., 1988). In the present study we chose to monitor nephrotoxicity by following urinary LDH activity, which is a relatively sensitive index of Cd-induced nephrotoxicity (Nomiyama and Nomiyama, 1984;

Bernard et al., 1992; Vestergaard and Shaikh, 1994).

Nomiyama and Nomiyama (1993) successfully depressed Cd-induced hepatic damage and renal dysfunction in rabbits by administration of a hepatoprotective Japanese drug, Stronger Neo-Minophagen C®. We reported similar findings in rats (Shaikh et al., 1998). In the present study, rats were treated with the Minophagen mixture and the individual components of the drug to determine the active component. The co-treatments were initiated before the onset of Cd-induced nephrotoxicity, which begins around week 10 under our exposure protocol (Shaikh and Hirayama, 1979; Tohyama and Shaikh, 1981). Vitamin E was used as a positive control since it has been shown to protect against Cd toxicity (Rana and Verma, 1996; Shaikh et al., 1998). The results showed that the Minophagen mixture was about as effective as vitamin E in protecting against Cd-induced hepatotoxicity as well as nephrotoxicity.

The biochemical mechanism of how Minophagen exerts its protective effect against Cd-induced hepatotoxicity is unknown. Chronic Cd administration induces oxidative stress (Bagchi et al., 1997). We have reported that oxidative stress plays an important role in Cd-induced hepatotoxicity (Shaikh et al., 1998). In the present study, after 15 weeks of Cd administration, the hepatic MDA levels were 2.3-fold higher than in the saline controls. Co-treatment with the Minophagen mixture significantly reduced Cd-induced hepatic lipid peroxidation, suggesting that the compounds in the mixture acted as antioxidants. These results support the contention that Minophagen is primarily a hepatoprotective drug and prevents Cd-induced nephrotoxicity by suppressing the Cd-induced hepatotoxicity (Nomiyama and Nomiyama, 1993).

Co-administration of glycine, but not cysteine or glycyrrhizin, also prevented the Cd-induced hepatic lipid peroxidation, elevation in serum enzymes, and the Cd-induced nephrotoxicity to roughly the same extent as the Minophagen mixture. Thus, the protective action of Minophagen mixture against the Cd-induced hepatotoxicity can be explained solely on the basis of glycine alone. Although the protection by glycine against Cd nephrotoxicity is possibly due to the reduction of lipid peroxidation in both liver and renal cortex, the primary site of its action could still be the liver; the magnitude of lipid peroxidation after Cd exposure was greater in the liver than in the renal cortex. Interestingly, although Deters et al. (1998) reported that in perfused rat liver glycine protected against acute Cd toxicity, they did not observe a decrease in Cd-induced lipid peroxidation. Besides the obvious differences in the experimental protocol, the reason for the disparity between their results and those obtained in the present study is unclear.

Glycine is easily transported into the cells. Glycine and cysteine can be combined in vivo to form cysteinylglycine by cysteinylglycine synthetase, and upon combination with glutamine further converted to GSH by GSH synthetase (Meister and Anderson, 1983). GSH is an antioxidant and is proposed to be the first line of defense against Cd toxicity (Singhal et al., 1987). In the present study, the hepatic GSH levels in the Cd-treated animals were already 2-fold higher than the saline controls and 9 weeks of glycine co-treatment could not further elevate the GSH levels. Although the GSH levels remained the same, Cd-induced lipid peroxidation in the liver was depressed by glycine. This suggested that glycine acted as an antioxidant and that this action was probably independent of GSH. Support for this conclusion is provided by a study in isolated renal tubules which found that the cytoprotective action of glycine on the renal tubular cells was not affected by GSH depletion (Weinberg et al., 1987).

Lipid peroxidation is an early intracellular event after Cd exposure (Muller and Ohnesorge, 1982). Cd interacts with critical subcellular sites such as the mitochondria, peroxisomes, and microsomes and results in the generation of free radicals (Ochi et al., 1987). Jagetia et al. (1993) reported that copper-glycine complex has the ability of scavenging free radicals produced by gamma radiation. It is possible that glycine inhibits Cd-induced lipid peroxidation by scavenging free radicals generated by Cd exposure. The protective action of glycine may also stem from its

ability to inhibit phospholipase A, which degrades cell membranes in hepatocytes (Schilling et al., 1994). It does not appear that glycine protects by reducing Cd accumulation, since hepatic and renal cortex Cd concentrations were unaltered.

In conclusion, the protective action of Minophagen in Cd toxicity is due to glycine. The biochemical mechanism of glycine protection appears to be related to the suppression of oxidative stress.

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