Carbon Tetrachloride-Induced Nephrotoxicity in Mice Is Prevented by Pretreatment with Zinc Sulfate

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Received January 26, 2016; accepted February 19, 2016

Carbon tetrachloride (CCl4) is commonly used as a chemical inducer of experimental liver injury. In addition, many studies showed that CCl4 can induce kidney damage. In the current study, we evaluated the protective effect of zinc (Zn) against CCl4-induced nephrotoxicity. We hypothesized that this protective effect would result from the ability of Zn to serve as an inducer of metallothionein (MT), a known endogenous scavenger of free radicals. We administered Zn (as ZnSO4) 50mg/kg subcutaneously once daily for 3 successive days prior to a single intraperitoneal administration of CCl4 4g/kg in male ddY mice. Our results showed that Zn pretreatment significantly decreased creatinine and blood urea nitrogen levels and reduced renal histopathological damage at 6h post-CCl4 injection, observations consistent with enhanced antioxidative activity in the kidney. Moreover, kidney MT levels in the Zn+CCl4-treated group decreased by greater than 70% compared with levels in the Zn-alone group, implying that MT was consumed by CCl4-induced radicals. These findings suggest that prophylaxis with Zn protects mice from CCl4-induced acute nephrotoxicity, presumably by induction of MT, which in turn scavenges radicals induced by CCl4 exposure.

Key words  carbon tetrachloride; kidney; zinc; metallothionein; radical scavenger

The molecular mechanism of CCl4-induced renal toxicity has been examined in detail. Specifically, CCl4 is metabolized through the cytochrome P450 CYP2E1 to produce the highly toxic chloromethyl free radical and/or chloromethyl peroxyl free radical. These radicals induce renal injury because free radicals bind to intracellular proteins, lipids of cell membrane, or DNA, leading in turn to protein denaturation, lipid peroxidation in the cell membrane, and oxidative damage (respectively), resulting in the death of kidney cells.1,7,13)

Many studies have reported that antioxidants can prevent hepatic damage and nephropathy by counteracting free radicals and preventing lipid peroxidation.2,4,6,11) Zinc (Zn) is well known as an essential trace element for a variety of biological activities. Zn is a component of more than 300 metalloenzymes and contributes to the maintenance of structure and catalytic activity in these enzymes. In addition, Zn plays regulatory roles in diverse cellular processes such as signaling transduction and gene expression.14,15) Aside from these physiological roles, Zn also may reduce oxidative stress by inducing metallothionein (MT), a small, cysteine-rich protein lacking aromatic amino acids and histidine16) that acts as a chelator or scavenger of heavy metals, reactive oxygen species (ROS), and free radicals.17) We previously reported that pretreatment with Zn prevented CCl4-induced lethal toxicity in mice and that Zn-induced MT may be involved in the detoxification mechanism18); however, we did not evaluate the potential renal protective effects of Zn prophylaxis. To our knowledge, previous studies have not examined the potential protective effect of Zn or MT against CCl4-induced nephrotoxicity.

Therefore, in the current study, we investigated whether pretreatment with Zn is sufficient to attenuate CCl4-induced acute nephrotoxicity.

MATERIALS AND METHODS

Animal Treatment Male ddY mice were purchased from Japan SLC (Shizuoka, Japan). Following arrival at our facility, mice were maintained under standard conditions of controlled temperature (24±1°C), humidity (55±5%), and light (12:12h light/dark cycles) with free access to water and food. Experimental treatments were performed using eight-week-old animals. Following the experiment, any surviving mice were sacrificed using pentobarbital. All experiments were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University (No. 110).

Experimental Protocol Mice were divided into four groups. At ~72, ~48, and ~24h, animals of the Group 2 (Zn group) and Group 4 (Zn+CCl4 group) were administered (by subcutaneous (s.c.) injection at 24-h intervals) with 50mg/kg ZnSO4 (Nacalai Tesque, Kyoto, Japan). Animals of Group 1 (control group) and Group 3 (CCl4 group) were injected s.c. with equivalent volumes of saline vehicle. Twenty-four hours after the final ZnSO4 or saline injection, both the CCl4 group and Zn+CCl4 group were administered intraperitoneally (i.p.) with 4g/kg (at 5mL/kg) CCl4. The control and Zn groups

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were injected i.p. with equivalent volumes of olive oil. At 6 h after the CCl₄ or olive oil injection, mice from each group were euthanized and bled for plasma. The resulting plasma samples were stored at −80°C pending creatinine and blood urea nitrogen (BUN) assays. Kidneys were harvested from each of these animals, and separate samples from each kidney were stored at −80°C (pending anti-oxidant power assay and MT determination) or fixed in 15% neutral buffered formalin (pH 7.2) (pending processing for histological evaluation).

**Measurement of Creatinine and BUN** Concentrations of plasma creatinine and BUN were measured using Creatinine Liquid Reagents Assay (DIAZYME, Poway, CA, U.S.A.) and BUN Wako Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (respectively) according to the manufacturer’s instructions. The absorbance of the assay reaction products was measured at 550 or 570 nm, respectively. For relative quantification, calibration curves were prepared using standard solutions.

**Determination of Total Antioxidant Power in the Kidney** Total antioxidant power levels in the kidney were examined by colorimetric total antioxidant power assay kit (FR40, Oxford Biochemical Research, Oxford, MI, U.S.A.) according to the manufacturer’s protocol. Aliquots (0.1 g each, including mixed cell types) of right renal tissue were homogenized with 900 µL ice-cold phosphate-buffered saline (PBS) containing protease inhibitor (Nacalai Tesque) and centrifuged at 18000×g for 20 min at 4°C. The resulting supernatant (diluted to yield consistent total protein concentrations) for each sample was used for further steps. The antioxidant level was determined by the reduction of Cu²⁺ to Cu⁺ to permit assessment of the combined action of all antioxidants present in the sample. Generated Cu⁺ was detected by determining the formation of complexes between Cu⁺ and bathocuproine; stable complex was detected at 450 nm. The obtained absorbance values were compared to a standard curve obtained using uric acid as the reductant.

**Measurement of Malondialdehyde (MDA) Levels in Kidneys** The total MDA levels in the kidney were examined by colorimetric thiobarbituric acid-reactive substance microplate assay kit (FR40, Oxford Biochemical Research, Oxford, MI, U.S.A.) according to the manufacturer’s protocol. Aliquots (0.1 g each, including mixed cell types) of right renal tissue were homogenized with 900 µL ice-cold PBS containing protease inhibitor and centrifuged at 18000×g for 20 min at 4°C. An aliquot (200 µL) of the resulting supernatant was combined with saturated ammonium sulfate (40 µL; Wako Pure Chemical Industries, Ltd.) and trichloroacetic acid (35 mg; Nacalai Tesque) and vortexed. After centrifugation (3000×g, 10 min, 4°C), supernatants were incubated at 65°C for 45 min. The absorbance of this reaction product was measured at 532 nm. For relative quantification, calibration curves were prepared using a standard solution of MDA.

**Measurement of MT Levels in the Kidney** The renal MT protein levels were determined by the cadmium saturation-hemolysate method (Cd-hem method). Aliquots (0.2–0.3 g each, including mixed cell types) of right kidney tissue were homogenized with 5 volumes of 0.25M sucrose (Nacalai Tesque). The homogenates were centrifuged at 18000×g for 20 min at 4°C to isolate the postmitochondrial supernatant; suitable aliquots then were used for MT assay by the Cd-hem method, as described previously.

**Histopathological Findings** For histological analysis, each left kidney was perfused with 15% phosphate-buffered neutral formalin (pH 7.2), dehydrated, and embedded in paraffin. Embedded tissues were sectioned at 4-µm thicknesses and stained with hematoxylin and eosin (H&E). Histopathological features in the slices were observed via light microscope.

**Statistical Analysis** All data from the control and treatment groups were obtained from the same numbers of replicated experiments. All experiments were performed independently at least two times. The results were analyzed using one-way ANOVA with post-hoc Tukey–Kramer’s test. All statistical analyses were performed using SPSS 19.0J software (Chicago, IL, U.S.A). Values of p<0.05 were considered statistically significant.

**RESULTS**

**Zn Attenuation of CCl₄ Acute Toxicity as Measured by Kidney Function Markers** Plasma concentrations of creatinine and BUN are known as markers for injury and dysfunction of the kidney. In the present study, the effect of CCl₄ administration on the blood concentration of these markers was tested. Systemic (i.p.) exposure of mice to CCl₄ significantly

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**Fig. 1. Effect of Pretreatment with Zn on Acute CCl₄ Toxicity as Measured by Creatinine and BUN Levels**

Mice were injected s.c. with 50 mg/kg ZnSO₄ (three times, q24h). Twenty-four hours after final pretreatment, mice were injected i.p. with 4 g/kg CCl₄. Creatinine (A) and BUN (B) levels in plasma were determined at 6 h after i.p. injection. Data are presented as the mean±S.D. of five or six mice. ** Significant difference between compared values (**p<0.01).
increased the plasma concentrations of creatinine (Fig. 1A) and BUN (Fig. 1B). In contrast, three-time pretreatment with Zn significantly attenuated the CCl₄-induced acute increases in creatinine and BUN levels. There was no significant difference in these parameters between the control group and the Zn group (i.e., in the absence of CCl₄ exposure).

**Zn Attenuation of CCl₄ Acute Toxicity as Measured by Renal Structure Histopathology** In parallel with the measurement of creatinine and BUN (Fig. 1), we evaluated histopathological changes in renal tissue. The morphology of tubules, glomeruli, and tubulointerstitial cells in the Zn-treated group (Fig. 2B) were similar to that seen in the control group (Fig. 2A). Exposure to CCl₄ induced swelling, degeneration, and the appearance of protein column (penetration of protein) in the renal proximal tubules (Fig. 2C). In contrast to the CCl₄-treated group, Zn+CCl₄-treated group showed almost normal morphology of the kidney (Fig. 2D).

**Zn Attenuation of CCl₄ Acute Toxicity as Measured by MDA Levels and Antioxidant Status** To investigate Zn-induced nephroprotective activity against CCl₄ in greater detail, we tested oxidative stress in the kidney tissue. CCl₄ treatment significantly increased renal MDA levels compared with those detected in the control group (Fig. 3A). In contrast, pretreatment with Zn of CCl₄-treated mice significantly attenuated the CCl₄-induced increase in MDA levels. No significant difference in the levels of MDA was detected between the control and Zn-treated groups.

Total antioxidant level is commonly used as an indicator of oxidative stress because this parameter inversely correlates with oxidant level. As shown in Fig. 3B, the total antioxidant

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**Fig. 2. Nephroprotective Effect of Zn Pretreatment on Acute CCl₄ Toxicity (H&E Stain)**

Mice were injected s.c. with 50mg/kg ZnSO₄ (three times, q24h). Twenty-four hours after final pretreatment, mice were injected i.p. with 4g/kg CCl₄. Kidneys were harvested 6h after i.p. injection, fixed, processed, and stained with H&E. (A), (B), (C), and (D) provide 20× magnification images of representative H&E-stained sections from kidneys obtained from control, Zn, CCl₄, and Zn+CCl₄ animals. Section for (C) reveals swelling, degeneration, and the appearance of protein column (penetration of protein) in renal proximal tubules in a CCl₄-exposed animal, in contrast to the mostly normal renal structure seen in (A), (B), and (D). Green arrows, black arrows and orange arrows indicate swelling, degeneration, and protein column, respectively.

**Fig. 3. Effect of Pretreatment with Zn against Acute CCl₄ Toxicity, as Measured by MDA Levels and Antioxidant Power**

Mice were injected s.c. with 50mg/kg ZnSO₄ (three times, q24h). Twenty-four hours after final pretreatment, mice were injected i.p. with 4g/kg CCl₄. MDA levels (A) and the antioxidant power (B) in kidney were determined 6h after i.p. injection. Data are presented as the mean±S.D. of five or six mice. **Significant difference between compared values (**p<0.01).
In the present study, pretreatment with Zn effectively protected mice from CCl₄-induced acute kidney damage by attenuating the CCl₄-induced elevation in function markers (creatinine and BUN) and alleviating renal histological changes. These results showed that Zn has protective effect against radical-induced renal dysfunction and histopathological damage. In addition, Zn attenuated increases in MDA levels and restored total antioxidant power in CCl₄-treated mouse kidney. These protective effects may be due to the powerful antioxidative activity of Zn itself. These results also suggest that Zn may attenuate oxidative stress by decreasing levels of lipid peroxide in CCl₄-exposed mouse kidney, an effect that may (at least partially) prevent renal damage.

The antioxidative activities of Zn may reflect the increased MT level that results from pretreatment with Zn, given that MT is a potent radical scavenger. It is well known that MT exhibits antioxidative activity against ROS via scavenging of free radicals with MT estimated to exhibit activity some 300-fold higher than that of glutathione (GSH). In addition, we showed in the present study that the Zn-induced increase of kidney MT levels was attenuated by more than 70% following CCl₄ injection, suggesting that MT is consumed by CCl₄-derived radicals. The Cd-hem assay, used to determine MT in our present study, is based on quantification of metal ions (Cd) bound to MT molecules. Since oxidized MT does not bind to Cd and is not detected in this assay, we speculate that CCl₄-derived free radicals preferentially attacked MT, resulting in a loss of Cd-binding activity.

Indeed, many researchers have reported that CCl₄ treatment significantly reduces the renal total content of reduced GSH, and that CCl₄ metabolites can react with sulfhydryl groups of GSH and protein thiol to alter the redox status of cells. Under oxidative stress (such as that induced under our experimental conditions), reduced GSH is likely to be oxidized directly and is converted to the oxidized forms of GSH. Therefore, antioxidant level is reduced in our current study (change 0.44±0.01 to 0.35±0.03 ms by CCl₄-injection). In contrast, although Zn-induced kidney MT levels was reduced by more than 70% by CCl₄-injection, no significant change in total antioxidant power between the Zn and Zn+CCl₄ groups was observed (0.50±0.01, 0.49±0.04 mm). In this context, total antioxidant level was poorly lowered despite marked MT increase in Zn-pretreatment group. Although we cannot exhibit rational assumption about the causative factor of low detecting sensitivity, a possible cause is that the detecting sensitivity to antioxidant potential of MT is considerably low. Hence, we speculate that total antioxidant power was comparable between Zn-treated group and control group because vast of antioxidant power of MT is not reflected in total antioxidant power, that also supports this hypothesis.

In conclusion, we have demonstrated that pretreatment with Zn suppresses CCl₄-induced acute nephrotoxicity; we hypothesize that the nephroprotective effect of Zn is due to the antioxidative role and free radical scavenging activity of MT, the expression of which is induced by Zn pretreatment. To our knowledge, this is the first evidence that Zn prophylaxis protects against CCl₄-induced acute nephrotoxicity. Although further investigation is needed to clarify the involvement of MT, these findings are expected to contribute to improved understanding of the protective effect of Zn against radical-induced organ injury and disease.
Acknowledgment The authors thank Dr. Kenichi Saeki (Kinjo Gakuin University, Japan) for his kind suggestions.

Conflict of Interest The authors declare no conflict of interest.

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