Protective role of L-2-oxothiazolidine-4-carboxylic acid in cisplatin-induced renal injury

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Abstract

Background. Oxidative stress and inflammation are implicated in the pathogenesis of cisplatin-induced nephrotoxicity. L-2-oxothiazolidine-4-carboxylic acid (OTC) is a cysteine prodrug, and increases cellular glutathione (GSH). OTC is converted to cysteine by the intracellular enzyme, oxoprolinase. To date, the protective role of OTC on cisplatin-induced renal injury has not been investigated. The purpose of the present study was to examine the protective effect of OTC on cisplatin-induced renal injury and to examine the mechanism of its protection.

Methods. Mice were treated with cisplatin with or without administration of OTC. The generation of reactive oxygen species (ROS), expression of intercellular adhesion molecule (ICAM)-1 and monocyte chemoattractant protein (MCP)-1 were determined in the kidney using 2',7'-dichlorofluorescein diacetate, immunostaining or western blot analysis. Nuclear factor (NF)-κB activity, infiltration of F4/80-positive cells and apoptosis were also investigated in addition to renal function and histology using electrophoretic mobility shift assay, immunostaining, western blot analysis, uridine triphosphate (dUTP) nick-end labeling or periodic acid–Schiff staining. The effect of OTC on superoxide dismutase activity and GSH level in cisplatin-treated normal adult human kidney (HK-2) cells were measured using assay kits.

Results. The administration of OTC resulted in a significant reduction of cisplatin-induced ROS production, the p65 subunit of NF-κB translocation into nucleus, expression of ICAM-1, caspase 3 activity, expression of MCP-1 and the infiltration of macrophages into renal tissue. OTC markedly ameliorated renal damage induced by cisplatin through antioxidant and anti-inflammatory effect.

Conclusions. These results suggest that OTC can be a potential therapeutic agent in cisplatin-induced renal injury through decreasing the ROS levels and activation of NF-κB.

Keywords: cisplatin; L-2-oxothiazolidine-4-carboxylic acid; nuclear factor-κB; oxidative stress

Introduction

Cisplatin is a potent anti-cancer agent for various types of tumours, including testicular, ovarian, head and neck and uterine cervical carcinoma [1]. However, cisplatin is a toxic agent to renal tubules and is associated with a decline in renal function. Oxidative stress is caused by various free-oxygen radicals including superoxide anion, hydrogen peroxide and hydroxyl radical. It has been suggested that oxidative stress plays a critical role in the pathogenesis of cisplatin-induced nephrotoxicity [2,3].

L-2-oxothiazolidine-4-carboxylic acid (OTC), a prodrug of cysteine, is the amino acid believed to be rate limiting for glutathione (GSH) synthesis [4]. OTC is a 5-oxoprolin analogue and a substrate for the ubiquitous intracellular enzyme called 5-oxoprolinase [5]. GSH is synthesized from cysteine and is a vital intracellular and extracellular protective antioxidant. Cystein derivatives, N-acetylcysteine and carbocysteine, which may act as antioxidants, have been shown to exhibit anti-inflammatory effects [6]. It has been reported that OTC replenishes cellular GSH stores and is more effective than N-acetylcysteine in replenishing intracellular GSH stores [7]. However, the protective role of OTC on cisplatin-induced renal injury has not been investigated.

The purpose of the present study was to determine the protective effect of OTC on cisplatin-induced renal
injury and to examine the mechanism of its protection. Our results show that oxidative stress and nuclear factor (NF)-κB activation are key elements in the upregulation of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) in cisplatin-induced renal injury. Administration of OTC suppresses reactive oxygen species (ROS) generation, apoptosis and NF-κB activation, leading to a decreased expression of ICAM-1 and MCP-1 by cisplatin. These results indicated that OTC exhibits protective effects on cisplatin-induced renal injury.

Materials and methods

Animals and drug treatment

Male C57BL/6 mice (Charles River Korea, Seoul, Korea) were given a standard laboratory diet and water *ad libitum*, and were cared for under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. At the start of the experiments, the mice were 8–10 weeks of age, weighing 25–30 g. To obtain an optimal dose of cisplatin and time of treatment, dose-dependent (10, 15 or 20 mg/kg) and time-dependent (24, 48, 72 or 96 h) experiments were performed. Renal injury examined by renal function and histological findings was clearly seen with a dose of 20 mg/kg cisplatin at 72 h after the cisplatin treatment. Therefore, 20 mg/kg cisplatin and 72 h treatment were applied throughout the study. In the case of OTC treatment, a dose-dependent experiment (40, 80 or 160 mg/kg OTC) was performed. Significant protective effects of OTC on the cisplatin-induced renal injury were obtained at a dose of 80 mg/kg OTC. This concentration of OTC was used throughout the experiment. The mice were divided into four groups: control (*n* = 12), OTC (80 mg/kg; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (*n* = 12), cisplatin (20 mg/kg; Sigma Chemical Co., St Louis, MO, USA) (*n* = 12) and cisplatin plus OTC (*n* = 12). Control buffer and cisplatin were injected intraperitoneally. OTC was administrated by oral gavage once a day until the mice were sacrificed at 72 h after cisplatin injection. Mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and sacrificed by cervical dislocation.

Cell culture and application of cisplatin and OTC in HK-2 cells

The immortalized proximal tubule epithelial cell line from the normal adult human kidney (HK-2) was purchased from the American Type Cell Collection and cultured. Briefly, cells were passaged every 3–4 days in 100 mm dishes (Falcon, Bedford, MA, USA) using Dulbecco’s modified Eagle’s medium (DMEM)-F12 (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Inc., Gaithersburg, MD, USA), insulin–transferrin–sodium selenite media supplement (Sigma Chemical Co.), 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma Chemical Co.). These cells were incubated in a humidified atmosphere of 5% CO₂, 95% air at 37°C for 24 h and subcultured at 70–80% confluence. For experimental use, HK-2 cells were plated onto 60 mm dishes in a medium containing 10% FBS for 24 h and then switched to DMEM-F12 with 2% FBS for 16 h. These cells were treated with or without cisplatin (1 µg/ml; Sigma Chemical Co.) in the presence and absence of OTC (1 mM; Sigma Chemical Co.) for 24 h. Control cells received only buffer instead of OTC and/or cisplatin. At the end of the treatment, the cells were harvested.

Renal function monitoring

On the day of the sacrifice, blood was collected immediately. Urea nitrogen and creatinine levels in blood were measured using an enzymatic method (SRL, Tokyo, Japan).

Histological examination

The mouse kidneys were sectioned in blocks and fixed in 4% paraformaldehyde, then dehydrated in graded concentrations of alcohols and embedded in paraffin. The kidney block was cut into 5 µm sections and stained with periodic acid–Schiff (PAS) reagents. Tubular damage in PAS-stained sections was graded using the percentage of cortical tubules showing epithelial necrosis: 0 = normal; 1 ≤ 10%; 2 = 10–25%; 3 = 26–75%; 4 ≥ 75%. Tubular necrosis was defined as the loss of the proximal tubular brush border, blebbing of apical membranes, tubular epithelial cell detachment from the basement membrane or intraluminal aggregation of cells and proteins. The morphometric examination was performed in a blinded manner by two independent investigators.

Western blot analysis

Western blot analysis was performed as described previously [8]. For western blot analysis, samples (50 µg of protein per lane) were mixed with sample buffer, boiled for 10 min, separated by 10% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis under denaturing conditions and electro-blotted to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h and incubated overnight at 4°C with anti-ICAM-1 monoclonal antibody (Santa Cruz Biotechnology, dilution 1:2000). The blots were washed with phosphate-buffered saline (PBS) and incubated with anti-rabbit horseradish peroxidase-conjugated IgG. Signals were visualized by chemiluminescent detection according to the manufacturer’s protocol (Amersham Pharmacia Biotech, London, UK). The membranes were reprobed with anti-actin antibody to verify equal loading of protein in each lane. All signals were visualized and analysed by densitometric scanning (LAS-1000, Fuji Film, Tokyo, Japan).

Cytosolic and nuclear protein extraction for analysis of NF-κB

HK-2 cells were lysed in a hypotonic buffer [10 mmol/l HEPES, pH 7.9, 1.5 mmol/l MgCl₂, 10 mmol/l KCl, 0.5 mmol/l dithiothreitol (DTT), 0.5 mmol/l phenylmethyl sulfonil fluoride and 0.6% NP-40] and centrifuged at 1500g.
for 15 min at 4°C. The supernatant was used as cytosolic protein. Pellets were lysed in 15 μl of a high-salt buffer (20 mmol/l HEPES, pH 7.9, 420 mmol/l NaCl, 25% glycerol, 1.5 mmol/l MgCl2, 0.2 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l phenylmethyl sulfonyl fluoride and 0.5 mmol/l DTT) for 20 min on ice. Storage buffer (75 μl; 20 mmol/l HEPES, pH 7.9, 100 mmol/l NaCl, 20% glycerol, 0.2 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l phenylmethyl sulfonyl fluoride and 0.5 mmol/l DTT) was added. The resulting nuclear pellets were agitated by vortex mixing, and then centrifuged at 6000g for 20 min. The resulting supernatant was used as soluble nuclear proteins.

For western blot analysis, samples (30 μg of protein per lane) were loaded on 10% SDS-polyacrylamide gel. After electrophoresis at 120 V for 90 min, separated proteins were electroblotted to nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% non-fat dry milk in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h and incubated overnight at 4°C in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h and incubated overnight at 4°C with anti-NF-κB p65 (Upstate Biotech, Lake Placid, NY, USA). The blots were detected with western blot analysis method as described earlier.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay (EMSA) was performed as described previously [9]. EMSA for NF-κB protein was performed with biotin-labelled NF-κB binding site oligomer 5'-AGTTGAGGGACTTCTCCAGGC. Signals were detected by chemiluminescent imaging according to the manufacturer's protocol (EMSA Gel-Shift Kit; Panomics, Redwood city, CA, USA).

**Immunohistochemical analyses for ICAM-1, MCP-1, NF-κB and F4/80**

Immunohistochemical stainings for ICAM-1, MCP-1, p65 and F4/80 were performed as described previously [10]. Isolated kidney tissues were fixed by immersion in 4% paraformaldehyde and blocked in paraffin. Tissue sections were deparaffinized with xylene and rehydrated with ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min, and the samples were then rinsed with PBS. To obtain an adequate signal, the slides were treated with pepsin at 42°C for 5 min. After treatment with the blocking buffer, the slides were incubated overnight at 4°C with a hamster anti-mouse ICAM-1 antibody (BD PharMingen, San Diego, CA, USA; dilution 1:100), with a rabbit anti-mouse MCP-1 antibody (Fitzgerald, Concord, MA, USA; dilution 1:100), with a rat anti-mouse F4/80 antibody (Serotec, Oxford, UK; dilution 1:50) or with rabbit polyclonal antibody against phospho-p65 (Santa Cruz Biotechnology; dilution 1:100). The primary antibody was localized using the Vectastain ABC-Elite peroxidase detection system (Vector Laboratories, Burlington, CA, USA), followed by reaction with diaminobenzidine as chromogen and counterstaining with haematoxylin (Sigma Chemical Co.,). Evaluation of all the slides was performed by two observers who were unaware of the origin of the slides. The numbers of F4/80-positive cells or NF-κB activated cells in each section were calculated by counting the number of positively stained cells in 10 × 400 fields per slide. The extent of MCP-1 immunostaining was graded on a scale from 0 to 4 under a micrometric ocular grid in 10 × 400 fields per slide of the cortical tubulointerstitium: 0 = normal; 1 ≤ 10%; 2 = 10–25%; 3 = 26–75%; 4 ≥ 75%.

**Detection of apoptosis**

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated uridine triphosphate (dUTP) nick-end labelling (TUNEL), and the number of apoptotic cells, as defined by chromatin condensation or nuclear fragmentation (apoptotic bodies), was counted. Apoptosis was detected in the specimen using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, CA, USA) according to the manufacturer's protocol. The morphometric examination was performed by two independent, blinded investigators. The number of apoptotic cells in each section was calculated by counting the number of TUNEL-positive apoptotic cells in 10 × 400 fields per slide.

**Measurement of caspase 3 activity**

The activity of caspase 3 in HK-2 cells was measured by fluorometric detection of free 7-aminocarboxyfluorescein (FAM) from the cleavage of 7-amino-4-trifluoromethylcoumarin, following the manufacturer's protocol (BD ApoAlert™ Caspase Fluorescent Assay Kits; BD Bioscience, Palo Alto, CA, USA) by using Synergy™ HT Multi-Detection Microplate Reader (Biotek, Woburn, MA, USA) as described previously [11]. Briefly, HK-2 cells were homogenized in 50 μl of chilled lysis buffer, incubated on ice for 10 min and centrifuged at 15 000 for 10 min at 4°C. The supernatant was incubated for 1 h at 37°C in the presence of a reaction buffer, 1 mmol/l DTT and 50 μmol/l Aminomethyl coumarin (AMC) substrate conjugates. The fluorescence was read at 400/505 nm (excitation/emission) for caspase 3 and the samples were run in triplicates. The activity of caspase 3 was expressed as percent increase compared with the saline-treated control group, and samples containing caspase inhibitors served as negative controls.

**Measurement of intracellular reactive oxygen species**

To measure the intracellular ROS, HK-2 cells were incubated for 10 min at room temperature with PBS containing 3.3 mM 2',7'-dichlorofluorescein diacetate (Molecular probes, Eugene, OR, USA), to label intracellular ROS. The HK-2 cells were then immediately observed under fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY, USA) and fluorescence-activated cell-sorting analysis (Partec, Münster, Germany).

**Measurement of glutathione (GSH)**

The GSH levels were measured in HK-2 cells using the Glutathione Assay kit II (Calbiochem, Darmstadt, Germany) according to the protocol provided by the manufacturer. Briefly, HK-2 cells were homogenized in 1 ml ice-cold lysis buffer and centrifuged at 10 000 g for 15 min at 4°C. The supernatant was deproteinized with 5% metaphosphoric acid (Sigma Chemical Co.) and 4 M triethanolamine (Sigma Chemical Co.). Samples were then mixed with the Assay Cocktail reagents. After incubation at 25°C for 25 min, absorbance was measured at 405 nm.
GSH levels were calculated using a standard curve generated by standards provided by the manufacturer.

**Superoxide dismutase (SOD) activity**

Total SOD activity was determined according to the protocol provided by the manufacturer. The principle of the method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine-xanthine oxidase system as a superoxide generator. HK-2 cells were carefully washed and homogenized in 60 μl of PBS. After centrifugation, the total SOD activity in the supernatant was measured using the Superoxide Dismutase Activity Assay kit (Chemicon International, CA, USA).

**Statistical analysis**

Data are expressed as mean ± SEM. Multiple comparisons were examined for significant differences using analysis of variance (ANOVA), followed by individual comparisons with the Tukey post-test. Statistical significance was set at \( P < 0.05 \).

**Results**

**OTC reduces renal dysfunction in mice with cisplatin-induced renal injury**

To investigate the effect of OTC on cisplatin-induced renal dysfunction, levels of blood urea nitrogen (BUN) and creatinine were measured at 72 h after cisplatin administration in the serum of both OTC-treated and untreated mice. As shown in Figure 1, cisplatin administration resulted in severe renal injury. In contrast, the treatment with OTC together with cisplatin significantly decreased BUN levels (158.2 ± 27.3 vs 58.2 ± 9.7 mg/dl, \( P < 0.05 \)) and serum creatinine levels (2.1 ± 0.3 vs 1.1 ± 0.2 mg/dl, \( P < 0.05 \)) compared with cisplatin alone. OTC alone had no effects on BUN and creatinine levels as compared with the control group (BUN, 18 ± 2.9 vs 19 ± 3 mg/dl; creatinine, 0.38 ± 0.09 vs 0.37 ± 0.06 mg/dl, \( P > 0.05 \)).

**OTC ameliorates tubular necrosis and apoptosis in cisplatin-induced renal injury**

Histological examination revealed necrosis, protein cast, vacuolation and desquamation of epithelial cells in the renal tubules of the cisplatin-treated control group. However, treatment of OTC together with cisplatin dramatically improved the cisplatin-induced damage of the renal tubules (Figure 2). Apoptosis of renal tubular epithelial cells was evaluated by TUNEL and caspase 3 activity. As shown in Figure 3, the caspase activity in HK-2 cells was increased markedly after cisplatin administration, and OTC treatment significantly reduced the enzyme activity. Morphologically, TUNEL-positive apoptotic cells were increased in cisplatin-treated mice, and OTC treatment significantly decreased the number of apoptotic cells.

**OTC decreases ROS production in cisplatin-treated HK-2 cells**

Oxidative stress is caused by various free-oxygen radicals and has been implicated in the pathogenesis of cisplatin-induced nephrotoxicity. Therefore, we evaluated the role of the OTC in cisplatin-induced oxidative stress. ROS generation in HK-2 cells was increased significantly after cisplatin treatment compared with the levels of the control. The increased ROS

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**Fig. 1.** Effect of OTC on BUN and serum creatinine in cisplatin-induced acute renal injury. Acute renal injury was induced by intraperitoneal injection of cisplatin (20 mg/kg). Mice were administrated OTC (80 mg/kg) orally once a day for 3 days after cisplatin injection. Blood samples were collected at 72 h after cisplatin administration, then BUN (A) and serum creatinine (B) were measured. Control mice were injected with saline. Values are expressed as mean ± SEM (n = 10–12 mice per group). *\( P < 0.05 \) vs saline, † \( P < 0.05 \) vs cisplatin. Con: saline; OTC, OTC treatment alone; Cis, cisplatin treatment alone; Cis + OTC, cisplatin with administration of OTC; BUN, blood urea nitrogen.
Fig. 2. Effect of OTC on renal histology in cisplatin-induced acute renal injury. PAS-stained sections of the kidney at 72 h after cisplatin injection. (A) Control without cisplatin treatment (Con), (B) OTC treatment alone (OTC), (C) treated with cisplatin without OTC treatment (Cis), (D) treated with cisplatin and OTC (Cis + OTC), (E) tubular necrosis was scored in harvested renal tissues. The cisplatin-treated kidneys (C) showed marked injury with cast formation, sloughing of tubular epithelial cells, loss of brush border and dilation of tubules. These changes were less pronounced in mice treated with cisplatin and OTC (D). OTC treatment alone (B) had no effect on renal histology. Data represent mean ± SEM from four independent experiments. *P < 0.05 vs saline; †P < 0.05 vs cisplatin. Magnification, 400×.

Fig. 3. Effect of OTC on apoptosis in cisplatin-induced acute renal injury. Terminal deoxynucleotidyl transferase-mediated TUNEL staining (A–D). Magnification, 400×. Kidneys from cisplatin-treated control group showed nuclear changes consistent with apoptotic cell death. Apoptotic cells were defined by chromatin condensation or nuclear fragmentation (apoptotic bodies). (A) Control without cisplatin treatment (Con), (B) OTC treatment alone (OTC), (C) treated with cisplatin without OTC treatment (Cis), (D) treated with cisplatin and OTC (Cis + OTC). (E) The number of apoptotic cells in each section was calculated by counting the number of TUNEL-positive apoptotic cells in 10 × 400 fields per slide. (F) Caspase 3 activity was expressed as percent increase compared with saline-treated control group. Data represent mean ± SEM from three independent experiments. *P < 0.05 vs saline; †P < 0.05 vs cisplatin.
Generation was significantly decreased by the treatment of OTC (Figure 4).

OTC restores GSH concentrations and counteracts the reduction in SOD in cisplatin-treated HK-2 cells

GSH is an important endogenous antioxidant that protects cells and tissues against oxygen radical damage. SOD catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. As such it is an important antioxidant defence in nearly all cells exposed to oxygen. We investigated whether OTC repletes GSH levels and inhibits the decrease of SOD in cisplatin-treated HK-2 cells. As shown in Figure 5A, GSH concentrations were greater in HK-2 cells treated with OTC and cisplatin together than in those treated with cisplatin alone. Cisplatin administration alone significantly reduced the SOD activity by 70%. However, cisplatin-induced decrease of SOD activity was restored by OTC (Figure 5B).

OTC inhibits NF-κB activation in cisplatin-induced renal injury

Western blot analysis showed that the levels of NF-κB p65 protein in nuclear extracts from HK-2 cells were increased after cisplatin treatment compared with the levels in the control. The increased NF-κB p65 levels in nuclear protein extracts from HK-2 cells after cisplatin treatment were decreased by the OTC treatment. In contrast, levels of NF-κB p65 protein in cytosol prepared from cisplatin-treated HK-2 cells were decreased compared with the levels in the control. The decreased NF-κB p65 levels in cytosol from HK-2 cells treated with cisplatin were increased by the OTC treatment (Figure 6A). EMSA revealed that NF-κB-DNA binding in nuclear extracts from HK-2 cells treated with cisplatin was decreased significantly by the administration of OTC (Figure 6B). Consistent with the western blot and EMSA data, the positive staining of NF-κB p65 was markedly increased in renal tubular cells from cisplatin-treated mice. The increased staining of NF-κB p65 was decreased significantly by the treatment with OTC (Figure 6C–G).

OTC reduces ICAM-1 and MCP-1 expression in cisplatin-induced renal injury

Chemokines and adhesion molecules play an important role in the migration and activation of inflammatory cells during inflammatory processes.
**Fig. 5.** Effect of OTC on GSH concentrations and SOD activity in cisplatin-treated HK-2 cells. GSH levels were measured in HK-2 cells using the Glutathione Assay kit II (A). SOD activities were measured in HK-2 cells using the Superoxide Dismutase Activity Assay kit (B). Control without cisplatin treatment, Con; OTC treatment alone, OTC; Treated with cisplatin without OTC treatment, Cis; Treated with cisplatin and OTC, Cis + OTC. Data were expressed as percent increase compared with saline-treated control group. Data represent mean ± SEM from four independent experiments. *P < 0.05 vs saline; †P < 0.05 vs cisplatin.

**Fig. 6.** Effect of OTC on NF-κB activation in cisplatin-induced acute renal injury. NF-κB p65 expression in nuclear and cytosolic protein extracts from HK-2 cells (A). Representative EMSA of nuclear extracts from HK-2 cells (B). NF-κB expression was measured in control without cisplatin treatment (Con), OTC treatment alone (OTC), HK-2 cells treated with cisplatin without OTC treatment (Cis), and HK-2 cells treated with cisplatin and OTC (Cis + OTC). Comp, cold competition control; NS, non-specific; FP, free probe. (C–F) Immunohistochemical detection of translocational NF-κB p65 in renal tissues. (C) Con, (D) OTC, (E) Cis, (F) Cis + OTC. Magnification, 400 x. In OTC-treated mice group with cisplatin, nuclear stainings in renal tubular cells were significantly reduced compared with cisplatin-treated group. (G) The number of NF-κB activated cells in 10 × 400 fields. Data represent mean ± SEM from four independent experiments. ND, not detected. *P < 0.05 vs saline; †P < 0.05 vs cisplatin.
In immunostaining, when compared with renal tissue obtained from vehicle-treated mice, renal tissue obtained from cisplatin-treated mice revealed a marked increase of ICAM-1 staining in the brush border of proximal tubules and interstitium. In renal tissues obtained from mice administered with cisplatin, OTC treatment demonstrated markedly reduced staining for ICAM-1 in tubular epithelial cells and interstitium by cisplatin treatment (Figure 7C). Administration with OTC significantly decreased the expression of ICAM-1 after cisplatin injection (D). (E) Western blots and corresponding densitometric analyses of ICAM-1 expression in renal tissue. Data represent mean ± SEM from four independent experiments. *P < 0.05 vs saline; †P < 0.05 vs cisplatin. Magnification, 400×.

**Discussion**

The present study clearly demonstrates that (i) cisplatin increases ROS generation and NF-κB activation in HK-2 cells and kidney; (ii) cisplatin up-regulates ICAM-1 and MCP-1 expression, resulting in the enhanced infiltration of macrophages to kidney; and (iii) administration of OTC results in a significant reduction of all pathophysiological features examined in cisplatin-induced renal injury through the restoration of the antioxidant system.

The production of ROS through oxidative stress in the kidney has been implicated in the pathogenesis of cisplatin-induced renal injury [2,3]. One report has suggested that renal lipid peroxidation is increased while the GSH level is decreased in cisplatin nephrotoxicity [12]. ROS have been shown to play an important role in renal tubulointerstitial inflammation and fibrosis. The generation of ROS is capable of activating the transcription factor NF-κB, leading to the synthesis of a wide range of pro-inflammatory adhesion molecules, cytokines and chemokines such as ICAM-1 and MCP-1. The activation of NF-κB also promotes immune and inflammatory responses [13]. Thus, the agents that can down-regulate the ROS generation and the activation of NF-κB are potential candidates for therapeutic intervention in cisplatin-induced renal injury [14].

An increase of the intracellular GSH concentration reduces the tissue damage caused by toxic

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**OTC inhibits the infiltration of macrophages in cisplatin-induced renal injury**

As shown in Figure 9, the infiltration of macrophages was markedly increased in cisplatin-induced renal injury, and the increase was significantly reduced by the administration of OTC (38 ± 6 vs 15 ± 4 in 10 × 400 fields, P < 0.05).
effects of free radicals. The generation of oxygen radicals depletes the supply of GSH, leaving tissues vulnerable to damage by oxygen radicals [5,15]. The rate of GSH synthesis is limited by the amount of cysteine, a GSH precursor. OTC is a cysteine prodrug and GSH replenishing agent, and is converted to cysteine by the intracellular enzyme, oxoprolinase. Thus, the administration of OTC replenishes the GSH level effectively. However, it has been reported that the activity of oxoprolinase in humans is significantly lower than that found in rats [16].

Consistent with the previous reports [2,3], in the present study, we have found that ROS production is markedly increased in cisplatin-treated HK-2 cells and that the administration of OTC decreases the production of ROS induced by the administration of cisplatin. In addition, OTC restores GSH concentrations and counteracts the reduction in SOD in cisplatin-treated HK-2 cells. These data have suggested that OTC might recover the antioxidant system in the kidney.

Henderson et al. [17] have reported that the development of oxidant/antioxidant imbalance in airway inflammation leads to the activation of redox-sensitive transcription factor NF-κB. In our study, consistent with immunostaining, NF-κB protein levels
in nuclear protein extracts from HK-2 cells is significantly increased in cisplatin-treated HK-2 cells. Since NF-κB activation has been known to induce chemokines and adhesion molecules [18], we have assessed whether ICAM-1 and MCP-1 are up-regulated in cisplatin-induced renal injury. As expected, cisplatin treatment significantly increases the expression of ICAM-1 and MCP-1. It has been suggested that the infiltration of inflammatory cells into injured tissue is one of the key steps in the inflammatory responses. ICAM-1 and MCP-1 play an important role in inflammatory cell migration and activation during an inflammatory response. Our study has shown that the infiltration of F4/80-positive cells is increased after cisplatin treatment. The administration of OTC results in a significant decrease in NF-κB activation, expression of ICAM-1 and MCP-1, and macrophages infiltration into the kidney.

Jo et al. [11] have demonstrated that cisplatin treatment activates multiple caspases, resulting in apoptotic cell death in renal tubular cells and induces renal tubular necrosis. Consistent with these observations, our results show that cisplatin treatment increases caspase 3 activity and the number of TUNEL-positive apoptotic cells, and induces tubular necrosis in renal tissue. However, cisplatin-induced renal tubular necrosis and apoptosis are significantly reduced by the treatment of OTC.

Several studies have suggested that ROS may be second messengers in NF-κB activation and that antioxidants suppress NF-κB activation by preventing IκB degradation [19,20]. Our results reveal that OTC indirectly suppresses NF-κB activation that is induced by ROS and subsequently inhibits the transcription of a variety of inflammatory genes in cisplatin-induced renal injury in mice (Figure 10).

In conclusion, our study provides evidence that OTC, which decreases the ROS levels and subsequently, the activation of NF-κB can have significant potential for therapeutic intervention in cisplatin-induced renal injury.

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Conflict of interest statement. None declared.

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