IN INVOLVEMENT OF ACTIVATION OF NADPH OXIDASE AND EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) IN RENAL CELL INJURY INDUCED BY ZINC

Yoshiko MATSUNAGA, Yoshiko KAWAI, Yuka KOHDA and Munekazu GEMBA

Division of Pharmacology, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

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ABSTRACT — Zinc is employed as a supplement; however, zinc-related nephropathy is not generally known. In this study, we investigated zinc-induced renal cell injury using a pig kidney-derived cultured renal epithelial cell line, LLC-PK1, with proximal kidney tubule-like features, and examined the involvement of free radicals and extracellular signal-regulated kinase (ERK) in the cell injury.

The LLC-PK1 cells showed early uptake of zinc (30 μM), and the release of lactate dehydrogenase (LDH), an index of cell injury, was observed 24 hr after uptake. Three hours after zinc exposure, generation of reactive oxygen species (ROS) was increased. An antioxidant, N, N'-diphenyl-p-phenylenediamine (DPPD), inhibited a zinc-related increase in ROS generation and zinc-induced renal cell injury. An NADPH oxidase inhibitor, diphenyleneiodonium (DPI), inhibited a zinc-related increase in ROS generation and cell injury. We investigated translocation from the cytosol fraction of the p67phox subunit, which is involved in the activation of NADPH oxidase, to the membrane fraction, and translocation was induced 3 hr after zinc exposure.

We examined the involvement of ERK1/2 in the deterioration of zinc-induced renal cell injury, and the association between ERK1/2 and an increase in ROS generation. Six hours after zinc exposure, the activation (phosphorylation) of ERK1/2 was observed. An antioxidant, DPPD, inhibited the zinc-related activation of ERK1/2. An MAPK/ERK kinase (MEK1/2) inhibitor, U0126, almost completely inhibited zinc-related cell injury (the release of LDH), but did not influence ROS generation.

These results suggest that early intracellular uptake of zinc by LLC-PK1 cells causes the activation of NADPH oxidase, and that ROS generation by the activation of the enzyme leads to the deterioration of renal cell injury via the activation of ERK1/2.

KEY WORDS: Zinc injury, Nephrotoxicity, Oxidative stress, NADPH oxidase, ERK, Renal epithelial cell

INTRODUCTION

Zinc shows the second highest content among transition metals that are present in the human body, following iron, and the level of zinc is markedly lower than levels of sodium, potassium, magnesium, and calcium. Therefore, zinc is classified as a biological trace element. It is known that zinc is involved in many biological phenomena, such as the biosynthesis of nucleic acid, neurotransmission, and gene expression, in vivo. Previously, it has been reported that many enzymes and transcription factors contain zinc (Vallee and Falchuk, 1993; Berg and Shi, 1996; Grummt et al., 1986; Petrie et al., 1991). Zinc is necessary for cellular proliferation and differentiation (Schmidt and Beyersmann, 1999), and zinc deficiency causes apoptosis in cultured cells and rodents (McCabe et al., 1993; Sunderman, 1995).

Zinc is essential for cranial nerve function; however, an excessive amount of zinc in the brain causes cranial nerve cell injury (Choi and Koh, 1998; Lobner et al., 1997; Manev et al., 1997). Antioxidants inhibit this type of cell injury, suggesting the involvement of reactive oxygen species (ROS) in the development of cell injury (Ryu et al., 2002). Furthermore, one study
has reported that activation of a mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)1/2, as an intracellular signal transmission molecule, is involved in the deterioration of zinc-related cranial nerve cell injury (Seo et al., 2001).

The kidney is readily influenced by metals, and it is speculated that free radicals are involved in mercury or cadmium-related renal toxicity (Goering et al., 2002; Thevenod, 2003). Recently, zinc has been recognized as a nutritional food in addition to magnesium and copper, and employed as a supplement. However, zinc-related nephropathy is not generally known. In this study, we investigated zinc-induced renal cell injury using a cultured renal epithelial cell line, LLC-PK₁, with proximal kidney tubule-like features, and examined the involvement of ROS and ERK activation in the deterioration of cell injury.

**MATERIALS AND METHODS**

**Cell culture**

A pig kidney-derived LLC-PK₁ cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The LLC-PK₁ cells were cultured in Dulbecco’s Modified Eagle Medium (D-MEM) and nutrient medium F-12 (1:1) (Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 5% fetal bovine serum (FBS, Trace Scientific Ltd., Melbourne, Australia) and 14.3 mmol/L sodium bicarbonate. The cells after proliferation were counted as described below. After the medium was aspirated, the cell surface was washed twice in phosphate buffered saline (PBS−; 137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na₃HPO₄, 1.5 mmol/L KH₂PO₄) that had been warmed to 37°C. Thereafter, trypsin-EDTA (0.05% trypsin, 0.53 mmol/L EDTA-4Na solution; Invitrogen Corp., Carlsbad, CA, U.S.A.) was diluted with PBS− 10-fold, and added to the cells. A few minutes later, D-MEM/F-12 containing 5% FBS was placed in a dish, and the cells were collected. The cell suspension was mixed with 0.3% trypsin blue solution at an equivalent volume, and the cell count was measured using a Burker-Turk blood cell counter.

Using a new medium, the cells were adjusted to a density of 3 × 10⁵ cells/ml, placed in the dish for cell passage, and cultured in a CO₂ incubator at 37°C. The interval until the confluence of the cells was 4 days, and then the cells were used for our experiment.

The medium containing the cells with the confluence was exchanged for D-MEM/F-12 without FBS. Two hrs later, the medium was mixed with zinc chloride (Wako Pure Chemical Industries, Co., Ltd.). Various agents, including antioxidants, were added when the medium was exchanged.

**Measurement of intracellular zinc content**

The medium containing the cells was exposed to zinc for a specific duration, and aspirated with an aspirator. Then the cells were washed 3 times in PBS− that had been cooled on ice. After the cells were treated with 0.05% trypsin-EDTA, a portion of the cell suspension was collected, and on trypsin blue staining, the cell count was measured using the cell counter. The residual cell suspension was centrifuged at 4°C and 3,000 r.p.m. for 10 min, and the supernatant was aspirated with an aspirator. The sediment was dried at 100°C overnight, mixed with 69% HNO₃, and placed at 60°C for 2 days to induce incineration. Measurement was performed using an atomic absorption instrument (AA-670/G V-4, Shimadzu Corp.). The zinc content was corrected with the cell count.

**Measurement of the rate at which lactate dehydrogenase (LDH) is released**

The medium containing the cells was centrifuged at 4°C and 3,000 r.p.m. for 10 min to isolate the supernatant. Thereafter, the precipitating cells were fused with 1% triton X-100, and ultrasonic treatment was performed for 15 sec to homogenize the cell suspension. We measured LDH activity in the supernatant and cell suspension, and calculated the rate at which LDH was released from the cells to the medium as an index of cell injury. For the measurement of LDH activity, we used a commercially available LDH-Cytotoxicity Test Wako kit (Wako Pure Chemical Industries, Co., Ltd.).

**Western blot analysis**

The cells collected from the medium were suspended in 100 μL of 20 mmol/L Tris-HCl (pH 7.5) containing 0.25 mol/L sucrose, 2 mmol/L EDTA, and 2 mmol/L EGTA, and then cracked by ultrasonic treatment. The cell suspension was centrifuged at 4°C and 2,900 r.p.m. for 10 min to remove untreated cells. The supernatant was centrifuged at 4°C and 4,500 r.p.m. for 5 min to remove nuclei. In addition, the supernatant was centrifuged at 4°C and 13,000 r.p.m. for 5 min to remove mitochondria. The supernatant was centrifuged at 4°C and 40,000 r.p.m. for 60 min, regarding the supernatant as cytosol fraction and the sediment as microsome fraction (Powner et al., 2002; West et al., 2001). The microsome fraction was mixed with the above Tris-HCl solution (pH 7.5) containing 1% triton.
NADPH oxidase and ERK in zinc injury of renal cells.

X-100, and solubilized by ultrasonic treatment for 20 sec. The solution was centrifuged at 4°C and 17,000 r.p.m. for 15 min, and the supernatant was used as a microsome fraction sample.

The cells were suspended in 200 µL of a hypertonic buffer (10 mmol/L HEPES (pH 7.6) containing 15 mmol/L KCl, 2 mmol/L MgCl₂, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 0.2% Nonidet P-40), and incubated on ice for 5 min. After centrifugation, the sediment was regarded as nuclear fraction. This was suspended in a hypertonic buffer (25 mmol/L HEPES (pH 8.0) containing 50 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% glycerol, and 0.4 mol/L NaCl), and solubilized by ultrasonic treatment for 20 sec. The suspension was centrifuged at 4°C and 18,000 × g for 15 min, and the supernatant was used as a nuclear fraction sample (Zoja et al., 1998). To prevent proteolysis, the above solutions were mixed with a 1% protease inhibitor (Nakalai Tesque, Inc.).

Using the cytosol, microsome, and nuclear fraction samples, as prepared above, 50 mA constant current electrophoresis with 10% polyacrylamide gel was performed (Celis and Gromov, 2000). Thereafter, transcription to nitrocellulose membrane (Amersham Biosciences Corp., NJ, U.S.A.) at 50 mA constant current was performed for 60 min using a semidry type transcription device (ATTO Corp.). The transcription membrane was immersed in TBS-T (50 mmol/L Tris-HCl (pH 7.5) containing 150 mmol/L NaCl, 0.1% Tween 20, and 1% bovine serum albumin), and placed at 4°C overnight for blocking. Then the transcription membrane was reacted with a mouse anti-p67phox antibody (B.D. Transduction Laboratories) and a mouse anti-phosphorylated ERK1/2 antibody (Sigma) at 37°C for 30 min. This membrane was washed in TBS-T 3 times, and reacted with a secondary antibody (sheep anti-mouse IgG-HRP conjugate, Amersham Biosciences Corp., NJ, U.S.A.) at 37°C for 30 min. After this membrane was washed in TBS-T 3 times, it was reacted with a luminescent substrate (Amersham Biosciences Corp., NJ, U.S.A.) for 5 min, and luminescence was detected using a lumino-image analyzer (LAS-1000, Fujifilm).

Measurement of the protein level

To measure the protein level, we used a commercially available kit (BCA Protein Assay Kit, Pierce Biotechnology, Inc., IL, U.S.A.), in which the bicinchoninic acid (BCA) method is employed, and calculated the protein level from a standard curve prepared using bovine serum albumin (Brenner and Harris, 1995).

Detection of ROS generation

For the culture of LLC-PK₁ cells, we used a slide glass (Matsunami Glass IND., LTD) with a sterile MAS coat. The medium was exchanged for D-MEM/F-12 without FBS, containing a fluorescence reagent for ROS detection, 5′,6′-chloromethyl-2′,7′ dichlorodihydro-fluorescein diacetate (CM-H₂DCFDA; Molecular Probes, Inc., OR, U.S.A.), at a concentration of 1 µmol/L, and reacted at 37°C for 15 min (Ubezio and Civoli, 1994). Thereafter, the cells were fixed in 10% formalin at room temperature for 25 min, washed in PBS(−) twice, and dried in a draft. The cells were included using an inclusion agent (Fluoro Guard Antifade Reagent (Bio-Rad Laboratories, CA, U.S.A.) and a cover glass (Matsunami Glass IND., LTD). Fluorescence was detected using a confocal laser microscope (LSM-510, Carl Zeiss). CM-H₂DCFDA was detected in the presence of fluorescence at 510 nm related to excitation of argon/krypton mixed-gas laser at 488 nm (Liu et al., 2001; Mahadev et al., 2001; Hua et al., 2003).

Statistical analysis

All data are expressed as the mean ± S.E.M. Values were compared between two groups using ANOVA software, followed by Tukey's test (Gad and Weil, 1986). p<0.05 was regarded as significant.

RESULTS

Uptake of zinc in cultured renal epithelial cells LLC-PK₁ and cell injury

We investigated intracellular uptake of zinc contained in the medium and the rate of LDH release as an index of zinc-related cell injury. The intracellular zinc content was significantly increased at a zinc concentration of 30 µM or more 24 hr after zinc exposure, and the rate of LDH release was increased (Figs. 1A and 1B). At a zinc concentration of 50 µM, the rate of LDH release was markedly increased with a further increase in uptake of zinc.

We serially investigated the relationship between uptake of zinc after exposure to 30 µM of zinc and the release of LDH (Figs. 2A and 2B). The intracellular zinc content was increased more than 1 hr after zinc exposure, and uptake of zinc reached a maximum 24 hr after zinc exposure. The incidence of cell injury (the rate of LDH release) serially increased more than 24 hr after zinc exposure.
Influence of zinc on ROS generation in LLC-PK₁ cells

We serially examined the influence of zinc (30 μM) on ROS generation. In the LLC-PK₁ cells, ROS generation increased from 3 hr until 24 hr after zinc exposure (Photo 1).

Influence of an antioxidant, DPPD, on zinc-related ROS generation and renal cell injury

In the LLC-PK₁ cells, we investigated the influence of an antioxidant, N, N'-diphenyl-p-phenylenediamine (DPPD), on a zinc exposure-related increase in ROS generation and cell injury (the release of LDH).

DPPD (1 μM) almost completely inhibited an increase in ROS generation related to exposure to zinc (30 μM), and reduced the rate of LDH release to the control level (Photo 2 and Figs. 3).

Examination of the source of zinc-induced ROS generation

It is suggested that the activation of NADPH oxidase is involved in free radical-associated cranial nerve cell injury related to zinc. In this study, to examine the source of zinc-induced ROS generation in the LLC-PK₁ cells, we investigated the influence of an NADPH oxidase inhibitor, diphenyleneiodonium (DPI), on a zinc-related increase in ROS generation and zinc-related release of LDH. DPI (0.5 μM) almost completely inhibited an increase in ROS generation 3 hr after exposure to zinc (30 μM) (Photo 3). DPI (0.1, 0.5 μM) markedly inhibited an increase in the rate of LDH release 24 hr after zinc exposure (Fig. 4).

Influence of zinc on the activation of NADPH oxidase

We serially investigated the influence of exposure to zinc (30 μM) on translocation (activation) from the cytosol fraction of the p67phox subunit, which is

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Fig. 1. Concentration-dependence of zinc effect on intracellular zinc content (A) and cell injury (B). LLC-PK₁ cells were incubated in the medium with zinc at the indicated concentrations for 24 hr at 37°C. Values represent means ± S.E. of at least 3 experiments. *p<0.01 vs. control.

Fig. 2. Time-course of zinc effect on intracellular zinc content (A) and cell injury (B). LLC-PK₁ cells were incubated in the presence of zinc (30 μM) or absence (controls) for the indicated time intervals at 37°C. Values represent means ± S.E. of at least 3 experiments. *p<0.01 vs. respective control.
involved in the activation of NADPH oxidase, to the membrane fraction. Zinc markedly decreased the level of \( \text{p67}^{\text{phox}} \) in the cytosol fraction 3 hr after exposure, but markedly increased that in the microsome fraction (Fig. 5). The zinc-related changes in \( \text{p67}^{\text{phox}} \) in the two fractions became less marked 6 hr or more after exposure.

**Influence of zinc on the activation of ERK1/2**

It is suggested that the activation of a mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK1/2), is involved in the pathogenesis of zinc-related cranial nerve cell injury. In this study, to determine the activation of ERK1/2 in the LLC-PK\(_1\) cells, we measured the level of phosphorylated ERK1/2 in the nuclear fraction by Western blotting. Twenty-four hours after zinc exposure, the level of phosphorylated ERK1/2 was significantly increased at a zinc concentration of 30 \( \mu \text{M} \) or more, and this parameter increased in a dose-dependent manner (Fig. 6A). Exposure to zinc (30 \( \mu \text{M} \)) significantly increased the level of phosphorylated ERK1/2 six hours after exposure, when there was no cell injury (no increase in the rate of LDH release), and then serially increased the level of phosphorylated ERK1/2 until 24 hr after exposure (Fig. 6B).

To further investigate the involvement of ERK1/2 activation in zinc-related renal cell injury, we employed U0126, an inhibitor against MAPK/ERK kinase (MEK1/2), which catalyzes the phosphorylation of ERK1/2. U0126 (1.10 \( \mu \text{M} \)) inhibited an increase in the rate of LDH release 24 hr after exposure to zinc (30 \( \mu \text{M} \)) in a dose-dependent manner. This agent at 10 \( \mu \text{M} \) almost completely inhibited it (Fig. 7). However, U0126 (10 \( \mu \text{M} \)) did not influence any zinc (30 \( \mu \text{M} \)) related increase in ROS generation (Photo 4).

![Time-course of zinc effect on ROS generation](Image)

**Photo 1.** Time-course of zinc effect on ROS generation.
LLC-PK\(_1\) cells were incubated in the presence of zinc (30 \( \mu \text{M} \)) for the indicated time intervals at 37\(^\circ\)C.
Photo 2. Effect of an antioxidant, DPPD, on zinc-induced ROS generation. LLC-PK₁ cells were incubated in the medium with 30 μM zinc at 37°C for 3 hr.

Photo 3. Effect of an NADPH oxidase inhibitor, DPI, on zinc-induced ROS generation. LLC-PK₁ cells were incubated in the medium with 30 μM zinc at 37°C for 3 hr.

Fig. 3. Effect of an antioxidant, DPPD, on zinc-induced cell injury. LLC-PK₁ cells were incubated in the medium with 30 μM zinc at 37°C for 24 hr. Values represent means ± S.E. of at least 3 experiments. *p<0.01 vs. control, #p<0.01 vs. "ZnCl₂ 30 μM".

Fig. 4. Effect of an NADPH oxidase inhibitor, DPI, on zinc-induced cell injury. LLC-PK₁ cells were incubated in the medium with 30 μM zinc at 37°C for 24 hr. Values represent means ± S.E. of at least 3 experiments. *p<0.01 vs. control, #p<0.01 vs. "ZnCl₂ 30 μM".
Influence of an antioxidant, DPPD, on the zinc-related activation of ERK1/2

An antioxidant, DPPD (0.1, 1 μM), inhibited the zinc (30 μM)-related activation of ERK1/2 in a dose-dependent manner. DPPD at 1 μM almost completely inhibited it (Fig. 8).

DISCUSSION

In this study, we investigated zinc-induced cell injury using a cultured renal epithelial cell line, LLC-PK₁, with proximal kidney tubule-like features, and examined the mechanism.

Many studies have reported the involvement of zinc in nucleic acid and protein metabolism, and zinc deficiency-related cellular dysfunction. With respect to cell disorders caused by an excessive amount of zinc,

Fig. 5. Time-course of zinc effect on p67phox level in cytosol fraction (A) and microsomal fraction (B). LLC-PK₁ cells were incubated in the presence of 30 μM zinc for the indicated time intervals. Values represent means ± S.E. of at least 3 experiments.

*p<0.01 and *p<0.05 vs. respective control.

Fig. 6. Effect of zinc on phosphorylated ERK (pERK) 1/2 in the nuclei fraction prepared from LLC-PK₁ cells. LLC-PK₁ cells were incubated in the medium with zinc at the indicated concentrations at 37°C for 24 hr (A) and in the presence of zinc (30 μM) for the indicated time intervals at 37°C (B). Values represent means ± S.E. of at least 3 experiments.

*p<0.01 vs. control.

Fig. 7. Effect of a MEK1/2 inhibitor, U0126, on zinc-induced cell injury. LLC-PK₁ cells were incubated in the medium with 30 μM zinc at 37°C for 24 hr. Values represent means ± S.E. of at least 3 experiments.

*p<0.01 vs. control, #p<0.01 vs. "ZnCl₂ 30 μM".
cranial nerve cell injury has previously been reported. Several studies have indicated that antioxidants and radical scavengers relieve these disorders (Kim et al., 1999; Kim et al., 1999), and that a decrease in the level of glutathione (GSH), which plays an important role in maintaining intracellular reduction, is involved (Ryu et al., 2002), suggesting the involvement of ROS. Zinc is used as a nutritional food; however, no study has reported zinc-related renal cell injury. This study showed that zinc damaged the LLC-PK1 tubular epithelial cells at 30 μM, which is 2 times higher than the normal plasma level of zinc, 24 hr after zinc exposure. Furthermore, renal cells showed extremely early uptake of zinc, and zinc increased ROS generation 3 hr after zinc exposure. An antioxidant, DPPD, almost completely inhibited it. In addition, DPPD inhibited zinc-related cell injury, suggesting the involvement of ROS in the deterioration of zinc-related disorders.

With respect to the source of ROS, which increases in the presence of zinc, the involvement of NADPH oxidase in cranial nerve cells has been reported (Noh and Koh, 2000; Kim and Koh, 2002). As the source of zinc-related ROS generation, we investigated the involvement of NADPH oxidase, which has been reported to be present in renal epithelial cells (Cu and Douglas, 1997). An NADPH oxidase inhibitor, DPI, inhibited a zinc-related increase in ROS generation and renal cell injury. Furthermore, the p67phox subunit, which is involved in the activation of NADPH oxidase, showed translocation to the membrane fraction (the activation of NADPH oxidase) 3 hr or more after zinc exposure. Therefore, zinc ingested by cells in the early phase may cause the activation of NADPH oxidase (translocation of the p67phox subunit to the membrane fraction), increasing ROS generation.

Previously, it has been reported that the activation of a MAPK, ERK1/2, is involved in zinc-related cranial nerve cell injury (Seo et al., 2001). In many kinds of cells, several studies have indicated that the activation of ERK1/2 contributes to cell proliferation, differentiation, and survival (Sinha et al., 2003; Derkinderen et al., 1999; Midwinter et al., 2001). However, the activation of ERK is involved in free radical-associated renal cell injury (Kohda et al., 2003). In this study, we examined the zinc-related activation of ERK1/2, and confirmed the activation (phosphorylation) of ERK1/2 more than 6 hr after exposure, prior to zinc-related cell injury. Furthermore, U0126, an inhibitor against MAPK/ERK kinase (MEK1/2), which catalyzes the phosphorylation of ERK1/2, relieved zinc-related cell

![Photo 4](http://example.com/photo4.png)

**Photo 4.** Effect of a MEK1/2 inhibitor, U0126, on zinc-induced ROS generation. LLC-PK1 cells were incubated in the medium with 30 μM zinc at 37°C for 3 hr.

![Fig. 8](http://example.com/fig8.png)

**Fig. 8.** Effect of an antioxidant, DPPD, on zinc-induced ERK1/2 activation. LLC-PK1 cells were incubated in the medium with 30 μM zinc at 37°C for 24 hr. Values represent means ± S.E. of at least 3 experiments.

* <p>0.01 vs. control, # p<0.01 vs. "ZnCl2 30 μM".
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Injury, suggesting that the activation of ERK1/2 is an important factor for zinc-related cell injury.

Previously, it has been reported that the activation of MAPK, including ERK, in the proximal tubular cells is involved in cell disorders related to an increase in ROS generation (Ramachandiran et al., 2002). In this study, we investigated the influence of DPPD on the activation of ERK1/2, and DPPD markedly inhibited the zinc-related activation of ERK1/2. Furthermore, U0126 did not influence any zinc-related increase in ROS generation. Therefore, a zinc-related increase in ROS generation via NADPH oxidase may cause the activation of ERK1/2, deteriorating renal cell injury.

In conclusion, zinc at 30 μM, which is 2 times higher than the normal plasma level of zinc, was immediately ingested by the LLC-PK1 tubular epithelial cells, causing the activation of NADPH oxidase and promoting ROS generation. This suggests that such ROS generation probably activates ERK1/2 to lead to the deterioration of renal cell injury.

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