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INVOLVEMENT OF RAF-1/MEK/ERK1/2 SIGNALING PATHWAY IN ZINC-INDUCED INJURY IN RAT RENAL CORTICAL SLICES

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ABSTRACT — Zinc is an essential nutrient that can also be toxic. We have previously reported that zinc-related renal toxicity is due, in part, to free radical generation in the renal epithelial cell line, LLC-PK_1 cells. We have also shown that an MEK1/2 inhibitor, U0126, markedly inhibits zinc-induced renal cell injury. In this study, we investigated the role of an upstream MEK/ERK pathway, Raf-1 kinase pathway, and the transcription factor and ERK substrate Elk-1, in rat renal cortical slices exposed to zinc. Immediately after preparing slices from rat renal cortex, the slices were incubated in medium containing Raf-1 and MEK inhibitors. ERK1/2 and Elk-1 activation were determined by Western blot analysis for phosphorylated ERK (pERK) 1/2 and phosphorylated Elk-1 (pElk-1) in nuclear fractions prepared from slices exposed to zinc. Zinc caused not only increases in 4-hydroxynonenal (4-HNE) modified protein and lipid peroxidation, as an index of oxidant stress, and decreases in PAH accumulation, as that of renal cell injury in the slices. Zinc also induced a rapid increase in ERK/Elk-1 activity accompanied by increased expressions of pERK and pElk-1 in the nuclear fraction. A Raf-1 kinase inhibitor and an MEK1/2 inhibitor U0126 significantly attenuated zinc-induced decreases PAH accumulation in the slices. The Raf-1 kinase inhibitor and U0126 also suppressed ERK1/2 activation in nuclear fractions prepared from slices treated with zinc. The present results suggest that a Raf-1/MEK/ERK1/2 pathway and the ERK substrate Elk-1 are involved in free radical-induced injury in rat renal cortical slices exposed to zinc.

KEY WORDS: Nephrotoxicity, Zinc injury, Raf-1 kinase, ERK, Elk-1, Kidney cortical slices

INTRODUCTION

The kidney, in particular the proximal tubule, is a major target site for the toxic effects of various metals, and is particularly susceptible to the toxic effects of cadmium and mercury (Goyer, 1996). Reported cases of zinc toxicity in humans, though few, suggest that zinc is potentially more toxic than once believed (McKinney *et al.*, 1994; Broun *et al.*, 1990; Chobanian, 1981; Potter, 1981). Very little is known about the renal effects of zinc after acute exposure.

Zinc is an essential nutrient that can also be toxic (Vallee and Falchuk, 1993). Recently, we have evaluated zinc toxicity using a pig kidney-derived cultured renal epithelial cell line, LLC-PK_1, with proximal kidney tubule-like features (Matsunaga *et al.*, 2005). We have also shown a mitogen-activated protein kinase (MAPK) /extracellular signal-regulated kinase (ERK) 1/2 kinase (MEK1/2) inhibitor, U0126, markedly inhibits zinc-induced renal cell injury in LLC-PK_1 cells (Matsunaga *et al.*, 2005).

Zinc is a ubiquitous element that is essential for normal enzymatic function in multiple metabolic pathways (Calesnick and Dinan, 1988). Excessive zinc ingestion causes severe reversible anemia in humans (Broun *et al.*, 1990; Bennett, 1997; Igic *et al.*, 2002). In animals, zinc toxicity leads to anemia as well as physiologic and morphologic damage to the pancreas, and often multisystem failure and death (Latimer *et al.*, 1989; Luttgen *et al.*, 1990; Smith and Embling, 1994). Zinc is essential for cranial nerve function; however, an excessive amount of zinc in the brain causes cranial nerve cell injury (Lobner *et al.*, 1997; Manev *et al.*, 1997; Choi and Koh, 1998). Antioxidants inhibit this

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type of cell injury, suggesting the involvement of free radicals in the development of cell injury (Ryu *et al.*, 2002). We have also previously reported that zinc-related renal toxicity is due, in part, to free radical generation in LLC-PK_1 cells (Matsunaga *et al.*, 2005).

Recent studies have identified zinc as an activator of ERK (Park and Koh, 1999). It has been reported that activation of the MAPK, ERK1/2, as an intracellular signal transmission molecule, is involved in the deterioration of zinc-related cranial nerve cell injury (Seo *et al.*, 2001). We previously reported that the MEK/ERK pathway plays a crucial role in zinc-mediated renal cell injury in LLC-PK_1 (Matsunaga *et al.*, 2005). Numerous studies have demonstrated that the ERK pathway is activated by a variety of growth factors, and the role of ERK pathway activation in the proliferation of cells has been well elucidated (Boulton *et al.*, 1991; Davis, 1993). Several *in vivo* studies have also shown that the ERK cascade is phosphorylated in the damaged brain caused by ischemia and hypoglycemia (Campos-Gonzalez and Kindy, 1992; Kindy, 1993). However, the ERK pathway has been known to play a role in giving a survival advantage to cells (Bonni *et al.*, 1999; Cobb, 1999). The biological outcome of ERK activation may be attributed to differences in stimuli and cell types. MAPK can transduce cellular signals into nuclear transactivation events. Members of the ERK1/2 of MAPK are phosphorylated and activated by MEK; on activation, ERK1/2 phosphorylates and activates the transcription factor Elk-1, among other substrates (Hill and Treisman, 1995). Activated Elk-1 interacts with the serum response factor to mediate transcription through serum response elements (Sukhatme, 1990). It has been reported that zinc activates ERK, and induces Elk-1-dependent gene expression in colorectal cancer cells (Park *et al.*, 2002). Wang *et al.* have observed that an MEK inhibitor, U0126, effectively attenuates ischemic brain injury through inhibition of ERK and Elk-1 activation (Wang *et al.*, 2004). We hypothesize that the activation of ERK and Elk-1 are involved in the zinc-induced renal cell injury.

The aims of the present study were firstly, to determine the toxicity of the inorganic metal compound zinc chloride ($ZnCl_2$) to the kidney and secondarily, to investigate the role of the Raf-1/MEK/ERK1/2 signaling pathway in zinc-related injury in rat renal cortical slices exposed to zinc.

MATERIALS AND METHODS

Chemicals

$ZnCl_2$ was purchased from Wako Pure Chemical Ind. (Osaka, Japan). The antioxidant, *N*, *N*'-diphenyl-*p*-phenylenediamine (DPPD), was obtained from Tokyo Kasei Industries Co. (Tokyo, Japan). The Raf-1 kinase inhibitor and MEK1/2 inhibitor U0126 were obtained from Calbiochem Novabiochem (Bad Soden, Germany). The anti-4-hydroxynonenal (4-HNE)-modified antibody was obtained from NOF Co. (Tokyo, Japan). The anti-phospho ERK1/2 antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA). The anti-phospho Elk-1 antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Protease inhibitor cocktails were obtained from Nacalai Tesuque, Inc. (Kyoto, Japan). All other chemicals used were of the highest purity available (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Zinc-induced nephrotoxicity in rats

Male Sprague-Dawley rats were used in all experiments. They were the progeny of rats obtained from SLC Inc. (Shizuoka, Japan) and were maintained in the central animal facility of our university. The animals were provided with a commercial diet and water ad libitum under temperature-, humidity- and lighting-controlled conditions ($22 \pm 2°$, $55 \pm 5\%$, and a 12:12-hr light-dark cycle, respectively). Six-week-old male rats received a single intravenous injection of 15 mg/kg $ZnCl_2$. The animals were sacrificed under pentobarbital anesthesia (50 mg/kg, i.p.), and their kidneys were removed at 24 hr after the injections. Blood samples were drawn from the abdominal aorta after zinc treatment. Urine was collected into bottles on ice after zinc treatment by placing the rats in metabolic cages for 18 hr. These samples were used to measure urinary *N*-acetyl-β-D-glucosamidase (NAG) levels by a colorimetric method using a spectrophotometer.

Preparation and incubation of renal cortical slices

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and their kidneys were rapidly removed, decapsulated and placed in ice-cold isotonic saline solution. Renal cortical slices were prepared freehand using a razor blade on an ice-cold petri dish, and were then placed in ice-cold medium containing 134 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ 11.5 mM glucose and 5.8 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), titrated with NaOH at pH 7.4, as described previously

(Gemba *et al.*, 1985). The slices were transferred to flasks containing the same medium. $ZnCl_2$, DPPD, an MEK1/2 inhibitor and a Raf-1 kinase inhibitor were added to the incubation medium, which was identical to the medium described above. The slices were incubated in the medium at 37°C with a gas phase of 100% oxygen for 90 min. The nuclear fraction was prepared from the slices after incubation. The slices and medium were analyzed for lipid peroxidation and *p*-aminohippurate (PAH) accumulation as described below.

Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) in the medium according to the method of Buege and Aust (Buege and Aust, 1985). Briefly, the incubation medium, centrifuged at 1,500×g for 10 min at 4°C, was measured by assaying the absorbance at 535 nm after the mixture of samples and thiobarbituric acid were heated in boiling water and then neutralized. The level of TBARS in the medium was expressed as nmol per g wet weight of slices incubated.

Determination of PAH accumulation

The slices were incubated in the medium with 0.074 mM PAH for the determination of PAH accumulation. After incubation, the slices were blotted on filter paper, weighed and homogenized with 10% trichloroacetic acid (TCA). Aliquots of samples obtained by centrifugation of the homogenate and medium, which were also treated with 10% TCA, were analyzed to determine the concentration of PAH by the method of Bratton and Marshall (Bratton and Marshall, 1939). PAH accumulation in the slices was expressed as the ratio of the level of PAH in slices (S) to medium (M). Accumulation of PAH in renal cortical slices was used as an index of renal cell injury (Hirsch, 1976; Cojocel *et al.*, 1983).

Preparation of nuclear fraction

Nuclear fractions were prepared from the kidney cortex exposed to zinc by a modification of the method of Dignam *et al.* (1983). Kidney cortical slices were rapidly placed in ice-cold hypotonic buffer, which consisted of 10 mM HEPES (pH7.6), 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.2% Nonidet P-40 with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) to prevent proteolysis and/or dephosphorylation. The slices were homogenized in a glass homogenizer and centrifuged at 850×g for 10 min at 4°C. The resultant pellets were suspended in hypotonic buffer, and recentrifuged at 850×g for 10 min at 4°C. The nuclear pellet was disrupted in hypertonic buffer, which consisted of 25 mM HEPES (pH8.0), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.4 M NaCl and protease inhibitors, for 30 min on ice for use as the nuclear fraction and recentrifuged at 18,000×g for 15 min at 4°C. Its supernatant was used as the soluble nuclei fraction and for Western blot analysis. The PIERCE protein estimation kit was used to determine the protein concentration with bovine serum albumin as the standard.

Western blotting analysis

1. ERK phosphorylation

Equal amounts of the soluble nuclear protein (10 μg protein) in the above fraction were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose membranes using a semi-dry blotting system. The membranes were blocked with 3% bovine serum albumin (BSA) in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20 (TBST) overnight at 4°C, and then incubated with the primary antibody specific against phospho-ERK (pERK) and phospho-Elk-1 (pElk-1) in 1% BSA-TBST for 30 min at 37°C. The membranes were washed three times in TBST to remove unbound antibodies and then incubated with a horseradish peroxidase-conjugated secondary antibody in 1% TBST for 30 min at 37°C. Enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech, NJ, USA) were used to detect the immunoreactive bands of pERK.

2. 4-HNE-protein adducts

Cortical tissues were homogenized in ice-cold precipitation buffer, which consisted of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.1 mM egtazic acid, 1.0% Nonidet P-40, 0.1% SDS and 0.5% sodium deoxycholate supplemented with protease inhibitor cocktail (Nacalai, Tesque), followed by centrifugation at 15,000×g for 30 min. The supernatant was collected; equal amounts of homogenate protein were separated by 12% SDS-PAGE under reducing conditions. The separated proteins were electrotransferred to nitrocellulose membranes. The membranes were blocked with 0.3% skimmed milk in TBST for 1 hr at 4°C, and then incubated with the primary antibody specific against the anti-4-HNE protein adducts

overnight at 4°C. After extensive washing with TBST, the bound antibodies were detected with a secondary antibody in TBST for 1 hr at room temperature. Detection of the immunoreactive bands of 4-HNE-protein adducts were adapted from protocols previously described.

Measurement of zinc contents in the renal cortex

The zinc contents of the renal cortex were investigated by atomic absorption analysis. The renal cortex (100 mg) was placed in a dryer at 100°C overnight, and incubated in 1 mL of 69% HNO_3. Zinc levels were quantified by atomic absorption spectrometry (Solaar A-880, Jarrell-Ash). The zinc standard solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) included equivalent amounts of HNO_3.

Statistical analysis

Data are presented as means ± S.E. The significance of the differences between groups was analyzed by Scheffe's *F* test (Scheffe, 1953) after analysis of variance. p values less than 0.05 were considered significant.

RESULTS

Effect of an antioxidant DPPD administration in zinc-induced nephrotoxicity

Acute renal failure was induced by 24 hr after the zinc (15 mg/kg $ZnCl_2$) administration. The urinary excretion rate of NAG was remarkably higher in zinc-administered rats compared with normal controls (Fig. 1).

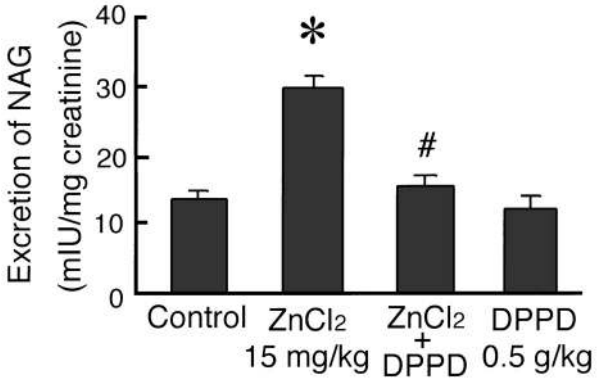

Fig. 1. Effect of an antioxidant, DPPD (0.5 g/kg, i.p.), on urinary excretion of *N*-acetyl-β-D-glucosamidase (NAG) in rats 24 hr after one i.v. injection of zinc chloride ($ZnCl_2$, 15 mg/kg).
Each value represents the mean ± S.E. obtained from 3 independent experiments. *p<0.01, compared with the control. #p<0.05, compared with "$ZnCl_2$".

Protective effect of an antioxidant DPPD, against the free radical-induced zinc nephrotoxicity was tested. Pretreatment with DPPD (0.5 g/kg, i.p.) at 2 hr before the zinc injection significantly decreased the zinc-induced increases in NAG levels normally seen at 24 hr after the zinc injection (Fig. 1). No significant differences in NAG levels were observed after the injection of DPPD alone.

Zinc levels in the kidney cortex and plasma showed no significant differences in zinc-treated rats and zinc and DPPD-treated rats (data not shown). Treatment with DPPD did not affect the accumulation of zinc in the kidney cortex.

Effect of an antioxidant DPPD on zinc-induced ERK phosphorylation in the nuclei

Rats treated intravenously with a single dose of 15 mg/kg $ZnCl_2$ were sacrificed 24 hr after treatment. To investigate the effect of DPPD on zinc-mediated ERK phosphorylation, nuclear fractions prepared from the kidney cortex of rats treated with zinc were subjected to Western blotting with anti-phospho ERK. The phosphorylation of ERK was clearly observed in nuclear fraction prepared from the kidney cortex of rats 24 hr after zinc administration. DPPD significantly suppressed the zinc-induced increase in phosphorylated ERK in nuclear fractions (Fig. 2).

Effects of zinc on lipid peroxidation and PAH accumulation in rat renal cortical slices

Next, *in vitro* experiments were carried out using rat renal cortical slices. We examined the effects of different $ZnCl_2$ concentrations on lipid peroxidation and the index of injury in the slices. The level of lipid peroxidation was measured indirectly to evaluate free radical generation; and PAH accumulation in the slices was assessed as an index of cell injury induced by zinc. Zinc caused not only increases in lipid peroxidation, as assessed by TBARS, but also decreases in PAH accumulation in renal cortical slices in concentration-dependent manners (Figs. 3A and 3B).

Expression of phosphorylated ERK1/2 in nuclear fractions prepared from rat renal cortical slices exposed to zinc

To elucidate if ERK1/2 was associated with the development of zinc-induced injury in rat renal cortical

slices, ERK1/2 activation was determined by Western blot analysis for phosphorylated ERK1/2 using a pERK1/2 specific antibody in nuclear fractions prepared from the slices exposed to zinc. As shown in Fig. 4, there was a significant expression of phosphorylated ERK1/2 (pERK1 and pERK2) in nuclear fractions from slices treated with zinc in a concentration-dependent manner.

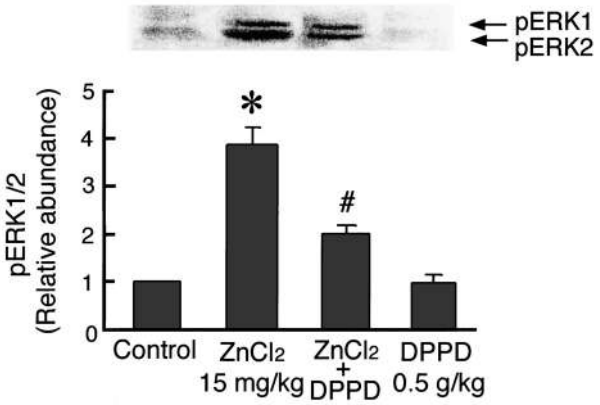

Fig. 2. Effect of an antioxidant, DPPD (0.5 g/kg, i.p.), on zinc-induced phosphorylation of ERK in the nuclei prepared from the kidney cortex of rats treated with zinc chloride ($ZnCl_2$,15 mg/kg) for 24 hr.
The phosphorylated ERK (pERK) bands were detected by Western blot analysis using each specific antibody against pERK1/2 and were quantitated by densitometric scanning. Blots are representative of three similar experiments. *p<0.01, compared with the control. #p<0.01, compared with "$ZnCl_2$".

Effect of the MEK1/2 inhibitor U0126 on zinc-induced renal cell injury and zinc accumulation in the rat renal cortex

To investigate whether the MEK/ERK pathway is involved in the development of renal damage induced by zinc, the MEK1/2 inhibitor U0126 was used. As shown in Fig. 5A, U0126 significantly attenuated zinc-induced decreases PAH accumulation in the slices. The MEK inhibitor alone had no effect PAH accumulation in the slices (Fig. 5A).

We investigated whether MEK inhibitor U0126 inhibits zinc-induced renal cell injury by reducing the zinc accumulation in the rat renal cortex. Zinc contents in the renal cortex exposed to $ZnCl_2$ were determined by atomic absorption spectroscopy. The MEK1/2 inhibitor U0126 had no effect on zinc accumulation in the slices (Fig. 5B).

Effects of the Raf-1 kinase and MEK1/2 inhibitors on zinc-induced renal cell injury and ERK phosphorylation in the nuclei

To investigate whether the Raf-1/MEK1/2 pathway is involved in the development of renal damage induced by zinc, the Raf-1 kinase and the MEK1/2

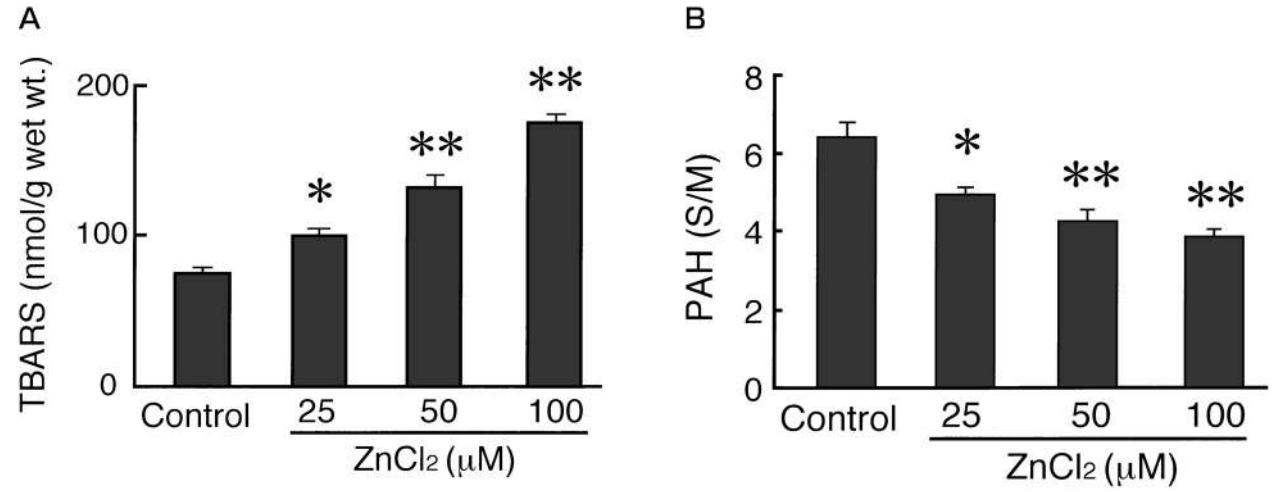

Fig. 3. Effect of zinc on lipid peroxidation and PAH accumulation in rat renal cortical slices.
The slices were incubated at 37°C for 90 min for the measurements of lipid peroxidation (TBARS) (A) and PAH accumulation (slice (S)/medium (M), S/M) (B) in the slices in medium containing zinc chloride ($ZnCl_2$). Each value represents the mean ± S.E. of five experiments. *p<0.05 and **p<0.01, compared with the control.

inhibitors were used. As shown in Fig. 6, the Raf-1 kinase and MEK1/2 inhibitors significantly attenuated zinc-induced decreases PAH accumulation in the slices. The Raf-1 kinase and MEK1/2 inhibitors alone had no effect on PAH accumulation in the slices (Fig. 6). ERK phosphorylation induced by zinc was blocked by the Raf-1 kinase and MEK inhibitors (Fig. 7).

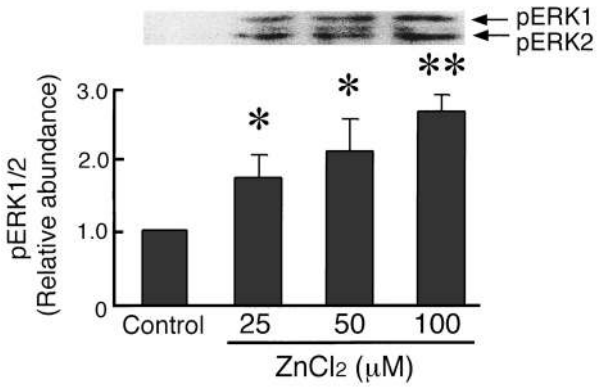

Fig. 4. Expression of phosphorylated ERK1/2 in nuclear fractions prepared from rat renal cortical slices exposed to zinc.
The slices were incubated at 37°C for 90 min in medium containing zinc chloride ($ZnCl_2$). Phosphorylated ERK1/2 (pERK) bands were detected by Western blot analysis using each antibody. The pERK bands were quantitated by densitometric scanning. Each value represents the mean ± S.E. of five experiments. *p<0.05 and **p<0.01, compared with the control.

Rapid expressions of 4-HNE-protein adducts and zinc accumulation in rat renal cortical slices exposed to zinc

Generation of 4-HNE was used as a marker of oxidant stress. Western blotting analysis was used to monitor 4-HNE-protein adduct in the kidney cortex. The slices were incubated at 37°C for 5, 10, 20 and 30 min in medium containing 100 μM $ZnCl_2$. As shown in Fig. 8A, several immunoreactive bands were observed in both control and zinc exposure groups, but the intensity of staining was greatest in the slices exposed to zinc.

Zinc contents in the renal cortex exposed to zinc chloride were determined by atomic absorption spectroscopy. The accumulation of zinc in the kidney cortex exposed to $ZnCl_2$ was rapidly observed (Fig. 8B).

Rapid expressions of phosphorylated ERK1/2 and Elk-1 in nuclear fractions prepared from rat renal cortical slices exposed to zinc

The above results indicate that the Raf-1/MEK/ERK1/2 pathway is involved in the development of renal cell injury induced by zinc. To investigate how these pathways might interact at the molecular level, we examined the status of ERK substrate Elk-1 after

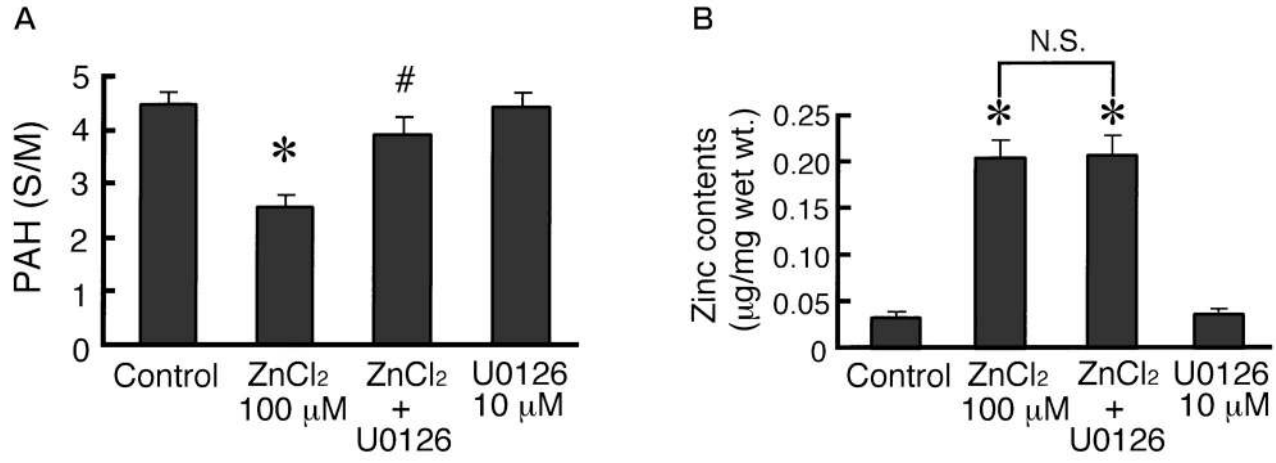

Fig. 5. Effects of MEK inhibitor, U0126, on zinc-induced renal cell injury and zinc accumulation in rat renal cortical slices exposed to zinc.
The slices were incubated at 37°C for 90 min for the measurements of PAH accumulation (slice (S)/medium (M), S/M) (A) and zinc accumulation (B) in the slices in medium containing zinc chloride ($ZnCl_2$) (100 μM) and U0126 (10 μM). Each value represents the mean ± S.E. of five experiments. *p<0.01, compared with the control. #p<0.01, compared with "$ZnCl_2$".

activation of the ERK pathway. As shown in Fig. 9A and 9B, ERK1/2 and Elk-1 were rapidly activated by zinc in nuclei prepared from renal cortical slices exposed to zinc.

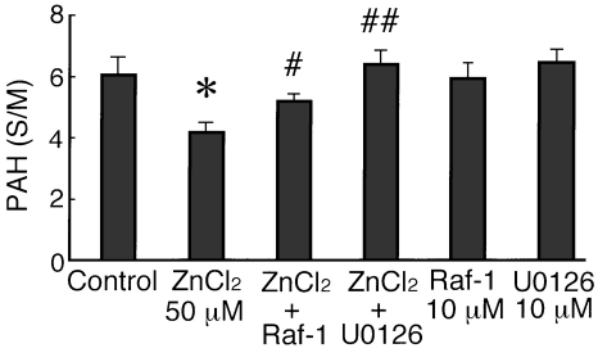

Fig. 6. Effects of Raf-1 kinase inhibitor and MEK inhibitor U0126 on PAH accumulation in rat renal cortical slices exposed to zinc.
The slices were incubated at 37°C for 90 min for the measurements of PAH accumulation (slice (S)/medium (M), S/M) in the slices in medium containing zinc chloride ($ZnCl_2$) (50 μM), Raf-1 kinase inhibitor (10 μM) and U0126 (10 μM). Each value represents the mean ± S.E. of five experiments. *p<0.01, compared with the control. #p<0.05 and ##p<0.01, compared with "$ZnCl_2$".

DISCUSSION

In the present study, we investigated the roles of the intracellular signaling pathways, Raf-1 kinase and MEK/ERK, in zinc-induced renal cell injury. Previous studies have demonstrated that the MEK1/2 inhibitor, U0126, markedly inhibits zinc-induced renal cell injury in LLC-PK_1 cells (Matsunaga *et al.*, 2005). ERK activation has been reported to be involved in the downstream Raf-1 kinase dependent pathway. We showed that zinc-induced lipid peroxidation as an index of free radical generation and cellular injury, assessed by PAH accumulation in rat renal cortical slices, were ameliorated by a Raf-1 kinase inhibitor, and U0126, an MEK inhibitor. We also demonstrated that zinc increased phosphorylation of ERK in isolated nuclear fractions prepared from rat renal cortical slices, and that ERK activation was markedly reduced by a Raf-1 kinase inhibitor, and U0126, an MEK inhibitor.

It has previously been suggested that free radical generation contributes to the development of zinc-induced renal cell injury (Matsunaga *et al.*, 2005). It is reported that free radicals are involved in mercury or cadmium-related renal toxicity (Goering *et al.*, 2002; Thevenod, 2003). However, the interrelationship between metal-induced generation of ROS and signal transduction pathways are under investigation. The signal transduction pathway responsible for the free radical-induced injury caused by zinc was examined

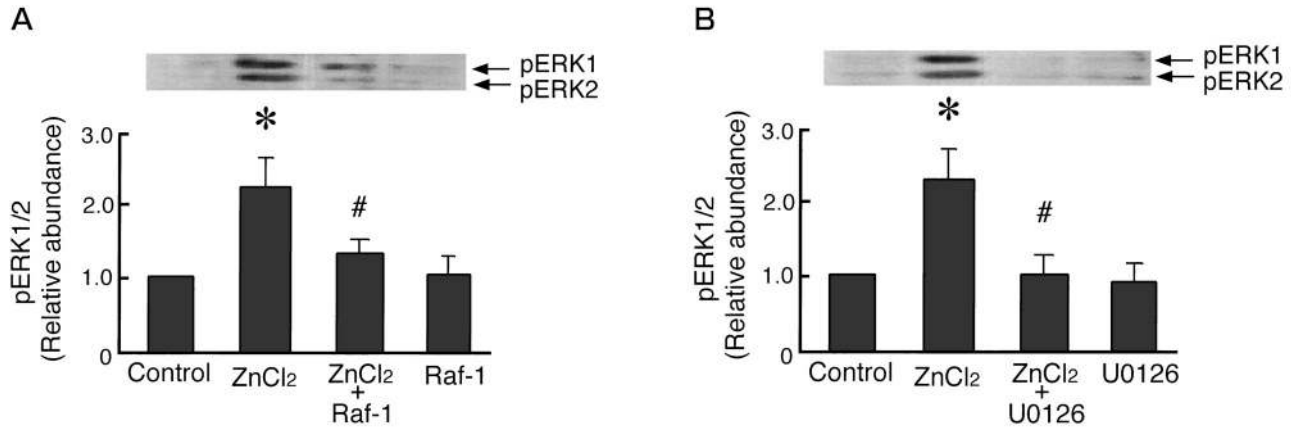

Fig. 7. Effects of Raf-1 kinase inhibitor and MEK inhibitor U0126 on zinc-induced ERK phosphorylation in the nuclei.
The slices were incubated at 37°C for 90 min in medium containing zinc chloride ($ZnCl_2$) (50 μM), Raf-1 kinase inhibitor (10 μM) (A) and U0126 (10 μM) (B). Phosphorylated ERK1/2 (pERK) bands were detected by Western blot analysis using each antibody. The pERK bands were quantitated by densitometric scanning. Each value represents the mean ± S.E. of five experiments. *p<0.01, compared with the control. #p<0.01, compared with "$ZnCl_2$".

using a renal cortical slices technique.

Zinc enhanced the expression of phosphorylated ERK1/2 (pERK1/2) levels in kidney cortex exposed to zinc. One possibility is to assume that MEK/ERK activation results in free radical-induced renal cell damage caused by zinc. Another possibility is that it results in renal cell survival. ERK is known to be regulated by several growth factors and cytokines and play an important role in cell proliferation (Segal and Greenberg, 1996; Derkinderen *et al.*, 1999). However, in our results, it is shown that the increases in ERK1/2 activation and urinary excretion of NAG caused by zinc

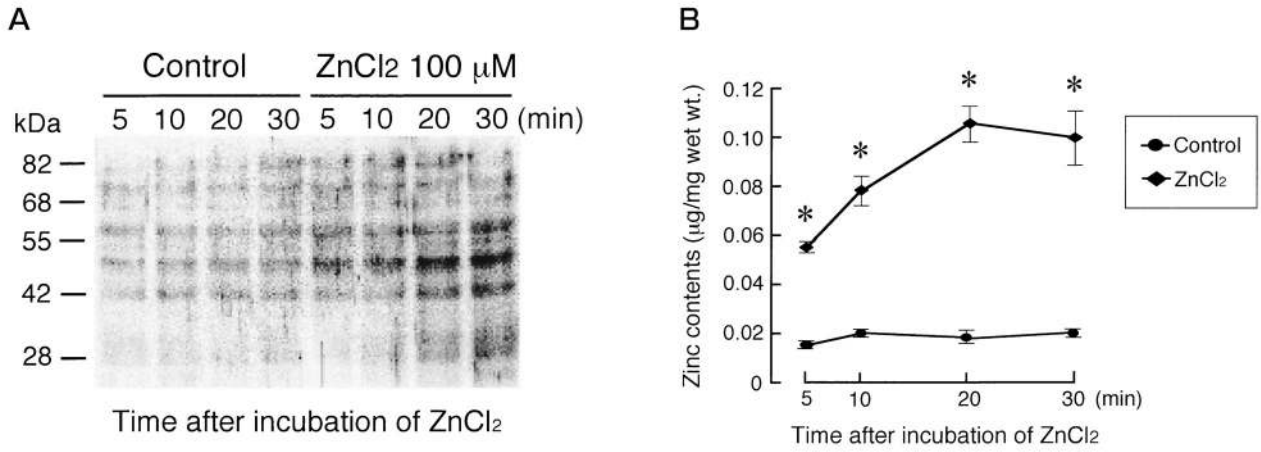

Fig. 8. Time-course of zinc effect on expressions of 4-HNE-protein adducts and zinc accumulation in rat renal cortical slices.
The slices were incubated at 37°C for 5, 10, 20 and 30 min in medium containing zinc chloride ($ZnCl_2$) (100 μM). Kidney proteins (15 μg) were separated by SDS-PAGE, transferred to a membrane, and probed with antiserum directed against 4-HNE-modified proteins (A). Zinc contents in the renal cortex exposed to $ZnCl_2$ were determined by atomic absorption spectroscopy (B). Each value represents the mean ± S.E. of three experiments. *p<0.01, compared with the respective control.

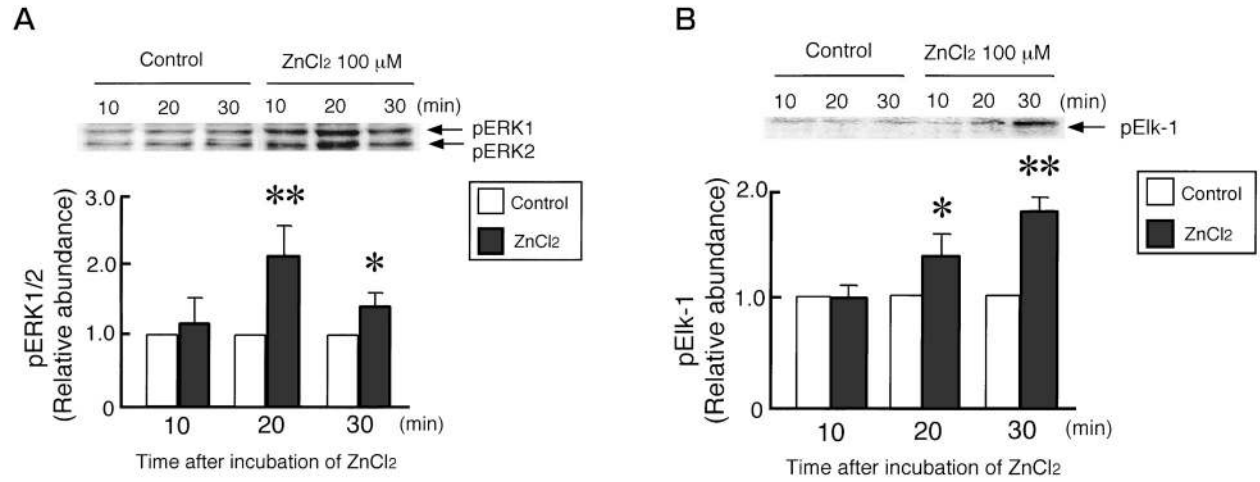

Fig. 9. Rapid expressions of phosphorylated ERK1/2 and Elk-1 in nuclear fractions prepared from rat renal cortical slices exposed to zinc.
The slices were incubated at 37°C for 10, 20 and 30 min in medium containing zinc chloride ($ZnCl_2$) (100 μM). Phosphorylated ERK1/2 (A) and pElk-1 (B) bands were detected by Western blot analysis using each antibody. The pERK (A) and pElk-1 (B) bands were quantitated by densitometric scanning. Each value represents the mean ± S.E. of three experiments. *p<0.05 and **p<0.01, compared with the respective control.

administration were suppressed by an antioxidant DPPD. Additionally, our findings clearly indicate that the Raf-1 kinase and MEK1/2 inhibitor U0126 ameliorated not only the observed increases in ERK activation but also the decreases in PAH accumulation in slices exposed to zinc. These findings suggest that activation of the Raf-1/MEK/ERK1/2 pathway is due to zinc-induced renal cell injury. To our knowledge, these are the first reported findings that the Raf-1/ERK1/2 signaling pathway is related to zinc-induced renal damage.

It remains unclear how the induction of ERK activation leads to zinc-induced renal cell injury. It has previously been reported that ERK activation contributes to cell death (Bhat and Zhang, 1999), and that ERK activation contributes to zinc toxicity in cortical neurons (Seo *et al*., 2001), and also to cisplatin-induced apoptosis in HeLa cells (Wang *et al*., 2000). Recently, it has been shown that intravenous administration of the MEK inhibitor, U0126, affords brain protection against forebrain ischemia and focal cerebral ischemia (Namura *et al*., 2001). It is shown here that zinc induced the phosphorylation (activation) of ERK in rat kidney cortex. In this study, zinc induced a rapid increase in Elk-1 phosphorylation as well as ERK activation. We have obtained preliminary data that the MEK inhibitor, U0126, attenuates the zinc-induced increase in Elk-1 phosphorylation. It has been suggested that ERK activation leads to activation of the transcription factor and ERK substrate Elk-1.

The level of pERK increased within 20 min to about two-fold in nuclear fractions prepared from rat renal cortical slices exposed to zinc. Zinc-induced ERK activation was decreased by 30 min, but, to our surprise, activation of ERK by zinc treatment for 90 min was similar to the level of pERK induced by treatment with zinc for 20 min. It has been reported that zinc induces the initial and prolonged activation of ERK in colorectal cancer cells (Park *et al*., 2002). We have hypothesized that biphasic activation of ERK by zinc may play a critical role in zinc-induced renal cell injury. Further studies are required to characterize the role of ERK activation involved in the renal damage.

Evidence for the involvement of an ERK-dependent pathway in zinc-induced renal cell injury is provided by the ability of zinc treatment to activate ERK and its substrate Elk-1, and the ability of the specific MEK inhibitor, U0126, to completely inhibit ERK activation and renal cell injury caused by zinc. Zinc probably activates ERK; specificity must reside in modulation of downstream signaling events by additional signals that, in aggregate, result in the upregulation of transcription contributing to renal cell injury.

We have previously reported that cephaloridine, a nephrotoxic antibiotic which induces ERK activation, may play a crucial role in free radical-induced nephrotoxicity (Kohda *et al*., 2003). We also demonstrated that ERK activation is involved in the cisplatin-induced renal dysfunction (Kohda *et al*., 2005). However, the effect of ERK activation in nephrotoxicity is still unclear. In this study, our data demonstrated that *in vitro* the close linking of Raf-1, MEK, ERK and Elk-1 in renal cells suggests that ERK activation is important in zinc-induced cytotoxicity.

The present data indicate that zinc activates the Raf-1/MEK/ERK1/2 pathway in rat renal cortical slices. ERK1/2 activation is further associated with the activation of the ERK substrate and transcription factor Elk-1, as determined by Western blot analysis, and these events mediate zinc-induced renal cell injury. The nature of the signaling events responsible for the transcriptional response of Elk-1 is under investigation. Taken together, these data suggest that zinc contributes to nephrotoxicity via Raf-1/MEK/ERK1/2 pathway- dependent mechanisms.

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