

## The protective effect of anti-oxidant in cadmium-induced hair cell death

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### SUMMARY

Cadmium (Cd<sup>2+</sup>) is a heavy metal and a potent carcinogen implicated in tumor development through occupational and environmental exposure. However, the auditory effect of Cd<sup>2+</sup> is not poorly understood. The purpose of the present study was to investigate whether prevent the ototoxic effects of Cd<sup>2+</sup> by antioxidant in auditory cell line, HEI-OC1. Rosmarinic acid is a naturally occurring phenolic compound and also known to possess marked antioxidant properties. We showed that antioxidant inhibited the the cytochrome c release induced by Cd<sup>2+</sup>. In addition, we showed that the regulatory effect of antioxidant on apoptosis is through the caspase-9 activation, and extracellular signal-regulated kinase activation in auditory cells. These results suggested antioxidant its therapeutic usefulness, against Cd<sup>2+</sup> induced activation of caspase-9 and ERK.

**Key words:** Cadmium; Antioxidant; Reactive oxygen species; Extracellular signal-regulated kinase

### INTRODUCTION

Cadmium (Cd<sup>2+</sup>) is a nonessential element that is widely used in industry, and is also found as a contaminant in many agricultural products (De Vries *et al.*, 2007). Cd<sup>2+</sup> contamination of the soil and water have raised concern because this metal bioaccumulates in the upper levels of the food chain, including in humans (Satarug *et al.*, 2003). Given its current rate of release into the environment, the Cd<sup>2+</sup> content in the human body is likely to

increase in the future and it has genotoxic effect (Bertin *et al.*, 2006), which can lead to a higher incidence of Cd<sup>2+</sup>-related diseases. The liver and kidneys have traditionally been considered the main targets of Cd<sup>2+</sup> toxicity (Horiguchi *et al.*, 2006; Nishijo *et al.*, 2006). Recently, it was reported that hair cells are more sensitive to cadmium than kidney tubule cells, and that the cochlear component of hearing is more vulnerable to cadmium toxicity than the other parts of the auditory system (Ozcaglar *et al.*, 2001).

Apoptosis plays not only an essential role in development and tissue homeostasis but is also involved in a wide range of pathological conditions (De Martinis *et al.*, 2007; Gatzka and Walsh 2007; Van Heemst *et al.*, 2007). In mammalian cells, there

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are two major caspase activation pathways: the extrinsic and intrinsic pathways. In the extrinsic pathway, binding of the death receptors causes the activation of caspase-8, which is an initiator caspase. In the intrinsic pathway, various forms of cellular stress cause mitochondrial alterations leading to mitochondrial membrane depolarization (MMP) and the release of cytochrome c (cyt c). In the cytosol, cyt c binds to and activates Apaf-1, which then activates pro-caspase-9. Active caspase-9 directly cleaves and activates the effector protease, caspase-3. The apoptotic pathway induced by Cd<sup>2+</sup> is controversial. Some studies strongly suggest that caspases play a key role in Cd<sup>2+</sup>-induced cell death (Kim *et al.*, 2000; Li *et al.*, 2000; Kondoh *et al.*, 2002), whereas other studies suggest that Cd<sup>2+</sup> induced the apoptosis through caspase-independent pathway (Harstad and Klaassen 2002; Shih *et al.*, 2003; Lemarie *et al.*, 2004). This apparently conflicting data suggests that the pathways of Cd<sup>2+</sup>-induced apoptosis differ according to the cell type or the exposure (cell treatment) conditions.

Mitogen-activated protein kinases (MAPKs) belong to a family of Ser/Thr protein kinases that transmit extracellular signals into the nucleus. There are three subfamilies of MAPKs including c-Jun NH2-terminal kinase (JNK; also known as stress-activated protein kinase), p38 MAPK, and extracellular signal-related kinase (ERK) (Schaeffer *et al.*, 1999). In general, the ERK signaling cascade is activated by growth factors and is associated with cell survival and proliferation (Lewis *et al.*, 1998; Puddicombe *et al.*, 2000). On the other hand, p38 and JNK are mainly activated by cellular stress and are often associated with inflammation and apoptosis. However, cumulative reports have indicated that these signaling pathways exhibit more complex roles in the regulation of distinct cellular effects. The cellular functions regulated by ERK, p38 or JNK seem to depend on the cell type, the stimulus, the duration and strength of kinase activities.

Rosmarinic acid (RA) is a naturally occurring phenolic compound. RA is an ester of caffeic acid

and 3,4-dihydroxyphenyllactic acid (Scarpati and Oriente, 1958) This compound is also known to possess marked antioxidant properties, acting as a reactive oxygen species scavenger and lipid peroxidation inhibitor (Kosar *et al.*, 2008). However, the protective effect of RA as an antioxidant on the auditory system is not completely understood. Thus, the aim of the present study was to investigate the ability of RA to prevent ototoxicity induced by Cd<sup>2+</sup> in auditory cell lines, HEI-OC1 cells.

## MATERIALS AND METHODS

### Reagents

Fetal bovine serum, and high-glucose Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO BRL (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and other reagents were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-human caspase-9, ERK, and cyt c antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The caspase assay kits were supplied by R & D system Inc. (Minneapolis, USA).

### Cell culture

The HEI-OC1 cell line was kindly donated by Dr. Federico Kalinec (House Ear Institute, Los Angeles, CA, USA). According to the report by Dr. Federico, establishment of immortal cell lines has been facilitated by the development of a transgenic mouse, Immortomouse<sup>TM</sup>, which harbors a temperature-sensitive mutant of the SV40 large T antigen gene under the control of an interferon- $\gamma$ -inducible promoter element. Cochlear half turns from Immortomice (Charles River Laboratories, Wilmington, MA, USA) at postnatal day 7 were cultured on uncoated plastic cultures dishes under permissive conditions (33°C) in DMEM (Gibco-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (JRH BIOSCIENCE, Lenexa, KS, USA) and 50 U/ml r-interferon without antibiotics. Cochlear explants

were moved to non-permissive conditions (39°C, 5% CO<sub>2</sub>) at different periods and allowed to differentiate for up to 180 days. Cultures were monitored on a daily basis by phase-contrast and video microscopy. Cells growing in the region formerly associated with the organ of corti were isolated by lifting them with a micropipette after a 2.5 min incubation of the explants with trypsin-EDTA. A cell line, termed HEI-OC1, was cloned using the limiting dilution method and characterized by phase-contrast light microscopy, Western blotting and immunofluorescence analysis. No antibiotics were used at any step of the cloning. HEI-OC1 cells, which express several molecular markers characteristic of the organ of corti sensory cells ( $\alpha$ -thyroid hormone, brain-derived neurotrophic factor, calbindin, calmodulin, Connexin 26, Math 1, Myosin 7a, organ of Corti protein 2, and prestin at permissive condition.), are extremely sensitive to ototoxic drugs. HEI-OC1 cells were cultured under permissive condition: 33°C, 10% CO<sub>2</sub> in DMEM supplemented (8 ml for 100 mm dish) with 10% fetal bovine serum. Cells were grown until they were 80.90% confluent. Confluent cells were incubated with various concentrations of chemicals. HEI-OC1 was cloned from cochlear explants via pathway of different culture stages (permissive condition and non-permissive condition). Cloned HEI-OC1 cells growing under permissive conditions (33°C, 10% CO<sub>2</sub>) express nestin, which suggests that they could retain multipotential stem cell properties (Kalinec *et al.*, 2003). Nestin makes HEI-OC1 cells retain their cell division activity.

#### Measurement of caspase-9 activity

The enzymatic activity of caspase-3 was assayed using a caspase colorimetric assay kit (R&D Systems) according to the manufacturer's protocol. Briefly, the cells were treated with CSCYT, then lysed in a lysis buffer. The lysed cells were centrifuged at 14,000 rpm for 5 min. The protein supernatant was incubated with reaction buffer (50  $\mu$ l) and caspase substrate (5  $\mu$ l) at 37°C for 2 h. The absorbance was

measured using a plate reader at a wavelength of 405 nm. Equal amounts of the total protein from each lysate were quantified using a bicinchoninic acid protein quantification kit (Sigma).

#### Western blot analysis

For analysis of the levels of cyt *c*, caspase-9, ERK, and p-ERK, stimulated cells were rinsed twice with ice-cold PBS and were then lysed in ice-cold lysis buffer (1% Triton, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate in PBS). Cell lysates were centrifuged at 15,000 g for 5 min at 4°C; the supernatant was then mixed with an equal volume of 2  $\times$  SDS sample buffer, boiled for 5 min, and then separated through 10% SDS-PAGE gels. After electrophoresis, the protein was transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% skim milk for 2 h, rinsed, and incubated overnight at 4°C with primary antibodies in PBS/0.5% Tween 20. Excess primary antibody was then removed by washing the membranes four times in PBS/0.5% Tween 20, and the membranes were incubated for 1 h with HRP-conjugated secondary antibodies (against mouse, goat, or rabbit). After three washes in PBS/0.5% Tween 20, the protein bands were visualized by an enhanced chemiluminescence assay (Amersham Pharmacia Biotech, NJ, USA) following the manufacturer's instructions.

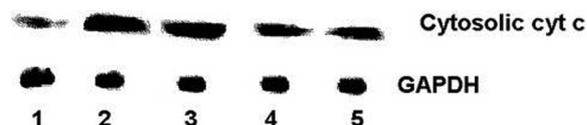
#### Statistical analysis

The experiments shown are a summary of the data from at least-three experiments and are presented as the mean  $\pm$  S.E.M. Statistical evaluation of the results was performed by ANOVA with Tukey post hoc test. The results were considered significant at a value of  $P < 0.05$ .

## RESULTS

#### The effect of RA on cyt *c* release in HEI-OC1 cell lines

Cyt *c* is released after mitochondrial membrane permeabilization, and induces apoptosis (Garrido *et al.*, 2006). Western blot analysis was performed



**Fig. 1.** The Effect of RA on cyt *c* release. Cells ( $5 \times 10^6$ /well) were preincubated for 2 h with RA (1 - 100 mM) and then treated with  $\text{Cd}^{2+}$  (10 mM) for 24 h, and then the cyt *c* was determined by Western blot analysis. 1) Control; 2)  $\text{Cd}^{2+}$  treatment; 3) RA (1 mM) +  $\text{Cd}^{2+}$ ; 4) RA (10 mM) +  $\text{Cd}^{2+}$ ; 5) RA (100 mM) +  $\text{Cd}^{2+}$ .

to assay the effect of RA on the release of cyt *c* induced by  $\text{Cd}^{2+}$ . The results showed that  $\text{Cd}^{2+}$  treatment increased cyt *c* levels in the cytosol, but RA suppressed the induction the cyt *c* levels (Fig. 1A). The relative expression level of cyt *c* was measured using an image analyzer (Fig. 1B).

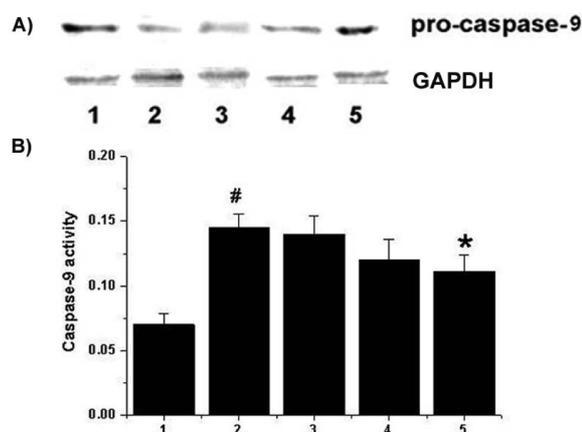
#### The effect of RA on caspase-9 activation in HEI-OC1 cell lines

To investigate whether RA regulate the caspase-9 activation, we performed the Western blot analysis. Cells were pretreated with RA (1 - 100 mM) for 2 h, and then treated with  $\text{Cd}^{2+}$  (10  $\mu\text{M}$ ) for 24 h. The results showed that  $\text{Cd}^{2+}$  reduced the pro-caspase-9 expression, which is an inactive form of caspase-9, but RA inhibited this phenomenon in a dose-dependent manner.

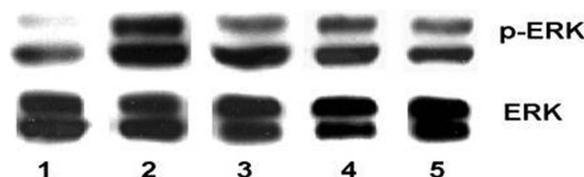
Next, we confirm whether RA inhibit the caspase-9 activation, we used caspase-9 assay. As shown Fig. 2B,  $\text{Cd}^{2+}$  induced the caspase-9 activation, these increase level was inhibited by the treatment of RA.

#### The effect of RA on ERK activation in HEI-OC1 cell lines

Previous study showed that the ERK inhibitor effectively suppressed cell death induced by  $\text{Cd}^{2+}$ . (Kim *et al.*, 2008) From this result, we can presuppose that  $\text{Cd}^{2+}$ -induced apoptosis occurs through ERK activation. To investigate the regulatory effect of RA on activation of ERK, Western blot analysis was performed. As shown Fig. 3, we showed that the pretreatment of RA inhibited the ERK activation in auditory cells.



**Fig. 2.** The Effect of RA on  $\text{Cd}^{2+}$ -induced caspase-9 activation. A) Cells ( $5 \times 10^6$ /well) were treated with various concentrations of RA (1 - 100 mM) for 2 h and then stimulated with  $\text{Cd}^{2+}$  (10 mM) for 24 h. The protein extracts were assayed by Western blot analysis for procaspase-9. GAPDH was used for loading control. B) Cells ( $3 \times 10^6$ /well) were treated with various concentrations of RA (1 - 100 mM) for 2 h and then stimulated with  $\text{Cd}^{2+}$  (10 mM) for 24 h. Caspase-9 activity was determined by a colorimetric kit using substrates. 1) Control; 2)  $\text{Cd}^{2+}$  treatment; 3) RA (1 mM) +  $\text{Cd}^{2+}$ ; 4) RA (10 mM) +  $\text{Cd}^{2+}$ ; 5) RA (100 mM) +  $\text{Cd}^{2+}$ . # $P < 0.05$ , significantly different from not-treated cells. \* $P < 0.05$ , significantly different from  $\text{Cd}^{2+}$  untreated cells. \* $P < 0.05$ , significantly different from  $\text{Cd}^{2+}$  alone-treated cells.



**Fig. 3.** The Effect of RA on ERK activation. Cells ( $5 \times 10^6$ /well) were treated with various concentrations of RA (1 - 100 mM) for 2 h and then stimulated with  $\text{Cd}^{2+}$  (10 mM) for 24 h, and then the phosphorylation of ERK was determined by Western blot analysis using specific anti-phospho-MAPKs. 1) Control; 2)  $\text{Cd}^{2+}$  treatment; 3) RA (1 mM) +  $\text{Cd}^{2+}$ ; 4) RA (10 mM) +  $\text{Cd}^{2+}$ ; 5) RA (100 mM) +  $\text{Cd}^{2+}$ .

## DISCUSSION

RA is a naturally occurring phenolic compound and it contributes to the beneficial and health-promoting effects of herbs (D'Amelio, 1999). This compound is also known to possess marked

antioxidant properties, (Kosar *et al.*, 2008), antiinflammatory (Osakabe *et al.*, 2004), antiangiogenic (Huang and Zheng, 2006), antitumor (McKay and Blumberg, 2006), antineurodegenerative (Lee *et al.*, 2008). However, the protective effect of RA on the auditory system is not completely elucidated. The findings herein show that antioxidant prevent the ototoxicity induced by  $\text{Cd}^{2+}$ . These results are expected to improve the understanding of the pharmacological mechanism and potential treatments for  $\text{Cd}^{2+}$ -induced ototoxicity.

$\text{Cd}^{2+}$  is a nonessential element widely used in industry, and is also found as a contaminant in agricultural products.  $\text{Cd}^{2+}$  contamination of soil and water have raised concern, because this metal is bioaccumulated in the upper levels of the food chain, including in humans, in which its biological half-life is approximately 20 years (Satarug *et al.*, 2003). Given its current rate of release into the environment,  $\text{Cd}^{2+}$  content in the human body is likely to increase in the future (Satarug *et al.*, 2003), which could lead to a higher incidence of  $\text{Cd}^{2+}$ -related diseases. Liver and kidney have traditionally been considered the main targets of  $\text{Cd}^{2+}$  toxicity (Horiguchi, *et al.*, 2006; Nishijo *et al.*, 2006). However, recent reports indicate that chronic exposure to low doses of  $\text{Cd}^{2+}$  may cause neurobehavioral problems in humans and other animals, despite a lack of detectable renal damage. These observations emphasize the importance of studying the effects of  $\text{Cd}^{2+}$  in other organs. Recently, it was reported that hair cells are more sensitive to cadmium than kidney tubule cells, and that the cochlear component of hearing is more vulnerable to cadmium toxicity than other parts of the auditory system.

Mitochondria factors such as cytochrome *c*, mediate the activation of pro-apoptotic proteases known as caspases. Activation of caspases-9 plays a central role in the execution-phase of cell apoptosis. After mitochondrial outer membrane permeabilization, intermembrane space proteins including cytochrome *c* are released to activate caspases in the cytoplasm. Cell death initiated by caspase activation is considered

to be the "classic" apoptotic pathway. Caspases, which are a family of cysteine-dependent aspartate-directed proteases, play important roles in initiating and executing apoptosis. Some studies strongly suggest that caspases play a key role in  $\text{Cd}^{2+}$ -induced cell death (Kim *et al.*, 2000; Li *et al.*, 2000; Kondoh *et al.*, 2002). Previously, we showed that the apoptosis mechanism of  $\text{Cd}^{2+}$  in auditory cells might occur, at least in part, through a caspase-dependent pathway (Kim *et al.*, 2008). Taken together, we supposed that the caspase-9 could be an effective target for anti-apoptotic therapy in auditory cells. Therefore, we investigated whether RA affects the cytochrome *c* release, and caspase-9 activation in induced by  $\text{Cd}^{2+}$ . We showed that RA abrogated  $\text{Cd}^{2+}$ -induced cytochrome *c* release, and caspase-3 activation. These results demonstrate that the anti-apoptotic effect of RA, at least in part, might be derived through the regulation of the mitochondria pathway such as cytochrome *c*, and caspase-9.

MAPKs pathways are central components of the intracellular signaling networks that control many aspects of mammalian cellular physiology including cell proliferation, differentiation, and apoptosis. In general, the ERK cascade is activated by growth factors and is associated with cell survival and proliferation (Lewis, *et al.*, 1998; Puddicombe *et al.*, 2000). On the other hand, p38 and JNK are mainly activated by cellular stress and are often associated with inflammation and apoptosis. However, cumulative reports have indicated that these signaling pathways exhibit more complex roles in the regulation of distinct cellular effects. The cellular functions regulated by ERK, p38 or JNK seem to depend on the cell type, the stimulus, the duration and strength of kinase activities (Nebreda and Porras, 2000). We showed that RA suppressed ERK activation induced by  $\text{Cd}^{2+}$ . These results suggest that the ERK pathway may be a potential therapeutic target to prevent ototoxic damage from  $\text{Cd}^{2+}$ . Although RA attenuated ERK activation, the effect of RA on other pathways involving MAPK upstream and downstream was not elucidated in the present

study. Thus, further investigation is necessary to clarify the role of RA on the MAPK pathway in the auditory system.

In conclusion, in the presence of Cd<sup>2+</sup>, an antioxidant counteracts the ototoxicity by suppressing the release of cyt c and activation of ERK and caspase-9. These results are expected to improve the understanding of the regulatory mechanism and potential treatments for Cd<sup>2+</sup>-induced ototoxicity.

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