Short-term depletion of catalase suppresses cadmium-elicited c-Jun N-terminal kinase activation and apoptosis: role of protein phosphatases

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The c-Jun N-terminal kinase (JNK) is a vital stress-activated signal that can be regulated differentially under oxidant or antioxidant conditions. Recently, we have reported that activation of JNK by cadmium chloride (Cd) contributes to apoptosis in CL3 human lung adeno-carcinoma cells. Although oxidative stress has been implicated in numerous biochemical effects altered by Cd, its role in Cd-elicited JNK activation has not been established. Here we report that catalase is crucial for the activation of JNK by Cd. Short-term treatment of 3-amino-1,2,4-triazole (3AT), a specific catalase inhibitor, completely suppressed the Cd-elicited JNK activation, conversely, exogenous addition of catalase increased the intensity and duration of JNK activation in Cd-treated CL3 cells. Co-administering high doses of H_2O_2 (500–1000 μM) with Cd also markedly decreased JNK activity, although at doses <200 μM H_2O_2 enhanced the Cd-elicited JNK activation in CL3 cells. 3AT also blocked JNK activation in Cd-treated normal human fibroblasts and Chinese hamster ovary cells, and in UV-irradiated CL3 cells. However, mannitol, a hydroxyl radical scavenger, did not alter the JNK activity in Cd-treated human and rodent cells. Intriguingly, sodium fluoride or okadaic acid, inhibitors for serine/threonine protein phosphatases (PP), recovered the JNK activity in CL3 cells exposed to Cd plus 3AT; however, the protein tyrosine phosphatases inhibitor sodium orthovanadate did not. Furthermore, 3AT decreased but catalase increased the Cd-induced cytotoxicity, apoptosis and procaspase-3 degradation in CL3 cells. Together, these results indicate that persistent activation of apoptotic JNK signal by Cd requires functional catalase and that short-term depletion of catalase activity may facilitate okadaic acid-sensitive PP to down-regulate the JNK activation and may predispose these cells to carcinogenic transformation upon Cd exposure.

Introduction

The mitogen-activated protein kinase (MAPK) family are vital signal transducers differentially activated in response to a wide diversity of extracellular stimuli including growth factors, cytokines and environmental stresses, thereby regulating cell proliferation, differentiation, survival and apoptosis (1–7). Activation of MAPKs is regulated through a three-kinase module composed of a MAPK, a MAPK kinase (MKK) and a MKK kinase (MKKK). These modules can be activated by small GTP binding proteins and MKKK kinases and inactivated by MAPK phosphatases. Activation of MAPKs requires a dual-phosphorylation of the Thr and Tyr residues in the catalytic domain, whereas dephosphorylation of one or two of these residues inactivates MAPKs through the action of serine/threonine protein phosphatase (PP), protein tyrosine phosphatase (PTP) or dual-specificity MAPK phosphatase (MKP) (8–11).

The c-Jun N-terminal kinase (JNK) is a member of MAPK family which can be activated by numerous environmental stresses including heavy metals, UV light, γ-radiation, DNA-damaging agents, cytotoxic drugs and protein synthesis inhibitors (5–7). The stress activated JNK has been shown to regulate several physiological and pathological processes, including growth arrest, DNA repair, apoptosis, transformation and survival (5–7). The multiplex roles of JNK primarily depend on the stimuli, cell types, duration of kinase activation and specific isoforms. The JNK is activated through upstream kinases MKK4 or MKK7, which can be activated by many MKKK (5–7). In addition to sequential physical interactions between members of a three-kinase module, scaffold proteins such as JNK interacting proteins organize the JNK into a specific signaling cassette to ensure the specificity in kinase activation (1,5–7). The JNK signal module can be inactivated by various MAPK phosphatases including MKP1, MKP2 and PP2A (8–10) and also negative regulated by redox-sensitive proteins such as thioredoxin and glutathione S-transferase π (GST) (12,13). In general, the activity of JNK reflects a balance between the actions of upstream kinases and negative control systems.

Cellular levels of reactive oxygen species (ROS) include hydrogen peroxide, superoxide, singlet oxygen and hydroxyl radical are influenced by endogenous redox processes as well as exogenous stimuli, conferring additional complexity for signal transduction (14). For example, intracellular H_2O_2 has been implicated to act as a signal molecule in the cascades triggered by platelet-derived growth factor, epidermal growth factor and cytokines (15,16). ROS is also involved in the activation of apoptotic JNK signals elicited by stresses, such as UV-irradiation and H_2O_2 (14). The roles of ROS in signal transduction have been implicated to directly alter the conformation and activity of kinases, phosphatases or redox-sensitive proteins in mammalian cells (14,17–19). For example, oxidative inactivation of the catalytically essential cysteine of PTP by H_2O_2 or UV contributes to the persistent activation of tyrosine kinases (18,20).

Cadmium (Cd) compounds are ubiquitous environmental toxicants that have been evaluated as human carcinogens

and can induce morphological transformations, chromosomal aberrations and gene mutations in cultured mammalian cells (21–23). ROS has been implicated in Cd genotoxicity (22–24). Recently, we have shown that Cd-elicited JNK contributes to cytotoxicity and apoptosis in CL3 human lung adeno-carcinoma cells (25). JNK activity can be induced by either antioxidants in Jurkat T lymphocytes (26), or H_2O_2 in HeLa (27) and CL3 cells (28), suggesting that JNK signaling is sensitive to a decreased or increased oxidative environment. To explore the roles of ROS in Cd-elicited JNK activation, in this study we co-exposed CL3 cells with Cd and ROS modulators including 3-amino-1,2,4-triazole (3AT; a catalase inhibitor), catalase, H_2O_2 and mannitol (a hydroxyl radical scavenger). Inhibitors for PP or PTP were adopted to examine the effects of phosphatases on JNK activation in cells treated with Cd and ROS modulators. Here we demonstrate that catalase is involved in the Cd-elicited persistent activation of apoptotic JNK and short-term depletion of catalase activity facilitates okadaic acid-sensitive PP to down-regulate the Cd-activated JNK.

Materials and methods

Cell culture

CL3 cells were established from a non-small-cell lung carcinoma tumor of a 60-year-old male patient (29). HFW human fibroblasts were initiated from the foreskin of a Taiwanese infant (30). Chinese hamster ovary-K1 (CHO-K1) cells were obtained from American Type Culture Collection (Rockville, MD). CL3, HFW and CHO-K1 cells were cultured in RPMI-1640, DMEM and F12/DMEM (1:1) media (Gibco, Life Technologies, Grand Island, NY), respectively, supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 U/ml), streptomycin (100 mg/ml) and fetal calf serum (10%). CL3 and CHO-K1 cells were maintained at 37°C in a humidified incubator containing 5% CO_2 in air, while HFW cells cultured in a 10% CO_2 incubator.

Treatment

Cells in exponential growth were plated 1 day before serum starvation for 16–18 h and exposed to $CdCl_2$ (Merck, Darmstadt, Germany), $NaAsO_2$ (Merck), H_2O_2 (Merck) or UV (254 nm) in serum-free media. The incident dose of UV was 2 J/m^2 per second measured using an UVX radiometer (UVP Inc., CA). In experiments to determine the effect of ROS on Cd-induced JNK activation and cytotoxicity, serum-starved cells were treated with 80 mM 3AT (Sigma, St Louis, MO), 80 mM D-mannitol (Merck) or 3000 U/ml catalase (Boehringer Mannheim, Germany) for 2 h followed by adding $CdCl_2$ for 3 h. In H_2O_2 co-treatment experiments, serum-starved cells were treated with $CdCl_2$ for 2.5 h before the addition of H_2O_2 for 30 min. To examine the effect of phosphatase, serum-starved cells were pre-treated with okadaic acid (Calbiochem, San Diego, CA) for 1 h or sodium fluoride or sodium ortho-vanadate for 30 min before exposure to $CdCl_2$ for 3 h.

Cytotoxicity

Immediately after treatment, cells were washed twice with phosphate-buffered saline (PBS) and trypsinized, diluted and plated at a density of 200–1000 cells/60-mm Petri dish in triplicate. The cells were cultured for 14 days and stained with 1% crystal violet solution. The colony numbers were counted for cytotoxicity determination that was calculated as the number of colonies in the treated cells were divided by those colonies obtained in the untreated cells.

Apoptosis

Annexin V-fluorescein isothiocyanate (FITC) binding assay (Medical & Biological Laboratories, Nagoya, Japan) was performed to determine the apoptotic cell death. After treatment, 2×10^5 cells were collected and followed by two washes with PBS. Next, the cells were suspended in a binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) and stained with 1 ng/ml of annexin V-FITC and 5 ng/ml of propidium iodide for 15 min in the dark. The stained cells were analyzed directly by flow cytometry using the Cell Quest program (Becton Dickinson, Franklin, NJ). Apoptotic cells were detected by those stained with annexin V-FITC but not with propidium iodide.

Whole cell extract preparation

Cells were rinsed twice with cold PBS and lysed at 4°C in a whole cell extract (WCE) buffer [20 mM HEPES pH 7.6, 75 mM NaCl, 2.5 mM $MgCl_2$,

0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride]. The samples were rotated at 4°C for 30 min, centrifuged at 10 000 r.p.m. for 15 min and the precipitates discarded. The BCA protein assay kit (Pierce, Rockford, IL) was adopted for the determination of protein concentrations using BSA as a standard.

JNK kinase assay

JNK in WCE (50 µg proteins) was interacted with GST–cJun(1-79) (5 µg) and glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech, Arlington Heights, IL) and the kinase reaction was performed in a buffer (20 mM HEPES pH 7.6, 20 mM $MgCl_2$, 2 mM DTT, 1 mM NaF and 0.1 mM Na_3VO_4) of a total volume of 30 µl containing 20 µM ATP and 2 µCi [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Pharmacia Biotech) as described previously (25). After kinase assay, the reaction mixture was separated on 12% SDS–polyacrylamide gel. The gel was dried and subjected to autoradiograghy. The relative radioactivity was determined using a computing densitometer equipped with the ImagQuant analysis program (Molecular Dynamics, Sunnyvale, CA).

Western blotting

Equal amounts of proteins in WCE from each set of experiments were fractionated on 10% SDS–polyacrylamide gel and subjected to western blot analysis as described previously (25). Rabbit polyclonal antibody against MKP-1 and mouse monoclonal antibody against α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against JNK1/2 was from Pharmingen (San Diego, CA). Antibody against procaspase-3 was from Transduction Laboratory (Lexington, KY).

Catalase activity assay

After treatments, cells were washed four times with PBS and harvested using a rubber policeman. The cells were centrifuged at 1000 r.p.m. for 5 min at 4°C and the precipitates were sonicated in 0.5 ml of 100 mM phosphate buffer (pH 7.0) followed by centrifuged at 10 000 r.p.m. for 30 min at 4°C. The supernatant was then used for the determination of catalase activity and protein concentration. Catalase activity assay was performed at room temperature in a 1-ml mixture containing clear cell lysate, 100 mM phosphate buffer (pH 7.0) and 10 mM H_2O_2. The decomposition of H_2O_2 was followed directly by a decrease in absorbance at 240 nm. Enzyme activity was expressed in micromole of H_2O_2 decrease per minute per milligram protein.

Measurement of intracellular peroxide

After treatments, cells were washed twice with PBS and trypsinized. Cells (1×10^6) were suspended in PBS and incubated with 80 µM of $2',7'$-dichlorofluorescin diacetate (Estman Kodak, Rochester, NY) for 30 min at 37°C in the dark. The cells were then centrifuged at 1000 r.p.m. for 5 min. The cell pellets were kept in ice and resuspended in 2 ml of cold PBS before fluorescence detection. The oxidation of intracellular peroxide with activated $2',7'$-dichlorofluorescin diacetate would result in dichlorofluorescein, which was detected using a fluorescence spectrophotometer (Hitachi F-4000) with excitation and emission wavelength at 502 and 523 nm, respectively.

Determination of cellular Cd level

Following treatment, the cells were washed three times with PBS and the numbers of cells were determined. One million cells were centrifuged and the cell pellet was sonicated in MilliQ-purified water. Total cellular Cd concentrations were analyzed by an inductively coupled plasma-mass spectrometer (SCIEX ELAN 5000, Perkin Elmer, Norwalk, CT). The conditions were as followed: power of 1000 W, plasma flow rate of 15 l/min, auxiliary flow rate of 0.8 l/min, and sample flow rate of 1 ml/min.

In vitro dephosphorylation of activated-JNK

Cd-treated CL3 cells (80 µM; 3 h) were lysed in an immunoprecipitation lysis buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstain]. JNK in the cell lysates (100 µg proteins) was immunoprecipitated using 1 µg anti-JNK1 antibody and collected with protein A–Sepharose beads. The Cd-activated JNK was then washed once with lysis buffer and twice with a phosphatase assay buffer (20 mM MOPS, pH 7.2, 25 mM 2-mercaptoethanol, 10 mM $MgCl_2$ and 100 µg/ml BSA) to serve as a substrate for phosphatase. PP2A in cell extract (100 µg proteins) was immunoprecipitated using a polyclonal antibody against the catalytic subunit of PP2A (PP2Ac) (Upstate Biotechnology, Lake Placid, NY) in the immunopre-cipitation lysis buffer without any phosphatase inhibitors. Dephosphorylation of Cd-activated JNK was performed by incubation with cellular PP2Ac immunocomplexes or purified active PP2Ac (Upstate Biotechnology) in the

phosphatase assay buffer in a volume of 50 µl at 30°C for 30 min. The reaction was terminated by adding 6 µl of a stop solution (50 mM EDTA, 1 M NaF, and 20 mM NaPPi) before two washes with the JNK kinase assay buffer. The samples were then subjected to JNK kinase activity assay.

PP2A activity assay

Endogenous PP2Ac immunocomplex was prepared as described above. The catalytic activity of PP2A was measured using KRpTIRR, a phosphopeptide as the substrate according to the manufacturer's instructions (Upstate Biotechnology). Free phosphate removed from the substrate by the cellular PP2Ac was measured by adding malachite green solution and a spectrophotometer at a wavelength of 650 nm. The buffer, substrate or immunocomplex were individually served as blanks.


Results

3AT blocks JNK activation elicited by Cd

To explore the role of ROS in the activation of JNK by Cd, serum-starved CL3 cells were left untreated or treated with 80 mM 3AT or 80 mM mannitol for 2 h before exposure to $CdCl_2$ for 3 h. The JNK activity in WCE was determined by *in vitro* kinase assay using GST–cJun(1-79) as a substrate. As shown in Figure 1A, 3AT (80 mM) pre-treatment completely blocked the JNK activity elicited by 80 µM Cd in CL3 cells. This dose of Cd reduced the colony-forming ability to 35% of the untreated control cells, while 3AT alone did not cause obvious cytotoxicity. Conversely, mannitol (80 mM) did not alter the JNK activity in Cd-treated CL3 cells (Figure 1A). The protein amounts of JNK in WCE derived from the treated cells were the same as the untreated control (Figure 1A), indicating the inhibitory effect of 3AT on the JNK activation by Cd is not due to suppression of protein expression. To determine whether 3AT could suppress the JNK activity induced by a higher concentration of Cd and whether post-administering 3AT could inhibit the JNK activation by Cd, serum-starved CL3 cells were left untreated or treated with 120 µM Cd for 1 h followed by adding 80 mM 3AT and kept in incubation for another 2 h. As shown in Figure 1B, the activity of JNK elicited by 120 µM Cd disappeared by 3AT post-treatment. The inhibitory effect of 3AT on the JNK activation by Cd was also observed in co-treatment experiments (Figure 1B).

To determine whether the inhibitory effect of 3AT on Cd-elicited JNK activation occurs in other mammalian cells, we performed similar experiments using HFW human diploid fibroblasts and CHO-K1 cells. HFW and CHO-K1 cells were pre-treated with 3AT (80 mM) or mannitol (80 mM) for 2 h followed by exposure to $CdCl_2$ for 3 h. The doses of Cd were 30 and 4 µM for HFW and CHO-K1 cells, respectively, which reduced the colony-forming ability to 30% of the untreated control cells. As shown in Figure 1C, the capability of 3AT to block the activation of JNK by Cd was also observed in serum-starved HFW human diploid fibroblasts and CHO-K1 cells, while mannitol had no effect.

Effect of 3AT on the intracellular catalase activity, peroxide level and Cd uptake

Table I shows the intracellular catalase activities in cells treated with 3AT (80 mM) in the presence or absence of Cd (80 µM, 3 h exposure) were significantly reduced to ~15% of the
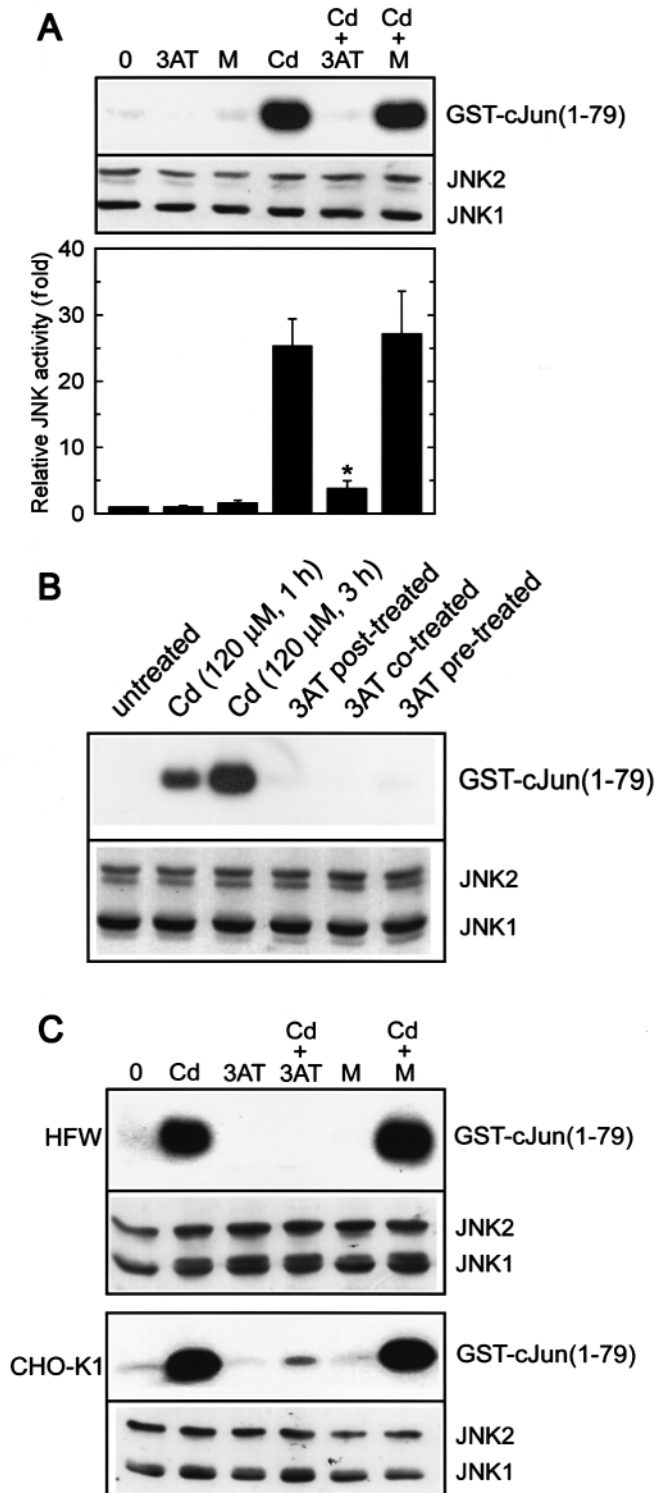
Fig. 1. 3AT abrogates the activation of JNK by Cd in mammalian cells. (**A**) CL3 cells were pre-treated with 3AT (80 mM) or mannitol (80 mM; indicated as M) for 2 h followed by exposure to $CdCl_2$ (80 µM) for 3 h. (**B**) 3AT (80 mM) was administered at different timings (pre-, co- or post-treated) in CL3 cells treated with 120 µM $CdCl_2$. The pre-treated experiment was performed as described above. In the post-treated experiment, CL3 cells were treated with Cd for 1 h before the addition of 80 mM 3AT and kept cultured for 2 h. In the co-treated experiment, 3AT and Cd were administered simultaneously. (**C**) HFW and CHO-K1 cells were pre-treated with 3AT (80 mM) or mannitol (80 mM) for 2 h followed by exposure to $CdCl_2$ for 3 h. The doses of Cd were 30 and 4 µM for HFW and CHO-K1 cells, respectively. WCE were prepared immediately after treatments and the JNK activity in equal amounts of proteins was examined using GST–cJun(1-79) as a substrate. The relative activities of JNK shown in (A) are densitometric analysis of seven experiments normalized by arbitrarily setting the densitometry of control cells to 1 (means ± SE; *$P < 0.01$ versus Cd, Student's *t*-test). Results shown in (B) and (C) are one representative of three independent experiments. Equal amounts of proteins were also subjected to western blot analysis of JNK1 and JNK2 and examples are shown in lower panels.
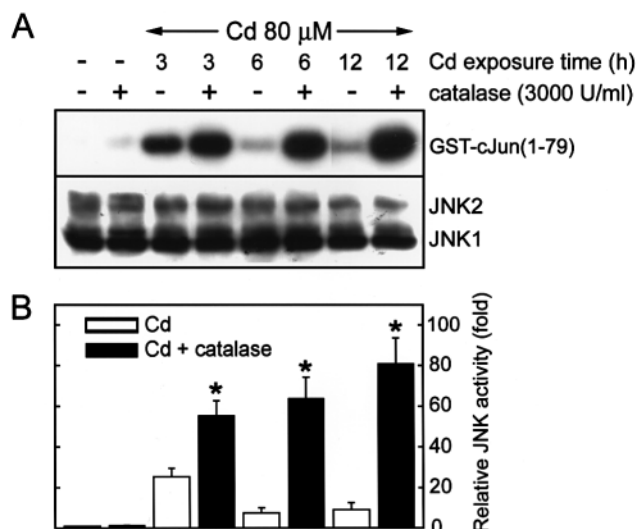
Fig. 2. Catalase enhances the activation of JNK elicited by Cd. CL3 cells were incubated with catalase (3000 U/ml) for 2 h and then treated with CdCl$_2$ (80 μM) for 3, 6 or 12 h. JNK activity in equal amounts of proteins was examined using GST–cJun(1-79) as a substrate. (**A**) An example of JNK activity measured in cells treated as indicated. Lower panel is western blot analysis of JNK1 and JNK2 in equal amounts of proteins. (**B**) Densitometric analysis of relative JNK activities averaged from three–nine independent experiments (*$P < 0.001$ versus Cd, Student's *t*-test).

Table I. Effects of 3AT on the intracellular catalase activity, ROS generation and Cd uptake in CL3 cells exposed to CdCl$_2$

Treatment[a]	Catalase activity[b] (μmol H$_2$O$_2$ decrease/min/mg protein)	Dichlorofluorescein fluorescence intensity[b] (per 10^6 cells)	Cd uptake[b] (ng per 10^6 cells)
Untreated	19.78 ± 3.17	12.61 ± 2.48	0.21 ± 0.09
Cd	19.51 ± 2.36	14.93 ± 1.29	47.59 ± 1.96
3-AT	2.91 ± 0.63[c]	19.38 ± 3.27[c]	0.14 ± 0.05
Cd + 3AT	2.90 ± 0.07[c]	20.85 ± 2.99[c]	71.74 ± 6.43[d]

[a]CL3 cells were left untreated or pre-treated with 3-AT (80 mM) in serum-free media for 2 h before adding CdCl$_2$ (80 μM) and kept in incubation for another 3 h.
[b]Data were obtained from three to four experiments, and represented as mean ± SE.
[c]$P < 0.05$ versus untreated cells, Student's *t*-test.
[d]$P < 0.05$ versus Cd-treated cells, Student's *t*-test.

untreated activity, although western blot analysis revealed that the catalase protein levels were similar in these treatments (data not shown). The result is consistent with the fact that 3AT covalently binds to the catalytic center of catalase and subsequently inhibits catalase activity (31). In the presence or absence of Cd, 3AT significantly increased the intracellular peroxide levels (Table I). Although Cd (80 μM, 3 h exposure) alone did not alter the catalase activity in CL3 cells, it slightly increased the peroxide level (Table I). Intriguingly, the uptake of Cd by CL3 cells was enhanced by 3AT co-treatment (Table I). These results suggest that insufficient catalase activity or an enhanced oxidative environment caused by 3AT may abrogate JNK activation by Cd in mammalian cells, while the intracellular level of Cd alone plays a limited role.

Catalase increases the activation of JNK elicited by Cd
To explore the role of catalase in regulating Cd-induced JNK activity, CL3 cells were left untreated or pre-treated with
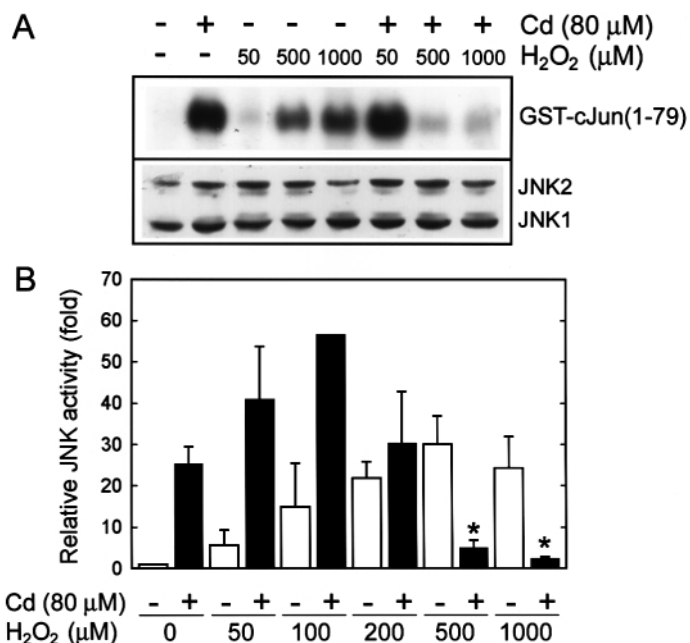

Fig. 3. Biphasic effects of H$_2$O$_2$ on the activation of JNK elicited by Cd. CL3 cells were treated with CdCl$_2$ (80 μM) for 2.5 h before adding various doses of H$_2$O$_2$ for 30 min. JNK activity in equal amounts of proteins was examined using GST–cJun(1-79) as a substrate. (**A**) An example of JNK activity measured in cells treated as indicated. Lower panel is western blot analysis of JNK1 and JNK2 in equal amounts of proteins. (**B**) Densitometric analysis of relative JNK activities averaged from three–nine independent experiments as indicated (*$P < 0.05$ versus Cd, Student's *t*-test).

catalase (3000 U/ml) for 2 h before they were exposed to CdCl$_2$ (80 μM) for 3–12 h. Western blot and enzymatic activity analyses revealed that the level and activity of catalase increased in WCE obtained from this exogenous catalase treatments (data not shown). As shown in Figure 2, exposure cells to catalase alone did not cause the activation of JNK. The levels of JNK activity induced by 80 μM Cd in CL3 cells decreased with prolonged exposure time, while catalase markedly increased the extent and lifetime of JNK activity elicited by Cd (Figure 2). The result indicates that catalase is crucial for persistent activation of JNK by Cd.

H$_2$O$_2$ exhibits biphasic effects on the activation of JNK elicited by Cd
Inhibition of catalase by 3AT subsequently resulted in increasing intracellular H$_2$O$_2$ levels (Table I). Also, exogenous catalase may reduce the intracellular H$_2$O$_2$ levels. We therefore examined the effect of H$_2$O$_2$ on the activation of JNK in Cd-treated cells. Serum-starved CL3 cells were treated with Cd for 2.5 h before adding various concentrations of H$_2$O$_2$ for 30 min. As shown in Figure 3, H$_2$O$_2$ alone dose-dependently induced JNK activation; at 500–1000 μM that reduced the viability to 60–40% of the untreated cells (data not shown) elevated JNK activity >20-fold of the control levels. Intriguingly, H$_2$O$_2$ exhibited biphasic effects on the JNK activation induced by 80 μM Cd; i.e. high doses (500–1000 μM) of H$_2$O$_2$ markedly suppressed the JNK activation by Cd, while low doses of H$_2$O$_2$ (<200 μM) additive increased its activation (Figure 3). Co-administering H$_2$O$_2$ (50–1000 μM) with 80 μM Cd decreased cellular viability by 10–20% as compared with that exposed to H$_2$O$_2$ alone (data not shown). These results indicate that enhanced oxidative stress can either increase or decrease JNK activation by Cd.
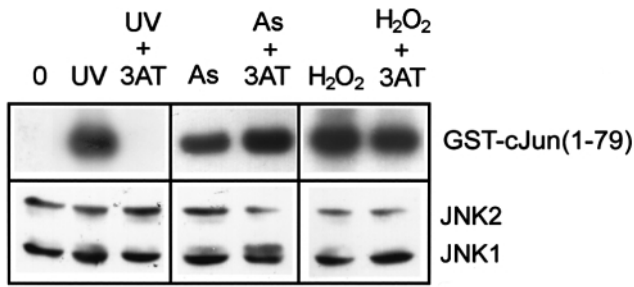
Fig. 4. Effect of 3AT on the JNK activation elicited by UV, sodium arsenite or H_2O_2. CL3 cells were pre-treated with 80 mM 3AT for 2 h before exposure to 40 J/m^2 UV, 50 µM sodium arsenite (3 h) or 200 µM H_2O_2 (30 min). WCE were prepared immediately after treatments except that for UV was obtained 30 min after irradiation. The JNK activity in equal amounts of proteins was examined using GST–cJun(1-79) as a substrate. Results shown are representative of at least three independent experiments. Equal amounts of proteins were subjected to western blot analysis of JNK1 and JNK2 and examples are shown in lower panels.

*Effect of 3AT on JNK activation elicited by other stress inducers*

Previously, we have reported that 3AT does not alter the levels of JNK activity induced by Cr(VI), a ROS-associated stress inducer (28). To explore whether 3AT can suppress the activation of JNK by stress inducers other than Cd, CL3 cells were left untreated or treated with 3AT for 2 h followed by exposure to UV (40 J/m^2), sodium arsenite (50 µM; 3 h) or H_2O_2 (200 µM; 30 min). As shown in Figure 4, 3AT pre-treatment also completely blocked the JNK activity induced by UV in CL3 cells. However, 3AT pre-treatment did not influence the levels of JNK activity induced by sodium arsenite or H_2O_2.

An okadaic acid-sensitive protein phosphatase participates in the suppression of Cd-elicited JNK activity by 3AT

To explore the mechanism by which Cd-elicited JNK activity is regulated upon 3AT or catalase co-exposure, we first examined whether 3AT or catalase directly target to JNK. The result shows that 3AT (20–80 mM) and catalase (1000–10 000 U/ml) *in vitro* did not suppress and enhance the JNK activity isolated from 80 µM Cd-treated cells, respectively (data not shown), suggesting that the Cd-elicited JNK activity is affected indirectly by 3AT or catalase in cells.

Activation of protein kinases is highly correlated with upstream kinase activators and down-regulated activity of protein phosphatases (8–10). We then examined the expression of MKP-1, an oxidative stress-inducible dual specific protein phosphatase for MAPK (10), in response to Cd in the presence or absence of 3AT or catalase. Immunoblot analysis revealed that no significant increases of MKP-1 protein expression upon Cd treatment, also, both 3AT and catalase slightly potentiated MKP-1 protein levels in Cd-treated cells (data not shown), suggesting that MKP-1 is not a causative for the dramatic changes on the Cd-elicited JNK activity by 3AT or catalase co-exposure.

To further investigate the role of protein phosphatases in Cd-elicited JNK activity under different catalase levels, we treated cells with 3AT (80 mM) or catalase (3000 U/ml) for 1.5 h and then added 5 mM sodium fluoride, an inhibitor for PP or 1 mM sodium orthovanadate, an inhibitor for PTP for 30 min followed by adding 80 µM Cd for 3 h. Figure 5A shows that sodium fluoride or sodium orthovanadate elevated spontaneous or Cd-elicited JNK activity, indicating functional
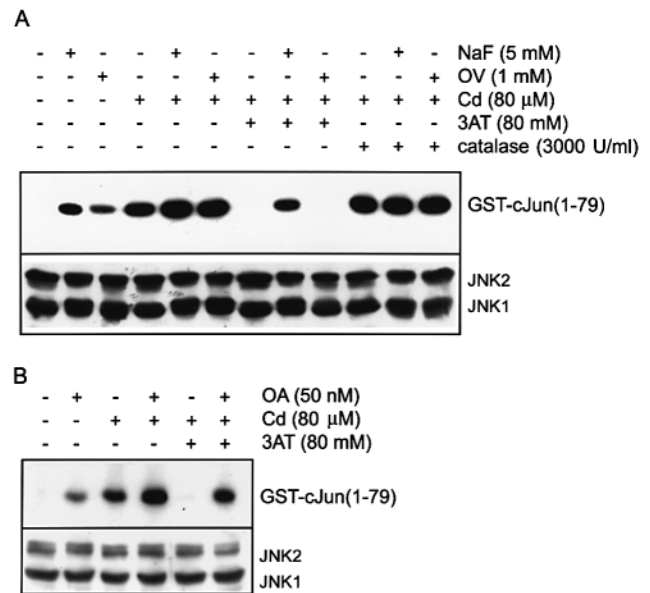

Fig. 5. Roles of protein phosphatases in the JNK activity in cells treated with Cd in the presence or absence of 3AT or catalase. (**A**) CL3 cells were treated with 3AT (80 mM) or catalase (3000 U/ml) for 1.5 h before adding 5 mM sodium fluoride (NaF) or 1 mM sodium orthovanadate (OV) for 30 min followed by exposure to 80 µM Cd for 3 h. (**B**) CL3 cells were treated with 3AT (80 mM) for 1 h followed by 50 nM okadaic acid (OA) for 1 h and 80 µM Cd for 3 h. The WCE was then assayed for the JNK activity using GST–cJun(1-79) as a substrate. Lower panels are examples of western blots of JNK1 and JNK2 in equal amounts of proteins. Results shown are one representative of at least three independent experiments.

JNK phosphatases exist in CL3 cells. Intriguingly, sodium fluoride significantly restored the Cd-induced JNK kinase activity suppressed by 3AT, but sodium orthovanadate did not (Figure 5A). These findings suggest that PP but not PTP participate in the down-regulation of JNK activity in cells exposed to Cd plus 3AT. On the other hand, sodium fluoride or sodium orthovanadate did not significantly alter the JNK activity induced in cells exposed to Cd plus catalase (Figure 5A).

To further evaluate the role of PP in the suppression of Cd-induced JNK by 3AT, the cells were left untreated or treated with 3AT (80 mM) for 1 h followed by adding okadaic acid (50 nM) for 1 h and Cd (80 µM) for 3 h. As shown in Figure 5B, okadaic acid could increase JNK activity in both untreated and Cd-treated cells, indicating an okadaic acid-sensitive PP down-regulates JNK in CL3 cells. Moreover, okadaic acid markedly restored the suppression effect of 3AT on the JNK activation elicited by Cd (Figure 5B). On the other hand, okadaic acid did not affect the JNK activity in cells treated with catalase plus Cd (data not shown).

It is known that okadaic acid suppresses PP2A and PP4 much more efficiently than acts on PP1 and PP5 (32,33). PP2A has been shown to involve in decreasing phosphorylation of c-Jun at Ser63 (34); also, activation of PP2A by an inflammatory stimulus is correlated with a reduced JNK activity (35). We therefore determined the PP2A activity in cells that had been left untreated or treated with Cd in the presence or absence of 3AT or okadaic acid. After treatment, the PP2A were immunoprecipitated using a PP2Ac polyclonal antibody and assayed for the ability to remove free phosphate from the KRpTIRR substrate. As shown in Figure 6A, the PP2A phosphatase activity markedly decreased in cells treated with okadaic acid as comparison with the untreated cells, confirming

A

B

IP: PP2Ac

none   untreated   Cd   Cd + 3AT

GST-cJun(1-79)

PP2Ac
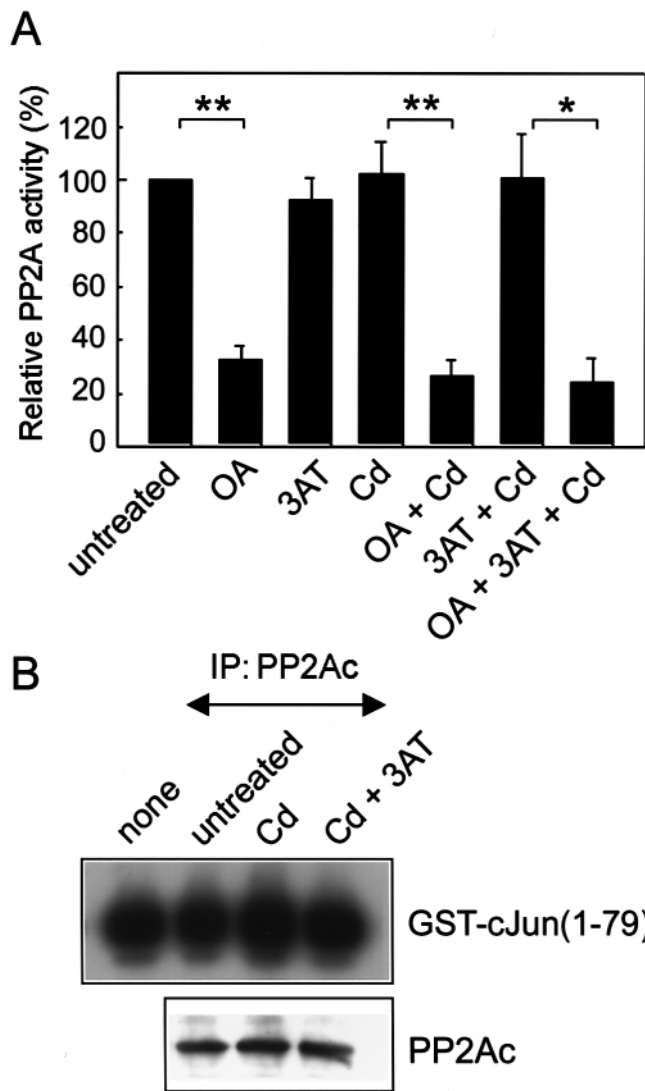
Fig. 6. PP2A may not be involved in the inhibitory effect of 3AT on the Cd-elicited JNK activation and does not dephosphorylate JNK *in vitro*. (**A**) Serum-starved CL3 cells were treated with 3AT (80 mM) for 1 h followed by 50 nM okadaic acid (OA) for 1 h and 80 μM Cd for 3 h. The endogenous PP2Ac was immunoprecipitated for the determination of PP2A activity using a synthetic phosphopeptide substrate as described in Materials and methods. The relative PP2A activities were normalized by arbitrarily setting the $OD_{650\,nm}$ absorbance of control cells to 100% (means±SE, $n = 3–6$; *$P < 0.05$, **$P < 0.01$, Student's *t*-test). (**B**) To determine the ability of PP2A to dephosphorylate JNK *in vitro*, PP2Ac immunocomplex (IP: PP2Ac) derived from Cd or Cd plus 3AT treatments or untreated control were reacted with Cd-activated JNK immunocomplex in a phosphatase assay buffer. After incubation at 30°C for 30 min, the JNK kinase assays were determined and a representative of three experiments is shown. Lower panel is western blot analysis of PP2Ac.

the inhibitory effect of okadaic acid. The PP2A activities in cells treated with Cd in the presence or absence of 3AT were the same as the untreated level and decreased significantly by co-administering okadaic acid (Figure 6A). Moreover, neither the PP2Ac immunocomplex derived from cells treated with Cd plus 3AT (Figure 6B) nor the purified active PP2Ac (data not shown) can dephosphorylate activated-JNK *in vitro*. The results indicate that PP2Ac activity is not elevated in cells treated with Cd plus 3AT and that PP2Ac is not directly involved in JNK dephosphorylation.

A

B
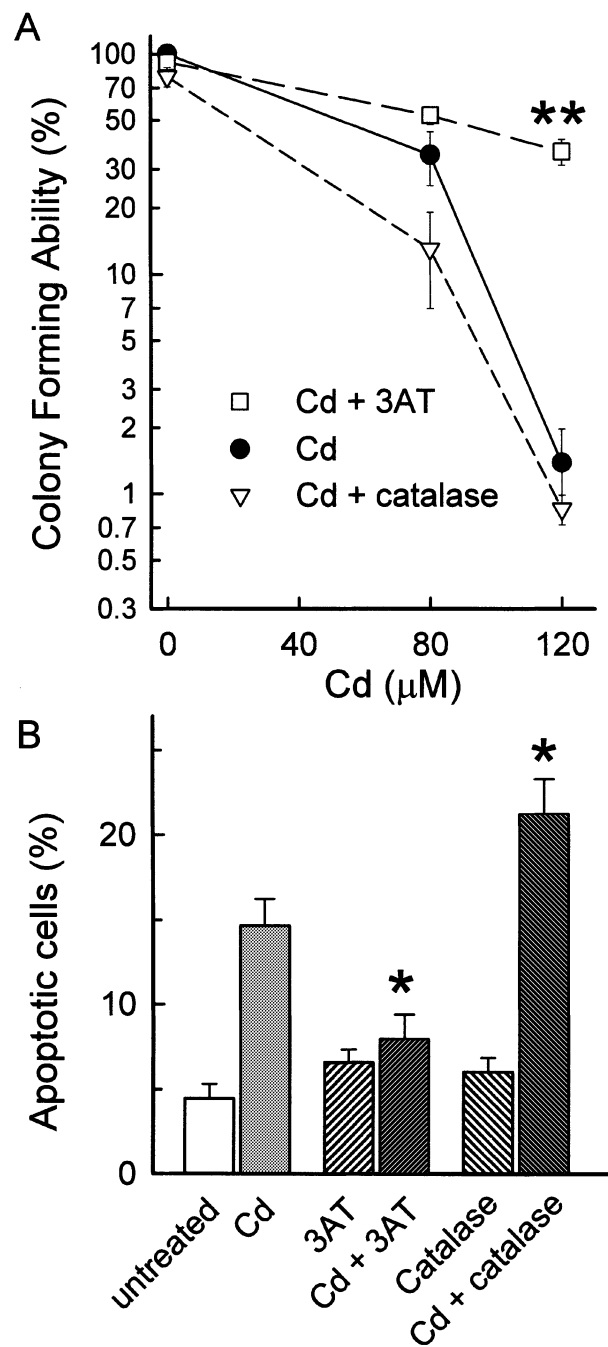
Fig. 7. Effects of 3AT or catalase on the Cd-induced cytotoxicity and apoptosis. CL3 cells were pre-treated with 3AT (80 mM) or catalase (3000 U/ml; CAT) before adding $CdCl_2$ (80 μM) for 3 h. (**A**) Cytotoxicity was determined by the colony-forming ability assay (means ± SE, $n = 4$; **$P < 0.001$ versus Cd, Student's *t*-test). (**B**) Apoptotic cells were examined 8 h after treatments using annexin V-FITC binding assay and flow cytometry analysis (means ± SE, $n = 3$; *$P < 0.05$ versus Cd, Student's *t*-test).

Effects of 3AT or catalase on Cd-induced cytotoxicity and apoptosis

To correlate JNK activation with Cd cytotoxicity, we treated serum-starved CL3 cells with Cd in the presence of 3AT or catalase as described above. The cytotoxicity and apoptosis were determined by the colony-forming ability and annexin V-FITC binding assays, respectively. As shown in Figure 7A, treatment cells with 3AT (80 mM) and catalase (3000 U/ml)
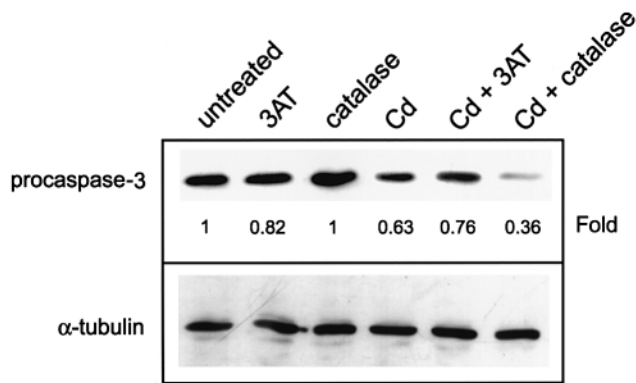
Fig. 8. Effects of 3AT or catalase on the Cd-induced degradation of procaspase-3 proteins in cells treated with Cd. CL3 cells were left untreated or pre-treated with 3AT (80 mM) or catalase (3000 U/ml) before adding $CdCl_2$ (120 µM) for 3 h. The cells were washed with PBS, kept cultured for 9 h and the amounts of procaspase-3 in WCE were detected by western blot analysis. Results were averaged from six individual experiments. After detection of procaspase-3 protein levels, antibodies were stripped from membranes by stripping solution containing 2% SDS, 62.5 mM Tris–HCl, pH 6.8 and 0.7% (w/w) β-mercaptoethanol at 50°C for 15 min and the membrane was reprobed with α-tubulin (lower panel) to serve as an internal control.

did not significantly decrease the colony-forming ability. Similarly, these ROS modulators alone did not increase the numbers of apoptotic cells (Figure 7B). Pre-exposure serum-starved CL3 cells to 3AT for 2 h significantly protected the cytotoxicity and apoptosis induced by Cd (Figure 7). Determination of the activity of mitochondrial succinate dehydrogenase in cells subjected to the above treatments by MTT assay (25) also revealed that 3AT and catalase indeed exhibited such protecting and enhancing effects on acute toxicity induced by Cd, respectively (data not shown). The protection effect of 3AT on Cd-induced toxicity and apoptosis may be due in part to the suppression of JNK activation, which is consistent with the finding that sustained activation of JNK participates in Cd apoptosis (25). Conversely, the toxicity and apoptosis of Cd were enhanced by catalase (Figure 7), which is correlated with the fact that catalase increases the activation of JNK by Cd.

Caspase-3 has been shown to play a pivotal role in the terminal, execution phase of apoptosis induced by diverse stimuli (36). We next examined the levels of procaspase-3 in Cd-treated cells in the presence of 3AT or catalase. As shown in Figure 8, the amounts of procaspase-3 decreased to 63% of the untreated levels in cells harvested 9 h after Cd treatment (120 µM, 3 h). Pre-treatment with 3AT increased the amounts of procaspase-3 in Cd exposed cells, suggesting that 3AT decreases Cd-elicited procaspase-3 degradation. In contrast, the amounts of procaspase-3 significantly decreased by the addition of catalase (3000 U/ml) to cells for 2 h followed by exposure to Cd for 3 h (Figure 8), suggesting that catalase enhances Cd-elicited apoptosis through a caspase-3 dependent pathway.

Discussion

In this study, we have explored the roles of ROS in the activation of JNK and apoptosis by Cd. We have demonstrated that 3AT, which significantly decreased the catalase activity and increased intracellular peroxide in Cd-treated CL3 cells, could completely suppress the JNK activation by Cd. In contrast, catalase markedly increased the extent and lifetime of JNK activity elicited by Cd in CL3 cells. Intriguingly, high

doses (500–1000 µM) of H_2O_2 markedly suppressed the JNK activation by Cd, while low doses of H_2O_2 (<200 µM) additive increased it. However, the hydroxyl radical scavenger mannitol did not influence the JNK activation elicited by Cd. These results indicate that JNK activation by Cd is highly associated with catalase activity but not hydroxyl radical; also, enhanced oxidative stress can decrease JNK activation by Cd. The inhibitory effect of 3AT on Cd-elicited JNK activation was also observed in normal human fibroblasts and CHO-K1 cells, indicating this may be a universal phenomenon. 3AT could also suppress JNK activation in CL3 cells irradiated with UV, yet it did not affect the levels of JNK activity induced by Cr(VI) (28), sodium arsenite or H_2O_2 (Figure 4). Moreover, it should be noticed that high doses of H_2O_2 synergistically enhance the JNK activity in Cr(VI) co-exposed cells (28), which is opposing to the finding of Cd. Taken together, the above results imply that oxidative stress can stimulate JNK activation in a catalase-dependent manner (UV and Cd) or not [sodium arsenite, Cr(VI) and H_2O_2].

Redox control is a general physiological mechanism to regulate protein–protein interaction and enzymatic function for cell signaling (14,17–19). Thioredoxin and GST are paradigms of cysteine-rich redox-sensitive proteins that direct association with apoptosis signal-regulating kinase 1 [an upstream activator of JNK and p38 MAPK (5–7)] and JNK, respectively, negatively regulating kinase activities in non-stressed cells (12,13). Under ROS stress, the formation of disulfide linkages between cysteines of thioredoxin or GST results in the dissociation of these redox-sensitive proteins from their associated stress-kinases, allowing activation of kinases (12,13). On the other hand, catalase is a heme/NADPH bound tetrameric enzyme which catalyses the conversion of H_2O_2 to water and oxygen (31). The crystallographic structure of the human catalase–3AT complex has recently been solved, indicating that 3AT inhibits catalase activity by forming a covalent adduct to the His75 residue of catalase which is the catalytic active center for H_2O_2 (31). In contrast to thioredoxin and GST, the present study indicates that catalase represents another category of redox-sensitive proteins that is crucial for positive regulation of JNK activation upon ROS stress generated by Cd or UV.

Numerous MAPK phosphatases including MKP, PTP and PP have been shown to mediate in a negative feedback regulation of three-kinase MAPK signal modules (8–11). Here, we further demonstrated that the inhibitory effect of 3AT on Cd-elicited JNK activation could be recovered by okadaic acid and was independent of PTP or MKP-1. These findings indicate that an okadaic acid-sensitive PP is crucial for the abrogation of Cd-elicited JNK activation by 3AT. Moreover, 3AT did not suppress the activation of ERK or p38 MAPK in Cd-treated cells (data not shown), indicating that the putative okadaic acid-sensitive PP acts specifically on JNK signaling pathway among MAPKs. It may be speculated that upon Cd stress functional catalase may associate with the JNK module acting not only as an enhancer of JNK activation but also as a physical blocker to obstruct the proximity of the putative PP, while depletion of catalase activity may facilitate the putative PP to down-regulate the activity of JNK.

PP2A, the major Ser/Thr phosphatase to be inhibited by okadaic acid at dose ranges used here, consisted of a catalytic subunit (PP2Ac) and a structural A subunit (PR65) forming as a core enzyme and a third regulatory B subunit (32,33). The three subunits are encoded by several genes to compose as many as 50–100 different PP2A trimeric holoenzymes for

a variety of specific regulation (32,33). The regulatory B subunits of PP2A determine substrate specificity and target holoenzymes to specific intracellular compartment as well as control the phosphatase activity (32,33). PP2A has been associated with dephosphorylation of JNK and its downstream c-Jun at Ser63 (34,35). However, an attempt to assay PP2A activity in CL3 cells treated with Cd in the presence or absence of 3AT indicated that this is not the case (Figure 6). Still, one cannot exclude the possibility of a potent regulatory B subunit of PP2A is enhanced in Cd plus 3AT-treated cells, whose substrate selectivity could not be detected by the short PP substrate used here. Moreover, okadaic acid has been implicated to decrease the activity of PP4, PP5 and PP1 (32,33), which warrants further study.

Intriguingly, short-term depletion of catalase by 3AT inhibited JNK activation and concurrently protected apoptosis and procaspase-3 degradation in Cd-treated CL3 cells. Conversely, co-administering catalase enhanced JNK activation, apoptosis and procaspase-3 degradation induced by Cd. These results are consistent with our previous report that JNK is an apoptotic signal in response to Cd (25). Moreover, we have examined the effect of 3AT on the cytotoxicity of UV-irradiated CL3 cells to correlate with the fact that 3AT blocked UV-elicited JNK activation. The result showed that pre-exposure to 3AT for 2 h markedly increased the colony-forming ability of UV (40 J/m^2)-irradiated cells from 0.92 to 71.8% of the control populations (data not shown). This finding further supports that down-regulation of the JNK activation by 3AT confers anti-cytotoxicity induced by stress inducers such as Cd and UV. Nevertheless, it should be noticed that Cd cytotoxicity is synergistically enhanced by a long-term (24 h) 3AT co-exposure in CL3 cells (data not shown) supporting that catalase is an essential cellular peroxidase.

Cd induces mitotic arrest in CL3 cells, which is associated with sustained activation of JNK (unpublished observation) as well as p38 MAPK (37). Short-term co-administering with 3AT may allow a portion of cells to bypass the Cd-induced mitotic arrest through suppression of the sustained JNK activity by a putative okadaic acid-sensitive PP, which subsequently protected the cytotoxicity of Cd. Several PP have been shown to regulate cell cycle at many points including the mitotic checkpoint control point due to their multiplicity and different substrate specificity (32,33). Identification of such a putative okadaic acid-sensitive PP and its role in the mitotic checkpoint control in cells co-exposure to Cd and 3AT are currently under investigation.

In conclusion, the major findings of this study are that persistent activation of apoptotic JNK signal by Cd requires functional catalase, which may obstruct the proximity of an okadaic acid-sensitive PP to inactivate JNK. The JNK activation elicited by UV, but not sodium arsenite or H$_2$O$_2$, may be regulated by catalase and JNK phosphatases in a similar mechanism as that for Cd. Moreover, short-term depletion of catalase activity can enhance cells escape from apoptosis induced by Cd or UV, which may predispose these cells to carcinogenic transformation. To our knowledge, this study demonstrates for the first time that catalase participates in cell death signaling generated by certain stress inducers.

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