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Potentiation of cadmium-induced cytotoxicity by sulfur amino acid deprivation through activation of extracellular signal-regulated kinase1/2 (ERK1/2) in conjunction with p38 kinase or c-jun N-terminal kinase (JNK) Complete inhibition of the potentiated toxicity by U0126 an ERK1/2 and p38 kinase inhibitor

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Abstract

The mechanisms of cadmium-induced toxicity may include oxidative stress, altered redox homeostasis, and injuries to organelles. The current study was designed to study the effect of decreased cellular glutathione (GSH) content by sulfur amino acid deprivation on cadmium toxicity and to identify the signaling pathways responsible for the cytotoxicity. GSH content was increased by cadmium in H4IIE cells prior to cell death, which was prevented by excess GSH or cysteine. Cell viability, however, was not improved by GSH or cysteine complexation of cadmium. Cadmium-induced cytotoxicity was 40-fold potentiated in cells with decreased GSH by sulfur amino acid deprivation. Cadmium in combination with decreased GSH markedly increased apoptotic cell death. Mitogen-activated protein kinases including extracellular signal-regulated kinase 1/2, p38 kinase and c-Jun N-terminal kinase (JNK) were all activated 1–12 hr after sulfur amino acid deprivation. U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), which inhibited activation of extracellular signal-regulated kinase1/2 and p38 kinase in cells under sulfur amino acid deprivation, completely prevented potentiation in Cd-induced cytotoxicity and apoptosis. Potentiation of cadmium toxicity by sulfur amino acid deprivation was prevented in part by either PD98059 or SB203580, or in cells stably expressing dominant negative mutant of JNK1, and to greater extents by PD98059 in combination with either SB203580 or JNK1(−) transfection. These results demonstrated that decreased cellular GSH content potentiated cytotoxicity induced by cadmium at the level of human exposure, and that the potentiation of cytotoxicity resulted from activation of extracellular signal-regulated kinase1/2 in conjunction with p38 kinase or JNK.

Keywords: Glutathione; MKK1/2; ERK1/2; p38 kinase; JNK; Cadmium; Cytotoxicity

1. Introduction

Cd is a heavy metal by-product produced from industry and has accumulated in environments. Chronic exposure to Cd results in renal tubular and pulmonary diseases as well as liver injury [1]. Cadmium toxicities include nuclear condensation, and dilation of endoplasmic reticulum, followed by mitochondrial swelling [2, 3]. Cd binds to sulfhydryl groups in cells with high affinity. Previous studies have shown that sulfhydryl groups are important in Cd-induced acute toxicity and detoxify Cd by sequestering Cd into an inert complex and preventing its reaction with target molecules [4, 5]. Cd-induced cytotoxicity involves apoptosis as a major mode of elimination of damaged cells [6].

The protective adaptive response to electrophiles and reactive oxygen species is mediated by cellular defense mechanisms. GSH as a cellular nonprotein sulfhydryl molecule plays a role as a protective substance and serves as an

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Abbreviations: Cd, cadmium; ERK, extracellular signal-regulated kinase; GSH, glutathione; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; PHAS-1, phosphorylated heat and acid-stable protein-1; p38 kinase, p38 mitogen-activated protein kinase; and SAAD, sulfur amino acid deprivation.

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effective oxygen radical scavenger [7]. A decrease in GSH would increase oxidative stress in cells. Disruption of the intracellular redox state by altered GSH content affects the state of activation of proteins and signaling pathways, which could subsequently affect susceptibility of cells to toxic insults.

In general, depletion of GSH increases susceptibility of cells to free radical-induced toxicity. Treatment of cells with GSH depleting agents (e.g. diethyl maleate, phorone, and buthionine sulfoximine) disrupts a dynamic equilibrium of the GSH pool by inhibiting the essential proteins involved in GSH synthesis or direct conjugation with GSH. It may be difficult to delineate the role of GSH in cells or animals after treatment with the inhibitors of GSH synthesis, because the GSH level would secondarily change as a consequence of adaptive responses. Sulfur amino acids are deficient in certain pathophysiological states such as protein-calorie malnutrition. Because cysteine is a direct precursor of GSH, the lack of sulfur amino acids in medium decreases cellular GSH. Previous studies showed that protein-calorie malnutrition decreases cellular GSH and persistently induces oxidative stress with activation of AP-1 [8,9]. Other studies from this laboratory have also shown that SAAD rapidly decreases the GSH content in cultured H4IIE cells and activates MAP kinase [10]. Hence, the physiology of cells under SAAD is similar to that in animals with protein-calorie malnutrition.

It is generally accepted that GSH provides a first line of defense against Cd prior to the induction of metallothionein [11]. The effect of Cd on the GSH level is variable in the liver probably due to compensatory GSH synthesis. Whether the altered GSH content is directly associated with Cd-induced cytotoxicity and GSH depletion is a requirement for Cd-induced cytotoxicity is still controversial. In view of this uncertainty, the present study was designed to establish the role of intracellular GSH for Cd-induced toxicity and to identify the signaling pathway(s) responsible for Cd-induced cytotoxicity in association with decreased GSH. The GSH level decreases in cells deprived of sulfur amino acids, which serves as an appropriate model to study the molecular event and the signaling pathway(s) for Cd-induced toxicity. In the present study, we showed that decreased cellular GSH substantially potentiated the toxicity of Cd by using this model. We were interested in establishing whether MAP kinase activation was responsible for SAAD-enhanced apoptosis. In the current study, activation of ERK1/2 in conjunction with either p38 kinase or JNK played a crucial role in Cd-induced cytotoxicity potentiated by decreased GSH.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (6000 mCi/mmol) was purchased from Perkin–Elmer. Minimum essential medium-select amine kit, recombinant protein A-agarose, and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium were obtained from Life Technologies. An antibody recognizing both metallothionein I and II was obtained from StressGen Biotechnologies. Anti-ERK1/2, anti-JNK, anti-p38 kinase, and anti-phospho ERK1/2 antibodies were supplied from New England Biolabs. Anti-SAPK2a p38α kinase antibody (Cat# 5–454) was purchased from Upstate Biotechnology. PD98059 and U0126 were obtained from Calbiochem. Phosphorylated heat and acid-stable protein-1 (PHAS-1) was purchased from Stratagene. SB203580 and other reagents in the molecular studies were supplied from Sigma. The JNK1 dominant negative mutant (KmJNK) vector was kindly provided from Dr. N. Dhanasekaran (Fels Institute for Cancer Research and Molecular Biology and Department of Biochemistry, Temple University, PA).

2.2. Cell culture

The H4IIE hepatoma cell line was obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37° in humidified atmosphere with 5% CO_2. The H4IIE monolaying cells were cultured in sulfur amino acid-deprived minimum, as described previously [10].

2.3. Determination of reduced GSH

Cells were washed twice in ice-cold PBS and then scraped into ice-cold 5% metaphosphoric acid. GSH was quantified using a commercially available GSH determination kit (Oxis International). The assaying kit specifically detects the reduced GSH content in cells without interference of other sulfhydyls.

2.4. MTT cell viability assay

H4IIE cells were plated at a density of 5×10^4 cells/well in a 96-well plate to determine cytotoxic concentrations of Cd. Cells were exposed to Cd at concentrations of 0.05–50 μM at 37° under 5% CO_2. After incubation of cells in the presence of Cd for the indicated time, viable cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (0.5 mg/mL) for 4 hr. The media were then removed and produced formazan crystals in the wells were dissolved by addition of 200 μL of dimethylsulfoxide. Absorbance was measured at 540 nm. Data represent the mean $\pm$ SEM with four separate experiments. Each experiment was performed with four replicates. If experiments were conducted on the separate day, then the data contained its own set of control.

2.5. Cell viability assay by crystal violet staining

When GSH or cysteine was present in culture medium, the sulfhydryl residues interfered with the MTT assay. In the case, cytotoxicity was assayed by staining viable adherent cells with crystal violet, as described previously [12]. After culturing cells at 37° in 5% CO_2 and 95% humidity in the presence or absence of Cd, non-adherent cells were removed by washing wells twice with PBS. The plates were then fixed with 0.1 mL of 5% formalin for 20 min. Attached cells were stained with a 0.05% solution of crystal violet for 20 min. The remaining dye incorporated into attached cells was eluted from the wells with 95% methanol and read at 570 nm in an enzyme-linked immunosorbent assay reader.

2.6. Terminal deoxythymidine transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed with an *in situ* cell death detection kit (Roche Diagnostics). Briefly, the cells on slides were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 hr at room temperature, and incubated in the solution containing 3% H_2O_2 in methanol for 10 min at room temperature. The cells were then permeabilized with 0.1% Triton® X-100 in 0.1% sodium citrate for 2 min on ice. Cells were washed with PBS, and incubated for 1 hr at 37° after addition of the reaction mixture containing 50 μL of TdT enzyme solution and 450 μL of fluorescein-labeled dUTP. The reaction mixture was incubated for 30 min at 37° following addition of 50 μL of anti-fluorescein antibody (Fab fragment from sheep conjugated with horse-radish peroxidase) and further incubated in the presence of 100 μL of 3,3′-diaminobenzidine solution for 5 min. Stained cells were analyzed under microscopy.

2.7. Immunoblot analysis

After washing cells twice with PBS, total cell lysates were prepared from the scraped cells after addition of 100 μL of Laemmli sample buffer. Cell lysates were collected, sonicated, and boiled for 5 min. The lysates were centrifuged at 15,000 *g* for 15 min. Cell lysates were separated by 12% gel electrophoresis [13] and electrophoretically transferred to nitrocellulose paper. Activation of ERK1/2 was immunochemically assessed by using the specific antibody (1:1000 dilution), which recognized the active-phosphorylated form, and developed by using ECL chemiluminescence system (Amersham). The levels of unphosphorylated ERK1/2, JNK, and p38 kinase were also measured using the respective antibody directed against each MAP kinase (1:1000 dilution). Metallothionein I and II expression in cell lysates was also immunochemically detected by using a specific antibody (1:500 dilution).

2.8. Immunocomplex kinase assay

H4IIE cells were lysed in the buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. Lysates were centrifuged at 10,000 *g* at 4° for 10 min. Anti-ERK1/2, anti-JNK, or anti-SAPK2a p38α kinase antibody (2 μg/sample) was added to the supernatant containing 500 μg of lysate, and the reaction mixture was incubated with gentle agitation at 4° for 2 hr. The immune complex was allowed to bind protein A-agarose (50 μL) for 2 hr and precipitated by centrifugation. The immune complex-protein A-agarose was washed twice with the lysis buffer and once with the kinase buffer containing 25 mM Tris·Cl (pH 7.4), 25 mM β-glycerophosphate, 25 mM magnesium chloride, 1 mM dithiothreitol, and 0.1 mM sodium orthovanadate. The immune complex was precipitated by centrifugation at 10,000 *g* for 2 min, and resuspended in 25 μL of the kinase buffer. The reaction was initiated by addition of 2 μg of PHAS-1 (for ERK1/2 or p38 kinase activity) or GST-c-Jun [1–79] (for JNK activity) and 5 μCi of [γ-^{32}P]ATP to the reaction mixture, continued at 30° for 30 min, and terminated by addition of 25 μL of 2× SDS-PAGE sample dilution buffer. Proteins were separated on 12% gel, which were autoradiographed after fixing and drying. Unphosphorylated ERK1/2, p38 kinase, and JNK in each sample were assessed as controls.

2.9. Stable transfection of a dominant negative mutant of JNK1

Cells were transfected by using Transfectam® according to the manufacturer's instruction (Promega). H4IIE cells were replated 24 hr before transfection at a density of 2×10^6 cells in a 10-cm^2 plastic dish. For JNK1(−) transfection, 20 μL of Transfectam® was mixed with 10 μg of a JNK1 dominant negative mutant plasmid (KmJNK1) in 2.5 mL of minimum essential medium. Cells were transfected by addition of minimum essential medium containing each plasmid and Transfectam®, and then incubated at 37° in a humidified atmosphere of 5% CO_2 for 6 hr. To establish a stable JNK1(−)-transfected H4IIE cell line, viable cells were subcultured at least five successive times in the medium containing 50 μg of geneticin (Life Technologies). Cells stably transfected with JNK1(−) were subjected to MTT or TUNEL assay.

2.10. Data analysis

Scanning densitometry was performed with Image Scan & Analysis System (Alpha-Innotech). One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for compari-

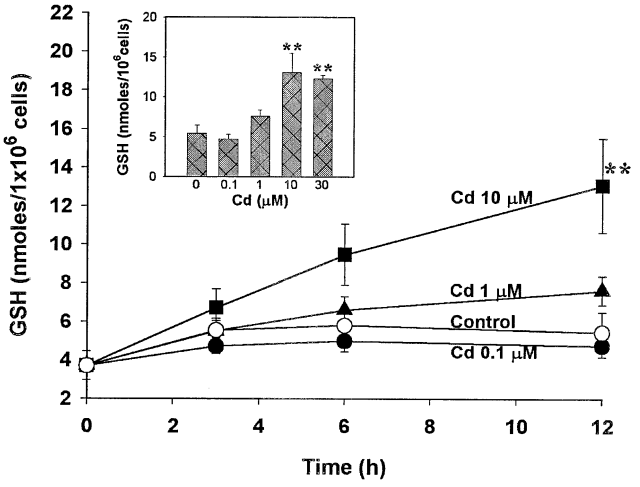

Fig. 1. The GSH contents in H4IIE cells cultured with $CdCl_2$. The GSH content was determined as a function of time in cells cultured with 0.1–10 μM of Cd. Inset shows the concentration-response at 12 hr after Cd treatment (0.1–30 μM). Data represent the mean ± SEM with four separate experiments (significant as compared to the initial GSH content, $^{**}P < 0.01$).

sons of multiple group means. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Effect of Cd on cellular GSH content

The GSH content was assessed 12 hr after treatment of H4IIE cells with varying concentrations of $CdCl_2$ (Fig. 1). Whereas $CdCl_2$ at a concentration of 0.1 μM caused no significant change in the GSH content in H4IIE cells, the GSH level was increased in a concentration-dependent manner after treatment with 1–10 μM $CdCl_2$. The GSH level was 1.3- to 1.5-fold increased by 1 μM $CdCl_2$, as compared to control. Treatment of cells with 10 μM $CdCl_2$ increased the GSH content by 1.8-, 2.6-, and 3.5-fold at 3, 6, and 12 hr, respectively, as compared to control. Cd at the concentration of 30 μM caused no further increase in GSH at 12 hr (Fig. 1, inset). An increase in GSH level by Cd may result from an adaptive response against Cd prior to cell death. Given the increase in GSH by $CdCl_2$, the effects of complexation of Cd with GSH or cysteine on cytotoxicity were monitored in the next experiment.

3.2. Effects of GSH or cysteine

Cd-induced cytotoxicity was assessed by crystal violet staining. In the preliminary study, we observed that MTT assay was interfered with excess thiol-containing molecules present in culture medium. Hence, we stained cells with crystal violet when cells were exposed to GSH or cysteine. The relative viability of cells was decreased by the presence

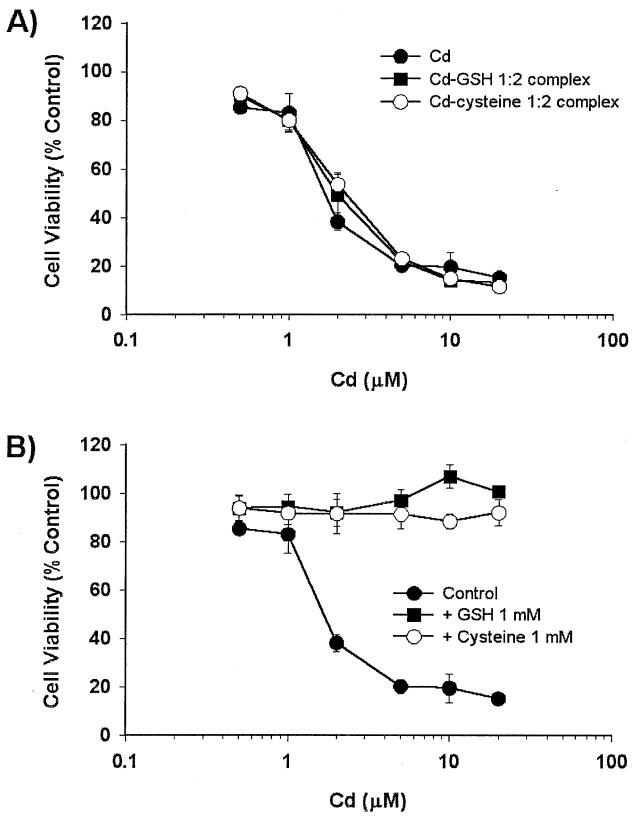

Fig. 2. Cytotoxicity of H4IIE cells by Cd in the presence of GSH or cysteine. (A) Cell viability after treatment with Cd-GSH or Cd-cysteine complex. After treatment with Cd-GSH or Cd-cysteine complex for 12 hr, cells were washed twice with incubation medium to remove the residual GSH or cysteine. (B) Cell viability after treatment with excess GSH or cysteine. H4IIE cells were treated with various concentrations of $CdCl_2$ for 12 hr in the presence or absence of 1 mM GSH or cysteine. Cells were then washed twice. The viability of cells was assessed by crystal violet staining. Data represent the mean ± SEM with four separate experiments.

of $CdCl_2$ in a concentration-dependent manner with an EC_{50} value of 7.9 μM (Fig. 2A). Cd makes complexation with GSH or cysteine in a 1:2 ratio [14]. To determine whether complexation of Cd with either GSH or cysteine altered cell viability, cells were treated with each Cd-complex. The extent of cell survival after treatment with either Cd-GSH (1:2) or Cd-cysteine (1:2) was identical to that caused by $CdCl_2$ (Fig. 2A). These data showed that cytotoxicity induced by Cd at the concentrations of 1 μM or above failed to be prevented by the amount of GSH or cysteine for complexation.

Studies were extended to determine whether an excess GSH or cysteine could prevent the cytotoxicity induced by $CdCl_2$. Crystal violet assay revealed that the presence of 1 mM GSH or cysteine in culture medium (i.e. 50- to 500-fold greater thiol concentrations than that of complexation) completely prevented cell death induced by $CdCl_2$ (Fig. 2B). Hence, the cytotoxicity inducible by 1–20 μM $CdCl_2$ was inhibited by excess GSH or cysteine, but not by complexation of Cd.

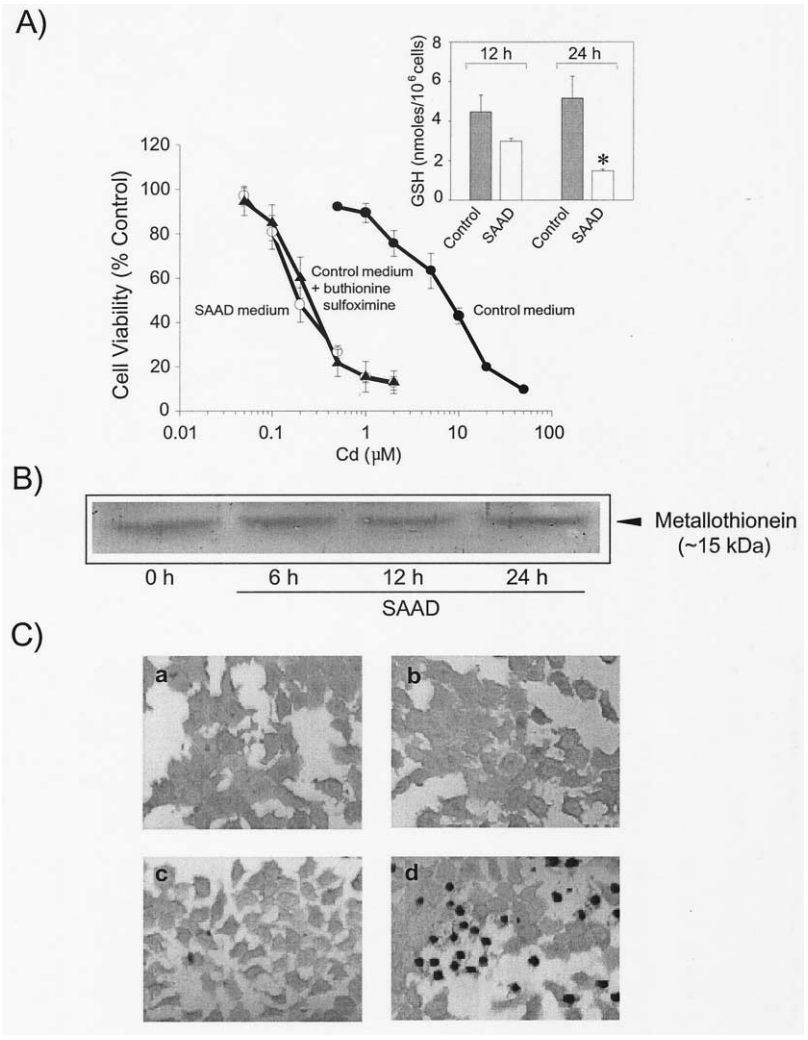

Fig. 3. Cd-induced cytotoxicity potentiated by GSH depletion. (A) Cell viability assessed by MTT assay. Cells were serum-starved for 12 hr, incubated in culture medium with or without sulfur amino acids for 12 hr, and subsequently treated with $CdCl_2$ for 12 hr. H4IIE cells were also incubated in the presence of 50 μM buthionine sulfoximine for 12 hr prior to addition of $CdCl_2$ and additionally incubated for 12 hr to assess cell viability. Inset shows the reduced GSH contents in cells 12 and 24 hr after SAAD. Data represent the mean $\pm$ SEM with five separate experiments. (B) The metallothioneins I and II expression in H4IIE cells under SAAD medium. A representative immunoblot shows metallothioneins levels in cells cultured in SAAD medium for 6–24 hr. Each lane was loaded with 70 μg of total cell lysates. (C) TUNEL assay of apoptosis in cells cultured under SAAD with or without 0.1 μM $CdCl_2$. Control cells showed no apoptosis (a). Representative photographs show minimal apoptosis in cells cultured with 0.1 μM $CdCl_2$ for 12 hr (b) or in cells cultured under SAAD for 24 hr (c). H4IIE cells cultured with 0.1 μM $CdCl_2$ for 12 hr after incubation under SAAD for 12 hr showed extensive apoptosis (d). Results were confirmed by multiple experiments. Magnification of 300×.

3.3. Effects of SAAD

A previous study has shown that the cellular GSH content decreased in a time-dependent manner when H4IIE cells were cultured in deficiency of cystine and methionine [10]. The first-order rate constant and the half-life time for the decrease in reduced GSH were 0.056 $\pm$ 0.007 per hr and 12.6 $\pm$ 1.5 per hr, respectively [10]. In the present study, cells were incubated in culture medium without sulfur amino acids for 12 hr prior to $CdCl_2$ treatment. MTT assay revealed that SAAD potentiated Cd-induced cytotoxicity. Incubation of cells in SAAD medium for 24 hr resulted in marginal cell death. The relative cytotoxicity to that by SAAD alone (i.e. 100%) was shown in Fig. 3A. Whereas the IC_{50} value of cell viability was 7.4 μM in cells cultured in control medium, the percent viability of H4IIE cells cultured without sulfur amino acids substantially decreased 12 hr after addition of $CdCl_2$ with the EC_{50} value of 0.2 μM (Fig. 3A). Hence, the cytotoxicity by $CdCl_2$ was about 40-fold potentiated in cells under SAAD for 12 hr. To further confirm the potentiation of Cd toxicity by decreased cellular GSH, cells were exposed to buthionine sulfoximine (50 μM) a GSH depleting agent for 12 hr prior to addition of $CdCl_2$. The IC_{50} value of cell viability was also greatly decreased (i.e. 0.25 μM) in cells exposed to buthionine sulfoximine (Fig. 3A). The GSH content rapidly decreased in cells incubated in SAAD medium (Fig. 3A, inset), which was consistent with the previous result [10]. Because me-

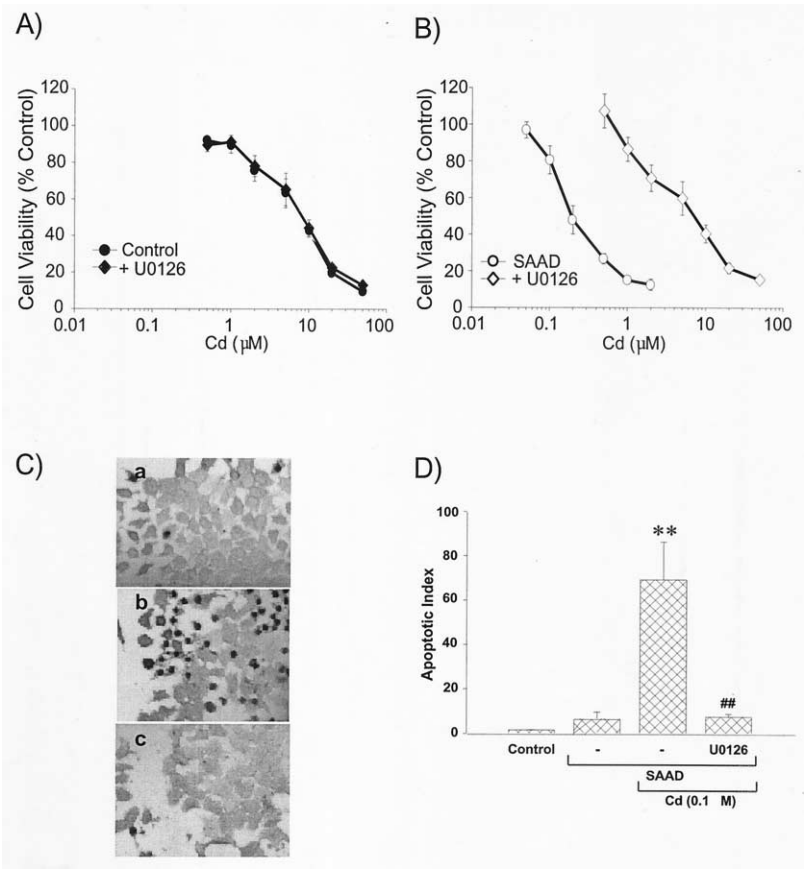

Fig. 4. Effects of U0126 on SAAD-potentiated Cd toxicity. (A) Effects of U0126 (10 μM) on Cd toxicity in control medium. (B) The effect of U0126 on Cd-induced cytotoxicity potentiated by SAAD. Eighty percent-confluent cells were incubated with or without sulfur amino acids in the presence of U0126 for 12 hr prior to addition of $CdCl_2$. Cell viability was assessed at 12 hr later. Data represent the mean ± SEM with five separate experiments. (C) Inhibition of SAAD and Cd-induced apoptosis by U0126. Apoptosis was assessed 12 hr after treatment of cells with Cd following preincubation under SAAD in the presence or absence of U0126 for 12 hr (a, control; b, SAAD + 0.1 μM $CdCl_2$; c, SAAD + 0.1 μM $CdCl_2$ + 10 μM U0126). Magnification of 300×. (D) The relative extent of apoptosis. The apoptotic index was obtained from the ratio of apoptotic cells to total cells × 100. Data represent the mean ± SEM with five independent experiments. (significant as compared to control, $^{**}P < 0.01$; significant as compared to SAAD + 0.1 μM $CdCl_2$, $^{\#\#}P < 0.01$).

tallothioneins play a role in the protection against Cd toxicity, the potential change in metallothionein I and II expression was assessed in cells under SAAD medium for 6, 12, and 24 hr (Fig. 3B). Immunoblot analysis revealed that the expression of metallothioneins was not changed in cells exposed to SAAD medium.

TUNEL assays were carried out to establish whether cell death induced by 0.1 μM of $CdCl_2$ in combination with SAAD involved apoptosis. Control cells showed no apoptosis (Fig. 3C-a). Either SAAD or $CdCl_2$ (0.1 μM) minimally induced apoptosis (<5%) (Fig. 3C-b and c). In contrast, cells treated with 0.1 μM $CdCl_2$ following incubation in SAAD medium for 12 hr showed substantial apoptosis (Fig. 3C-d). The apoptotic index was 69%. This indicated that TUNEL assay was more sensitive than that of MTT assay.

3.4. Effect of U0126 on potentiated cytotoxicity

Previous studies have shown that U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), serves as a potent inhibitor of MKK1/2. We were interested in whether U0126 prevented the Cd toxicity potentiated by SAAD. U0126 was not active in reducing Cd-induced toxicity in cells cultured in control medium (Fig. 4A). Cell viability was further determined at a variety of concentrations of Cd in cells cultured under SAAD with or without U0126. The concentration-response curve of cell viability right-shifted in the presence of 10 μM U0126 (Fig. 4B). The EC_{50} value was identical to that observed in control cells. TUNEL assay confirmed that U0126 inhibited apoptosis induced by SAAD in combination with a minimal concentration of $CdCl_2$ (Fig. 4C and D). Whereas cells incubated with 0.1 μM $CdCl_2$ for 12 hr following SAAD for 12 hr exhibited 69% of apoptosis, the presence of U0126 (10 μM) almost completely prevented apoptosis (i.e. 7.6% of apoptotic index). We confirmed that U0126 did not restore the GSH level in cells under SAAD medium.

3.5. Effects of U0126 on MAP kinase activation

A previous study has shown that SAAD increases phosphorylation of p38 MAP kinase and its activity [10]. In the

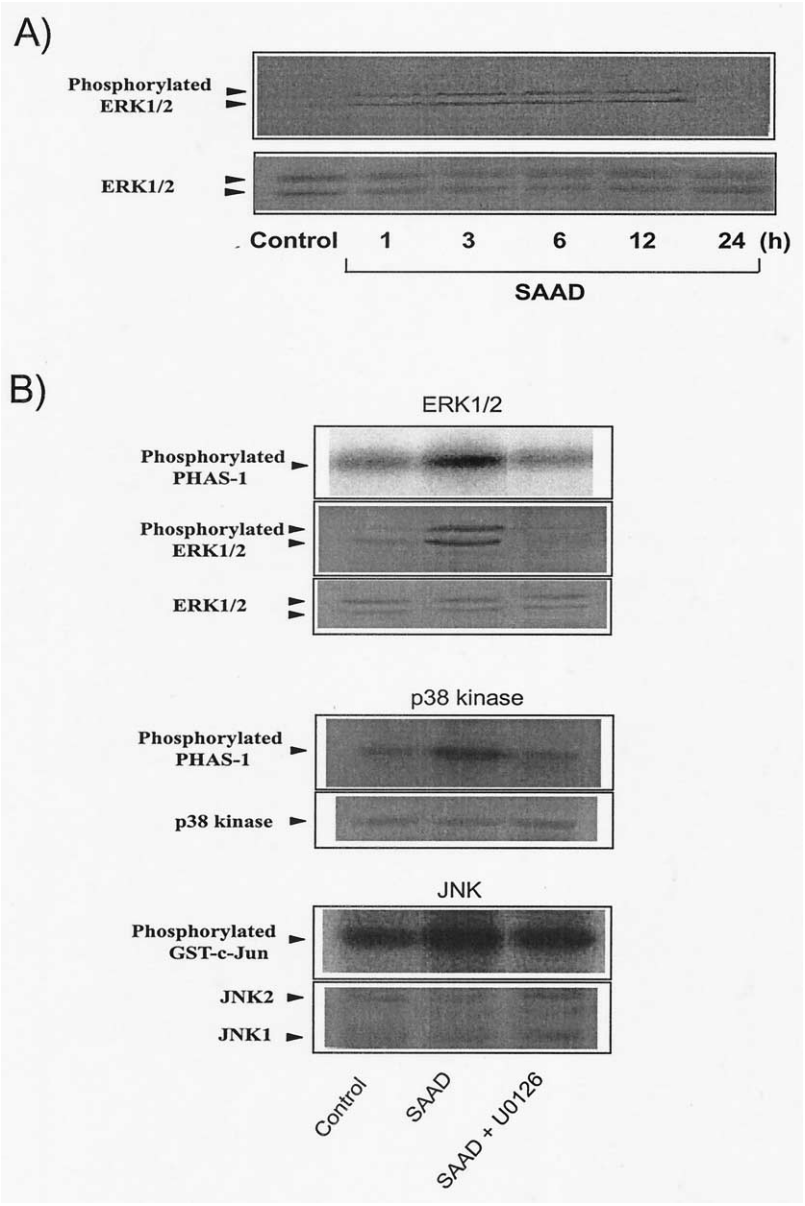

Fig. 5. ERK activation by SAAD and the effect of U0126 on MAP kinase activation. (A) ERK1/2 activation by SAAD. Time-dependent activation of ERK was assessed by immunoblotting of phosphorylated ERK-1 (44 kDa) and ERK-2 (42 kDa). The levels of unphosphorylated ERK were shown as controls. (B) The effects of 10 μM U0126 on the activation of ERK1/2, p38 kinase, and JNK in cells incubated under SAAD for 1 hr. Activity of ERK1/2 and phosphorylation of ERK1/2 were measured in cells 1 hr after SAAD with or without 10 μM U0126. Activation of p38 kinase was assessed as described previously. JNK activity was monitored by phosphorylation of GST-c-Jun.

current study, activation of ERK1/2 was measured in cells under SAAD (Fig. 5A). The level of phospho-ERK1/2, monitored by Western blot analysis, was increased from 1–12 hr after SAAD. Although ERK was activated at early times (10 or 30 min) after SAAD, the activation of ERK was variable (data not shown). The activation of ERK1/2 turned off at 24 hr (Fig. 5A).

U0126 effectively prevented the activation of ERK1/2 (Fig. 5B). Furthermore, U0126 inhibited the activation of p38 kinase by SAAD, the extent of which was comparable to that elicited by SB203580. JNK activation, which occurred at 1 hr after SAAD, was not inhibited by U0126 (Fig. 5). Hence, U0126 was active in inhibiting activation of ERK1/2 and p38 kinase, but not JNK, in cells under SAAD for 1 hr. Effective inhibition of SAAD-potentiated cytotoxicity by U0126 might result from concomitant inhibition of ERK and p38 kinase.

3.6. Effect of PD98059 on potentiated cytotoxicity

To determine the role of ERK pathway in the Cd-induced cytotoxicity potentiated by SAAD, cells were incubated with $CdCl_2$ in the presence or absence of PD98059 a selective ERK1/2 inhibitor [15]. Whereas treatment of cells in

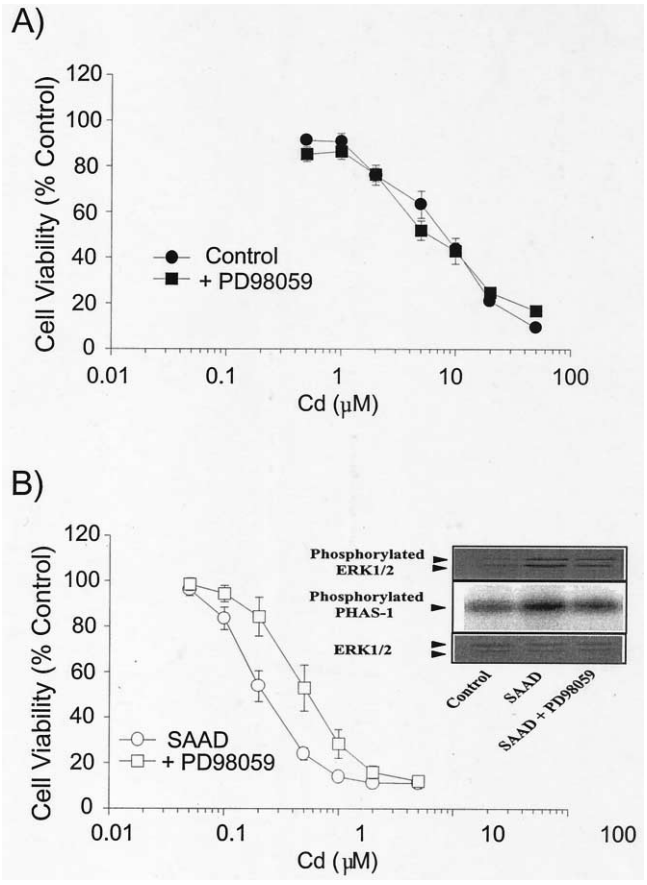

Fig. 6. The effect of PD98059 on SAAD-potentiated Cd-induced cytotoxicity. (A) Viability of cells incubated in control medium in the presence or absence of PD98059. (B) The effect of PD98059 on Cd-induced cytotoxicity potentiated by SAAD. Cells were incubated with or without sulfur amino acids in the presence or absence of PD98059 for 12 hr prior to addition of 0.5 μM $CdCl_2$. Cell viability was assessed at 12 hr later. Data represent the mean ± SEM with five separate experiments. The phosphorylated ERK1/2 was immunoprecipitated with the specific antibody and the activity of ERK1/2 was determined toward the substrate PHAS-1. The level of phosphorylated ERK and the activity of ERK1/2 were both measured in cells exposed SAAD for 1 hr with or without 50 μM PD98059. Unphosphorylated ERK was also assessed as a control. Results were confirmed by repeated experiments.

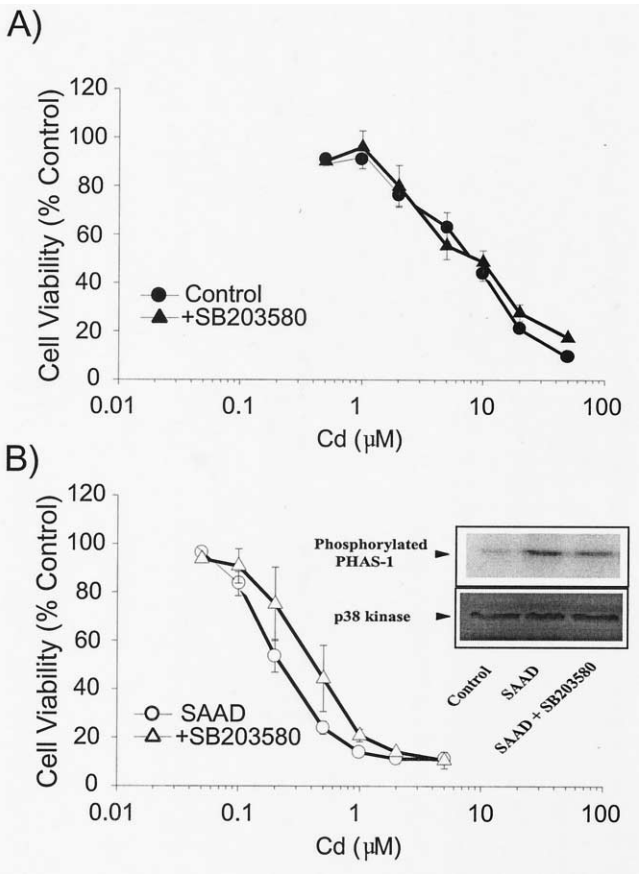

Fig. 7. The effect of SB203580 on Cd-induced cytotoxicity. (A) The effect of SB203580 (10 μM) on the viability of cells in control medium. (B) The effect of SB203580 on Cd-induced cytotoxicity potentiated by SAAD. Cell viability was assessed, as described previously. Activation of p38 kinase by SAAD (1 hr) was inhibited by 10 μM SB203580.

control medium with PD98059 for 12 hr prior to $CdCl_2$ treatment did not reduce cytotoxicity (Fig. 6A), PD98059 at the concentration of 50 μM significantly shifted the concentration-response curve to right in cells under SAAD (Fig. 6B). The EC_{50} value of SAAD-potentiated Cd toxicity was 2.5-fold increased by the presence of PD98059 (i.e. 0.20 vs. 0.50 μM, $P < 0.05$). Because PD98059 at 100 μM showed toxicity in H4IIE cells, the concentration-response effect of PD98059 could not be further assessed. Phosphorylation of ERK1/2 and activity of immunoprecipitated ERK1/2 toward PHAS-1 were both increased by SAAD (Fig. 6B). The effect of PD98059 on ERK1/2 activation was assessed in cells under SAAD for 1 hr (Fig. 6B). PD98059 inhibited ERK1/2 activation by SAAD, although the extent of inhibition was not complete. These results supported the notion that SAAD-potentiated Cd toxicity was associated at least in part with activation of ERK1/2.

3.7. Role of p38 kinase in SAAD-potentiated toxicity

Studies were carried out to determine the effect of SAAD plus Cd on the activation of p38 kinase in the presence or absence of SB203580 a p38 kinase inhibitor. SB203580 at the concentration of 10 μM failed to affect the concentration-response in cells incubated in control medium (Fig. 7A). Although SAAD-potentiated Cd toxicity was slightly reduced by SB203580 (Fig. 7B), the EC_{50} value was not statistically significant (0.20 vs. 0.34 μM). Activation of p38 kinase was inhibited by SB203580 in H4IIE cells (Fig. 7B, right panel). The data showed that activation of p38 kinase by itself marginally contributed to the enhanced toxicity.

3.8. Role of JNK in potentiated cytotoxicity

Given the activation of JNK by SAAD, the role of JNK in potentiated cytotoxicity was assessed. Cells stably trans-

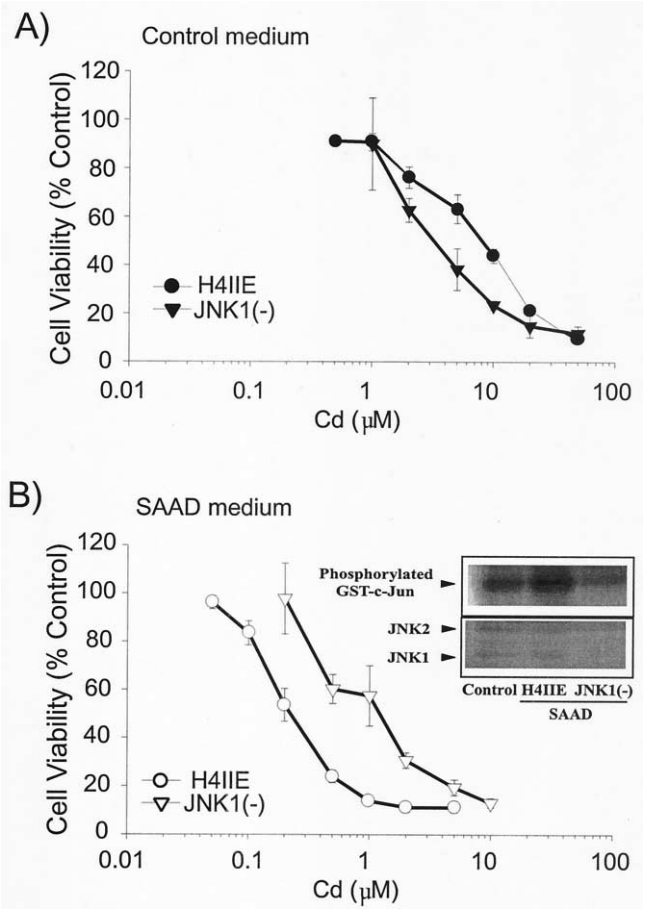

Fig. 8. Role of JNK in SAAD-potentiated Cd toxicity. Viability of cells or cells stably transfected with a dominant negative mutant of JNK1 [JNK1(−)]. (B) Cd-induced cytotoxicity in control or JNK1(−) cells. Cells stably transfected with JNK1(−) were cultured in SAAD medium in the presence of $CdCl_2$. Cell viability was assessed 12 hr later. Data represent the mean ± SEM with five separate experiments. The activity of JNK was determined by phosphorylation of GST-c-Jun 1 hr after SAAD. The levels of unphosphorylated JNK were also monitored in cells exposed to SAAD.

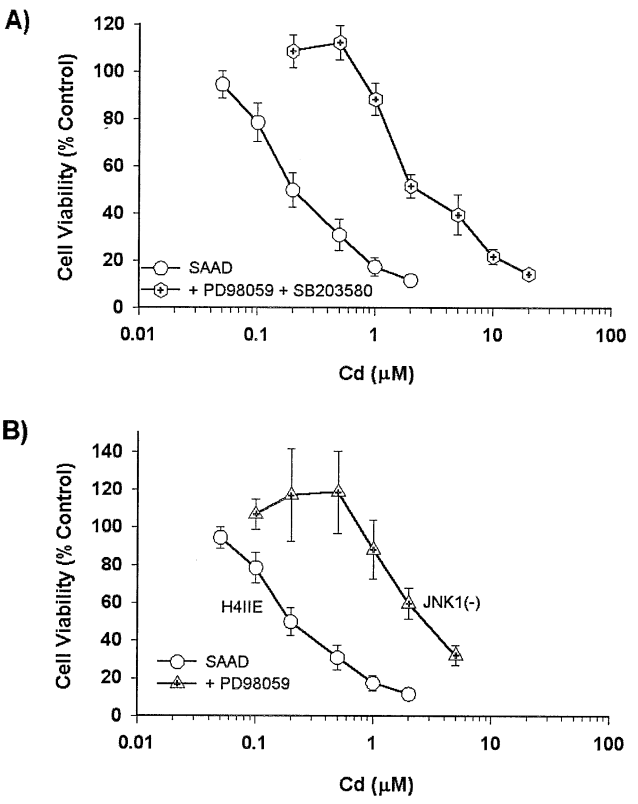

Fig. 9. Effects of PD98059 in combination with SB203580 or JNK1(−) transfection on SAAD-potentiated Cd toxicity. (A) The MTT cell viability assay. Cell viability was increased by the presence of both PD98059 and SB203580 to a greater extent than that by each inhibitor alone. (B) Effects of PD98059 on viability of JNK1(−) cells.

fected with a dominant negative mutant vector of JNK1 were incubated in control or SAAD medium in the presence of Cd. We found that Cd-induced toxicity was not affected by JNK1(−) transfection (control medium) (Fig. 8A). The EC_{50} value of SAAD-potentiated Cd cytotoxicity was, however, 5-fold increased in JNK1(−) stably transfected cells than in control cells (0.20 vs. 1.0 μM, $P < 0.05$) (Fig. 8B). JNK1(−) cells showed no activation of JNK1 (Fig. 8B, right panel). Hence, the JNK pathway was responsible for SAAD-potentiated Cd toxicity.

3.9. Role of ERK1/2 in combination with p38 kinase or JNK

We showed that inhibition of ERK1/2 and p38 kinase by U0126 effectively blocked the potentiated cytotoxicity of Cd. Hence, we were further interested in assessing whether inhibition of ERK1/2 in combination with either p38 kinase or JNK was more active in reducing SAAD-potentiated Cd toxicity. Treatment of cells with both PD98059 and SB203580 for 12 hr prior to $CdCl_2$ treatment decreased the potentiated toxicity to a greater extent than that with either inhibitor alone (Fig. 9A) (i.e. EC_{50} = 1.64 μM). PD98059 (50 μM) was more effective in inhibiting the toxicity in JNK1(−) cells than in control cells (Fig. 9B) (EC_{50}, 0.34 vs. 2.9 μM, $p<0.05$). Therefore, activation of ERK1/2 in conjunction with either p38 kinase or JNK greatly contributed to SAAD-potentiated Cd toxicity. This was further supported by the effect of U0126, which inhibited both ERK1/2 and p38 kinase. SB203580 (10 μM) further prevented the potentiated toxicity in JNK1(−) cells (EC_{50}, 1.0 vs. 2.8 μM, $P < 0.05$).

4. Discussion

The levels of cellular metallothionein and GSH affected severity of Cd-induced cytotoxicity [2,3]. However, the exact mechanism for Cd-induced cytotoxicity in the state of decreased GSH content has not been studied yet. The present study showed that the level of cellular GSH increased prior to cell death after treatment of cells with toxic

concentrations of Cd. A compensatory increase in GSH may provide the first line of defense against Cd. This was supported by prevention of Cd-induced cytotoxicity by excess GSH or cysteine. Previous studies have shown that Cd makes complexation with GSH or cysteine in a 1:2 ratio [14, 16]. The transport and intracellular accumulation of Cd was not affected by complexations with sulfhydryl residues [16]. In the present study, complexations of Cd with sulfhydryl-containing molecules such as GSH and cysteine failed to prevent Cd toxicity in cells cultured in control medium. Hence, blocking the toxicity of Cd by excess sulfhydryls might be mediated not with complexation, but with change in cellular redox-state and protection of protein thiols.

Studies have shown that GSH depletion may not be a requirement of acute Cd toxicity or that GSH depletion *per se* by Cd may not be a major mechanism of toxicity [17]. This would result from a compensatory change in GSH content. In the present study, the toxicity of Cd was 40-fold potentiated by deficiency of cellular GSH. The crucial role of cellular GSH for enhancement of Cd toxicity was further supported by the experiment using cells pretreated with a GSH depleting agent. Oxidative stress elevated by a decrease in cellular GSH would affect the redox state, and subsequent activation of signaling pathway(s) may alter the sensitivity of cells to toxic substances.

Depending on the exposure level, Cd may exert cytotoxicity in diverse ways. Many of the studies on Cd toxicity have been conducted with relatively high concentrations or doses of Cd [18–20]. Although the daily human exposure to Cd via dietary and respiratory absorption is picomolar levels [21], continued retention would lead to progressive accumulation of Cd to submicromolar concentrations in tissues particularly liver and kidney. The present study revealed that nontoxic submicromolar concentrations of Cd in combination with a decrease in cellular GSH synergistically increase cytotoxicity. Submicromolar concentrations of Cd, which are relevant to sub-chronic or chronic human exposure, could cause tissue damage if the exposure occurs in combination with a diminished cellular GSH content. Chromium (IV) alone (10–80 μM) activates MAP kinases including ERK1/2, p38 kinase, and JNK without decreasing the cellular GSH content [22]. Treatment of cells with 30 μM chromium (IV) prior to addition of Cd failed to markedly enhance Cd toxicity (Kim and Park, unpublished data), supporting the notion that activation of MAP kinases alone in the absence of decrease in the GSH level does not potentiate Cd toxicity.

Three distinct MAP kinase modules ERK, p38 kinase, and JNK have been characterized [23, 24]. The present study showed that three MAP kinases are all activated by deprivation of sulfur amino acids [10]. Hence, the SAAD experiment serves as an appropriate model to assess the molecular events and the signaling pathways for toxicity induced by heavy metals. A number of cellular stresses and lethal insults engage MAP kinases [25,26]. ERK1/2 is activated by a variety of growth factors, cytokines, and phorbol esters, and regulates cellular proliferation and differentiation [27,28]. Oxidative stress stimulates ERK1/2 [29,30]. In rat neonatal cardiomyocytes, hydrogen peroxide specifically activates ERK through activation of the Src family of tyrosine kinases, Ras, and Raf-1 [31]. Activation of ERK prevented hydrogen peroxide-induced apoptosis of these cells. On the contrary, ERK activation may also be involved in signaling of cell death. For example, activation of ERK1/2 in macrophage cells increased apoptosis induced by toxins [32]. In the present study, we demonstrated for the first time that SAAD stimulated activation of ERK1/2 from 1–12 hr and potentiated the toxicity of Cd at the levels of human exposure. U0126 failed to prevent Cd-induced toxicity in cells in control medium. Activation of ERK1/2 was in part responsible for the toxicity of Cd potentiated by SAAD. The activation of ERK1/2 was suppressed to a greater extent by U0126 than by PD98059, which paralleled with the extent of inhibition of SAAD-potentiated Cd toxicity. U0126 is an inhibitor of MKK1/2 and thus activation of ERK1/2. It has been shown that the intrinsic efficacy and potency of U0126 for the inhibition of ERK1/2 were higher than those of PD98059. We showed that activation of p38 kinase was also inhibited by U0126, which is in agreement with Davies *et al.* [15]. A recent study showed that U0126 could block the activity of p70S6 kinase [33], which affects cell cycle control. Presence of rapamycin, an upstream inhibitor of p70S6 kinase, failed to prevent the Cd toxicity potentiated by SAAD (data not shown). This excludes the possibility that the beneficial effect of U0126 against potentiated Cd toxicity be mediated with the inhibition of p70S6 kinase. Taking the observation into consideration that treatment of cells with both PD98059 and SB203580 further inhibited SAAD-potentiated Cd toxicity, the protective effect of U0126 might result from the inhibition of p38 kinase as well as ERK1/2 activation. While we were preparing the current manuscript, Iryo *et al.* reported that ERK1/2 was responsible for toxicity of Cd, in which experiment U0126 was used as an ERK1/2 inhibitor [34]. In their study, pretreatment with U0126 suppressed Cd-induced ERK activation and the apoptosis, whereas the inhibition of p38 kinase activity with SB203580 did not protect cells from apoptosis. The results were in agreement in part with those in the present study. However, U0126 failed to protect cells against Cd in control medium. This discrepancy may be due to the cell type difference. The ERK1/2 pathway was not responsible for Cd-induced cytotoxicity of CL3 a lung carcinoma cell line [35].

The p38 kinase is a recently identified member of the MAP kinase family and represents the distinct stress-activated pathway. Activation of p38 kinase is an early response of cells upon exposure to a variety of stresses [36–38]. Activity of p38 kinase is involved in apoptosis [39]. An increase in p38 kinase activity was paralleled by antioxidant-induced activation of AP-1 [37] and was activated by prooxidants such as hydrogen peroxide or nitric oxide [29, 40]. SAAD markedly decreased the cellular GSH content,

elicited oxidative stress, and activated p38 kinase [10]. It is noteworthy that the inhibition of p38 kinase and ERK1/2 activities by SB203580 and PD98059 markedly reduced potentiation of Cd-induced cytotoxicity by SAAD, although SB203580 alone marginally prevented SAAD-enhanced toxicity. The extent of inhibition by the inhibitors was comparable to that by U0126. Therefore, ERK particularly in combination with the activation of p38 kinase served as the toxic signaling pathway for Cd in cells with decreased GSH.

Stress-activated protein kinase cascade involves activation of JNK. JNK-induced phosphorylation of c-Jun activates AP-1, and increases the expression of AP-1-targeted genes [26,38]. In this study, the stable transfection of dominant negative mutant of JNK1 altered the toxicity of Cd enhanced by SAAD to a certain extent. Hence, activation of ERK in combination with JNK appeared to contribute to the potentiated Cd toxicity. While we studied the signaling pathways, the role of JNK in Cd-induced toxicity was reported [35]. In their study, activation of JNK was studied at much higher concentrations of Cd (i.e. 80–130 μM).

The mechanistic basis for the activation of MAP kinases by SAAD has also been studied in this laboratory. We revealed that SAAD activated PI3-kinase and stimulated phosphorylation of PDGF receptor tyrosine kinase (Kang and Kim, unpublished data). Nonetheless, the activation of the kinases associated with the plasma membrane was not directly related with the activation of MAP kinases. Other studies in this laboratory revealed that SAAD induces apoptosis with an increase in cytochrome *c* release from mitochondria at 72 hr, but not with caspase-3 activation (Kang and Kim, unpublished data).

The results of the current study provided evidence that SAAD activated three MAP kinases including ERK1/2, p38 kinase, and JNK and markedly potentiated the cytotoxicity induced by Cd at the concentrations of human exposure. SAAD-potentiated Cd-induced toxicity might result from the activation of ERK1/2 in combination with other MAP kinases such as p38 kinase or JNK concomitantly activated by decreased GSH.

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