CADMIUM TOXICITY INVOLVES OXIDATIVE STRESS, CALCIUM AND MAP KINASES: IMPLICATION FOR APOPTOSIS IN MURINE MACROPHAGES

by

JIYOUNG KIM

(Under the Direction of Raghubir P. Sharma)

ABSTRACT

Cadmium is a well-known carcinogen and immunotoxic metal commonly found in cigarette smoke and industrial effluents. It has been reported that smokers have 4-5 times higher level of cadmium in blood than non-smokers. Inhalation of cadmium will directly effect on macrophages in respiratory system without first-pass elimination and be a factor of immunodepression of smokers. The objectives of the present study are to define the important signaling mediators in cadmium-induced cell death and growth arrest in murine macrophages.

Cadmium elevated intracellular calcium ($[Ca^{2+}]_i$) and reactive oxygen species (ROS) at early time point of 6 h in J774A.1 murine macrophage cells. Caspase-3 activation at 8 h and thereafter DNA fragmentation were detected and initiator caspase-8 and -9 were involved to activate executor caspase-3 in cadmium-induced apoptosis. Phosphorylation of c-Jun NH_2-terminal kinase (JNK) and extracellular signal-related kinase (ERK) were activated and p38 mitogen-activated protein kinase (MAPK) was down-regulated by cadmium.

We show here that $[Ca^{2+}]_i$ elevation and oxidative stress by cadmium are interrelated each other. Both Ca^{2+} chelators and antioxidants inhibited cadmium-induced caspase-3 activation, DNA fragmentation and growth arrest. Chelating intracellular and extracellular Ca^{2+} inhibited

cadmium-induced JNK activation and inhibition of JNK reduced apoptotic response suggesting elevated $[Ca^{2+}]_i$-JNK-caspase-3 signaling pathway leading to apoptosis by cadmium.

Antioxidants decreased cadmium-induced ERK activation and cell death. We found that cadmium induces ROS-ERK-$p21^{WAF1/CIP1}$ signaling pathway leading to G2/M arrest and cell death. Inhibition of ERK recovered cadmium-induced necrosis but did not show any effect on caspase-3 activation and apoptosis. Activation of ERK was dependent on free Cd^{2+}, while JNK and p38 MAPK were not. Chelating Cd^{2+} was able to inhibit cadmium-induced necrosis but failed to recover cadmium-induced apoptosis. Chelating Cd^{2+} was still able to elevate $[Ca^{2+}]_i$ and hydrogen peroxide generation. These results demonstrate that free Cd^{2+} plays an important role in ERK-necrosis signaling. Altered $[Ca^{2+}]_i$ level or redox system by cadmium complexes is capable of initiating toxic action leading to apoptosis, mitochondrial impairment and growth arrest.

INDEX WORDS: Cd^{2+}, Ca^{2+}, ROS, JNK, ERK, p38 MAPK, caspase-3, apoptosis, necrosis, proliferation, cell cycle arrest, chelation, J774A.1

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DEDICATION

to my father, Kwang Joon Kim and to my mother, Young Sook Lee

for their invaluable support, enduring love, understanding, encouragement and inspiration

to my only brother, Jung Woo Kim and my dear friends

who were always there for me

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS……..……..……..……v

LIST OF TABLES……..……..……..…….....viii

LIST OF FIGURES……..……..……...…..ix

CHAPTER

1 INTRODUCTION……..……..……1

2 LITERATURE REVIEW……..15

3 EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK)-SIGNALING-DEPENDENT G2/M ARREST AND CELL DEATH IN MURINE MACROPHAGES BY CADMIUM.……..…….....42

4 CALCIUM-MEDIATED ACTIVATION OF C-JUN NH_2-TERMINAL KINASE (JNK) AND APOPTOSIS IN RESPONSE TO CADMIUM IN MURINE MACROPHAGES…78

5 CADMIUM-INDUCED APOPTOSIS IN MURINE MACROPHAGES: ROLE OF REACTIVE OXYGEN SPECIES AND CASPASE-3 ACTIVATION…….……...113

6 FREE VERSUS CHELATED CADMIUM IN CADMIUM-INDUCED CYTOTOXICITY IN MURINE MACROPHAGES……..……..148

7 SUMMARY AND CONCLUSIONS…….....……174

LIST OF TABLES

Page

Table 3.1: The Effect of inhibition of ERK activity on cadmium-altered cell cycle progression

…….....68

LIST OF FIGURES

Page

Figure 3.1: Effect of $CdCl_2$ on cell viability and proliferation……..……...……69

Figure 3.2: Effect of $CdCl_2$ on cell cycle progression……...……...70

Figure 3.3: Flow cytometric analysis of necrotic and apoptotic cell death…...……..…..…71

Figure 3.4: Effect of $CdCl_2$ on phosphorylation of ERK..…….……...72

Figure 3.5: Effect of PD98059 on $CdCl_2$-induced ERK activation, $p21^{WAF1/CIP1}$ induction, p27 down-regulation and inhibition of proliferation……..73

Figure 3.6: Effect of PD98059 on $CdCl_2$-induced necrotic and apoptotic cell death...……74

Figure 3.7: Effect of PD98059 on $CdCl_2$-induced caspase-3 activation and DNA fragmentation……...……75

Figure 3.8: Effect of antioxidants on $CdCl_2$-induced ERK activation, $p21^{WAF1/CIP1}$ induction, and p27 down-regulation……76

Figure 3.9: Confirmation of necrosis and apoptosis by fluorescence microscopy……...77

Figure 4.1: Effect of $CdCl_2$ on $[Ca^{2+}]_i$ level observed by fluorescence microscopy…..…..…..101

Figure 4.2: Effect of $CdCl_2$ on phosphorylation of JNK……102

Figure 4.3: Effect of $CdCl_2$ on phosphorylation of p38 MAPK…..…….……..103

Figure 4.4: Effect of SP600125 on $CdCl_2$-induced caspase-3 activation and DNA fragmentation……...……..104

Figure 4.5: Effect of SP600125 on $CdCl_2$-induced necrosis……..…….……106

Figure 4.6: Effect of BAPTA-AM and EGTA on $CdCl_2$–induced $[Ca^{2+}]_i$ elevation, growth arrest, mitochondrial activity impairment and necrosis……...107

Figure 4.7: Effect of BAPTA and EGTA on $CdCl_2$-induced caspase-3 activation and DNA fragmentation……..……..……..109

Figure 4.8: Effect of BAPTA and EGTA on $CdCl_2$-induced JNK activation and down-regulation of p38……..…111

Figure 4.9: Interrelationship of ROS and $[Ca^{2+}]_i$ elevation……112

Figure 5.1: Effect of $CdCl_2$ on H_2O_2 generation.……..……..…138

Figure 5.2: Effect of $CdCl_2$ on caspase-3 activation and DNA fragmentation by TUNEL…....139

Figure 5.3: Inhibition of caspases on $CdCl_2$-induced caspase-3 activation and apoptosis…....140

Figure 5.4: Effect of antioxidatns on $CdCl_2$-induced caspase-3 activation and DNA fragmentation.……....141

Figure 5.5: Effect of antioxidants on $CdCl_2$–induced growth arrest, mitochondrial activity impairment, necrosis and $[Ca^{2+}]_i$ elevation.……..…….143

Figure 5.6: Concentration-dependent effect of antioxidants on $CdCl_2$-induced JNK activation..……...……...145

Figure 5.7: Concentration-dependent effect of antioxidants on $CdCl_2$-induced ERK activation……146

Figure 5.8: Relation to caspase-3 and MAPKs activation on $CdCl_2$–induced H_2O_2 generation…….……..147

Figure 6.1: Effect of TPEN on $CdCl_2$-induced necrosis…….…….……...168

Figure 6.2 Effect of TPEN on $CdCl_2$-induced caspase-3 activation and DNA fragmentation...169

Figure 6.3: Effect of TPEN on $CdCl_2$–induced mitochondrial activity impairment and growth arrest …..……..…….170

Figure 6.4: Effect of TPEN on $CdCl_2$-induced activation of ERK and JNK and down-regulation of p38…..…….…..……171

Figure 6.5: Effect of TPEN on $CdCl_2$-induced generation of H_2O_2 and $[Ca^{2+}]_i$ elevation……..…..……..……..173

Figure 7.1: Possible signal transduction mechanism of cadmium toxicity leading to apoptosis and growth arrest in J774A.1 murine macrophages……..178

CHAPTER 1

INTRODUCTION

Cadmium is a naturally occurring nonessential and toxic heavy metal, which belongs to transition metal group IIB of the periodic table. The toxicity of cadmium as an industrial pollutant and a food contaminant, and as one of the major components in cigarette smoke is well established (Morselt, 1991). Cadmium is known to have a biological half-life exceeding 20 years and may accumulate in various target organs such as liver and kidney (Goering *et al.*, 1995). It has been reported that cadmium induces apoptosis in leukemia and lymphoma cells (Bagchi *et al.*, 2000; el Azzouzi *et al.*, 1994; Tsangaris *et al.*, 1998), renal epithelial cells (Matsuoka *et al.*, 1995), proximal tubule cells (Hamada *et al.*, 1997), lung epithelial cells (Hart *et al.*, 1999), liver (Habeebu *et al.*, 1998), testis (Xu *et al.*, 1996; Zhou *et al.*, 1999), and ventral prostate (Zhou *et al.*, 1999). However, the mechanism of toxicity and the signal transduction pathways leading to apoptosis exposed to cadmium have not been clarified.

Cadmium is known to increase lipid peroxidation and to decrease activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (del Carmen *et al.*, 2002). The involvement of reactive oxygen species (ROS) as intracellular messengers to induce DNA single strand breaks and fragmentation in the toxicity of cadmium has been proposed (Bagchi *et al.*, 1998; Hassoun *et al.*, 1996). A number of recent studies also demonstrate that cadmium interacts with the function of Ca^{2+}-dependent enzymes such as endonuclease and regulatory proteins such as protein kinase C (PKC) and phospholipase C, thus interfering with the Ca^{2+}-signaling pathways (Lohmann *et al.*, 1993; Long, 1997; Misra *et al.*, 2002). Calcium ions are central to multiple signal transduction pathways to accomplish a variety of biological functions. The spatial and temporal regulation of intracellular calcium ($[Ca^{2+}]_i$) serves as a

modulator of pathways involved in learning and memory, fertilization, proliferation and development (Berridge *et al.*, 2000). Ca^{2+}-dependent processes by cadmium (< 1 μM) activate $p21^{ras}$-dependent mitogen activated-protein kinases (MAPKs) pathways, and nuclear factor-κB (NF-κB)-dependent gene expression, to stimulate proliferation in peritoneal macrophages (Misra *et al.*, 2002). Further the direct interaction of cadmium with intracellular molecules, oxidative stress or altered $[Ca^{2+}]_i$ homeostasis is considered as initiation of toxic action by cadmium. If cadmium interacts with cell surface receptors affecting intracellular signals, free Cd^{2+} ion will not be required to enter cells in order to exert toxic effects (Beyersmann *et al.*, 1997). Cadmium in culture or tissues exists in several different forms, however, which type of cadmium is responsible for toxicity and the role of free Cd^{2+} has not been clearly determined.

MAPKs are a family of Ser/Thr protein kinases that transmit extracellular signals into the nucleus (Robinson *et al.*, 1997; Schaeffer *et al.*, 1999). Three subfamilies of MAPKs including extracellular signal-related kinase (ERK), c-Jun NH_2-terminal kinase (JNK, also known as stress-activated protein kinase) and p38 MAPK have been shown to participate in a diverse array of cellular functions such as cell growth, cell differentiation and cell death (Robinson *et al.*, 1997; Schaeffer *et al.*, 1999). However, it is not clear if cadmium-altered MAPKs activity is interrelated with cell growth and apoptosis. Cadmium-induced phosphorylation of JNK has been reported in LLC-PK_1 porcine renal epithelial cells (Matsuoka *et al.*, 1998), in Rat-1 fibroblasts (Iordanov *et al.*, 1999), and ERK as well as JNK in rat mesangial cells (Ding *et al.*, 2000). On the other hand, ERK and p38 MAPK but not JNK were activated in response to cadmium exposure in LMH chicken hepatoma cells (Elbirt *et al.*, 1998) and 9L rat brain tumor cells (Hung *et al.*,

1998), suggesting that MAPKs are differentially activated by cadmium exposure depending on the cell type.

Based on substantial evidence, mostly from in vivo animal models, cadmium is able to damage both the humoral immune response and cell mediated immunity (Descotes, 1992). J774A.1 cells are a commonly used murine macrophage cell line possessing similarities to mature macrophages making them an alternative to primary cells (Yan *et al.*, 2004). J774A.1 cells originated from BALB/c mouse reticulum cell sarcoma, and due to the tumor-like property, these cells may be more resistant to cadmium toxicity. Even though macrophages are considered terminally differentiated, studies using macrophages on cadmium-induced inhibition of cell cycle progression and subsequent cell death would be helpful to understand cadmium effect on the immune system.

The objective of this research comprising in the dissertation is to test the hypothesis that **Cadmium toxicity involves oxidative stress, calcium and MAPKs in murine macrophages.** The following specific aims were attempted to accomplish the objectives:

1. **To investigate the effect of cadmium on cell cycle progression and cell death via ERK in J774A.1 murine macrophage cells.**
2. **To evaluate cadmium-mediated calcium signaling in activation of JNK and caspase-3 in J774A.1 murine macrophage cells.**
3. **To determine the time frame of ROS generation and apoptosis induced by cadmium in J774A.1 murine macrophage cells.**

4. **To examine the role of free versus chelated cadmium in cadmium-induced cytotoxicity in J774A.1 murin macrophages.**

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CHAPTER 2

LITERATURE REVIEW

Cadmium : Occurrence, Exposure and Dose

Cadmium is a naturally occurring metallic element, one of the components of the earth's crust. Its presence in the environment results mainly from gradual phenomena such as rock erosion and abrasion, volcanic eruptions. Cadmium is therefore present everywhere in air, water, soil and foods. Its existence was revealed in 1817. Unlike most metals, cadmium use began fairly recently with its large-scale application dating from 1940s, based on its unique chemical and physical properties (Goering *et al.*, 1995). The most remarkable characteristics of cadmium are its great resistance to corrosion, its low melting point and excellent electrical conduction. For these reasons, cadmium is widely used in special alloys, pigments, coatings, and stabilizers and, above all (almost 70% of its use) in rechargeable nickel-cadmium batteries.

Major occupational exposure occurs in non-ferrous metal smelters, in the production and processing of cadmium, its alloys and compounds and, increasingly, in the recycling of electronic waste. Non-occupational exposure is mainly from cigarette smoke, which contains relatively high concentration of cadmium; for non-smokers who are not occupationally exposed, diet is the main route of exposure to cadmium (WHO, 1992). Breathing high levels of cadmium in contaminated air (from battery manufacturing, metal soldering, welding or burning of fossil fuels or municipal waste) severely damages the lung and can cause death (Andersen *et al.*, 1988; Manca *et al.*, 1994). Eating contaminated water or foods such as shellfish, liver, and kidney meats severely irritates the stomach leading to vomiting and diarrhea (Andersen *et al.*, 1988). Gastrointestinal absorption of cadmium may be influenced by nutritional factors, such as iron status (Flanagan *et al.*, 1978).

The cadmium concentration in blood generally reflects current exposure, but partly also lifetime body burden (Jarup *et al.*, 1983). Biological monitoring has shown that cigarette

smoking may cause significant increases in blood cadmium levels. The concentration on smokers were average 4-5 times higher than those in non-smokers (Jarup *et al.*, 1998). The cadmium concentration in urine is manly influenced by the body burden (Jarup, 2003). Smokers and people living in contaminated areas have higher urinary cadmium concentration, smokers having about twice as high concentration as non-smokers (Jarup *et al.*, 1998).

Cadmium : Health Effect

In most studies, the half-life in human is estimated to be between 15 and 20 years (Jin *et al.*, 1998). Long-term exposure to lower levels of cadmium in air, food, or water leads to a buildup of cadmium in the kidneys and possible kidney disease (Manca *et al.*, 1991; Shaikh *et al.*, 1999). Animals given cadmium in food or water had high blood pressure, iron-poor blood, liver disease, and nerve or brain damage (Andersen *et al.*, 1988; Fariss, 1991; Kumar *et al.*, 1996; Shaikh *et al.*, 1999). Other long-term effects are lung damage and fragile bones (Andersen *et al.*, 1988; Manca *et al.*, 1994). The classical example of the importance of cadmium as an environmental contaminant is the outbreak at the Jinzu River in Japan of a severe disease (itai-itai, ouch-ouch disease) characterized by severe pain, bone fractures, osteomalacia and osteoporosis, which appeared mainly among women. It seems that the exposure was caused by the ingestion of cadmium-contaminated rice and water used, originating in a mine slag, in combination with nutritional factors (Agency for Toxic substances and Disease Registry, 1999).

Cadmium : Cancer

Cadmium is a potent human carcinogen and occupational exposure to it has been associated with cancers of the lung, the prostate, the pancreas, and the kidney (Waisberg *et al.*, 2003). Because of its characteristics as a lung carcinogen, the IARC (International Agency for Research on Cancer and the National Toxicology Program of the USA) has classified cadmium

as a human carcinogen (group I) on the basis of sufficient evidence in both humans and experimental animals (IARC, 1993). Cadmium predominantly is a non-genotoxic carcinogen (Waisberg *et al.*, 2003). It is essentially non-mutagenic in bacterial tests and only weakly mutagenic in mammalian cells in vitro (IARC, 1993). On the other hand, cadmium compounds have been proven to be comutagenic in mammalian cell tests when combined with genotoxic agents, and this property has been explained by the inhibition of DNA repair processes by this metal (Hartwig *et al.*, 2002; Liu *et al.*, 2000). Non-genotoxic mechanisms up-regulating intracellular signaling pathways leading to increased mitogenesis are suggested as major mechanisms for the interpretation of the carcinogenic activity by chronic cadmium exposure.

Cellular Uptake and Distribution

Cadmium uptake occurs by an active transport rather than passive diffusion mechanism in Chinese Hamster Ovary (CHO) cells. The marked reduction of cadmium uptake by thiol binding agents indicates the involvement of –SH groups in the transport of Cd^{2+} ions (Klug *et al.*, 1988). Cd^{2+} ions are taken up primarily through voltage-gated Ca^{2+} channels in an established secretory cell line, GH4C1 (Hinkle *et al.*, 1987) and in rat hepatocytes, about a third of the cadmium enters the cell through the receptor-operated Ca^{2+} channels (Blazka *et al.*, 1991). Cadmium is accumulated intracellularly due to its binding to cytoplasmic and nuclear material. Most of toxic effect of cadmium are attributed to the intracellular reactions of the Cd^{2+} ion; However, Cd^{2+} need not enter cells at all in order to exert intracellular effects if it interacted with cell surface receptors which affect intracellular signals (Beyersmann *et al.*, 1997).

Cadmium and Reactive Oxygen Species (ROS):

Various studies have shown that cadmium toxicity seems to be crucially mediated by the production of ROS. In cultured cells, cadmium induces the production of hydroxyl radicals (O'Brien *et al.*, 1998), superoxide anion, nitric oxide, and hydrogen peroxide (Galan *et al.*, 2001; Stohs *et al.*, 2001). Cadmium-increased lipid peroxidation was observed in the liver, kidney, brain, lung, heart, and testes of rat (Manca *et al.*, 1991). Because cadmium is not a Fenton metal, indirect mechanisms for the generation of free radicals have been proposed.

Short-term exposure to cadmium has been shown to decrease the activities of cellular antioxidant enzymes in vitro and in vivo (Waisberg *et al.*, 2003). Cadmium can decrease the level of cellular glutathione, a scavenger of intracellular ROS by a direct reaction, or the activities of antioxidant enzymes, including superoxide dismutase, glutathione peroxidase and catalase (del Carmen *et al.*, 2002; El Maraghy *et al.*, 2001; Ochi *et al.*, 1987; Tatrai *et al.*, 2001). The decrease in the activity and/or intracellular levels of antioxidants caused by cadmium, together with the generation of radicals that are produced during normal metabolism, may explain the increase in lipid peroxidation and DNA damage in cells (Waisberg *et al.*, 2003).

On the other hand, more elevated doses and extended exposure of cadmium have been reported to increase the levels of metallothionein and glutathione, and to enhance the activities of antioxidant enzymes, superoxide dismutase, glutathione reductase and glutathione peroxidase in various tissues (Casalino *et al.*, 1997; El Maraghy *et al.*, 2001; Gupta *et al.*, 1991; Liu *et al.*, 2001; Shaikh *et al.*, 1976), probably because of adaptive induction of genes. Metallothioneins are involved in the control of apoptosis and metallothionein null cells are more susceptible to apoptotic death after exposure to a variety of anticancer agents (Kondo *et al.*, 1997; Shimoda *et*

al., 2003), suggesting a possible role of cadmium metallothionein in induction as an anti-apoptotic agents, which could lead to aberrant cell survival (Waisberg *et al.*, 2003).

Another possible mechanism which helps to explain the increase in ROS caused by cadmium is the displacement of iron and copper from various intracellular sites (cytoplasmic and membrane proteins). Cadmium interferes with the utilization of iron and copper and abolishes their function in biological system (Nath *et al.*, 1984), because of similar physical properties. Increased concentration of ionic iron and copper, which was displaced by cadmium, can then cause oxidative stress through fenton reactions (Waisberg *et al.*, 2003). Rats exposed to cadmium had increased testicular Fe content 12 h after exposure (Koizumi *et al.*, 1992). Desferrioxamine (a ferric chelator) prevented cadmium-induced ROS formation and cytotoxicity (Pourahmad *et al.*, 2000). Because ROS can act as signaling molecules, the increase in ROS probably is involved in the modulation of apoptosis and gene expression by cadmium. Both the oxidative damage to DNA and the induction of proto-oncogenes by signaling oxidative metabolites by cadmium may contribute to the initiation and promotion of tumor growth.

Cadmium and Calcium (Ca^{2+})

The maintenance of cellular calcium homeostasis is a pre-requisite for the role of Ca^{2+} ions in mediating signals from cell surface to the nucleus. Since Ca^{2+} serves as an almost universal messenger in cell activation, the intracellular free Ca^{2+} concentration has to be regulated tightly (Alkon *et al.*, 1988; Berridge, 1993; Clapham, 1995). Perturbation of Ca^{2+}-mediated signal transduction have been considered as underlying mechanisms for the action of various chemical toxicants (Nicotera *et al.*, 1992; Pounds, 1990). Transient increases in intracellular Ca^{2+} and subsequent activation of protein kinases modulate cell proliferation and/or

differentiation (Cerutti *et al.*, 1991). Whereas persistent elevation of the Ca^{2+} concentration might be associated with necrotic or, in certain systems, apoptotic processes (Fawthrop *et al.*, 1991; Orrenius *et al.*, 1989).

Cd^{2+} and Ca^{2+} are two closely related elements with similarity in many aspects, partially due to their similar ionic radii; the radius of a common form of free Cd^{2+} in the body and that of Ca^{2+} are 0.099 and 0.097 nm, respectively (Weast *et al.*, 1982). Some of Cd^{2+} ions are taken up by the voltage-operated Ca^{2+} channel to enter mammalian cells (Blazka *et al.*, 1991; Hinkle *et al.*, 1987). However, Cd^{2+} is also a well-known Ca^{2+} channel blocker (Chow, 1991; Thevenod *et al.*, 1992). Cd^{2+} inhibits receptor-operated Ca^{2+} channels in hepatocytes as well as voltage-operated Ca^{2+} channels in neuronal cells (Hughes *et al.*, 1989; Thevenod *et al.*, 1992). Once Cd^{2+} has reached cytosol, it interacts with all known Ca^{2+} transport systems. Cadmium decreases Ca^{2+} efflux by competitive inhibition of the Ca^{2+} extruding Ca^{2+}-ATPase pump, leading to increased cytosolic free Ca^{2+} (Verbost *et al.*, 1988; Verbost *et al.*, 1989). Similarly, Cd^{2+} inhibits the intracellular sequestration of Ca^{2+} into microsomal stores (Hechtenberg *et al.*, 1991; Zhang *et al.*, 1990), and even uptake into nuclear compartments (Hechtenberg *et al.*, 1994). With regard to cell surface effects, Cd^{2+} causes a large but short-lasting Ca^{2+} mobilization through the formation of inositol triphosphate in human skin fibroblasts (Smith *et al.*, 1989).

Cadmium can also affect components of the Ca^{2+} messenger system. Cd^{2+} displaces and binds with high affinity for and activates calmodulin, a Ca^{2+} binding protein that regulates a variety enzymes and cell progresses (Behra *et al.*, 1991). Moreover, a number of recent studies demonstrated that cadmium interacts with the function of Ca^{2+}-dependent enzymes such as endonuclease and regulatory proteins such as protein kinase C (PKC) and phospholipase C, thus

interfering with the Ca^{2+}-signaling pathways (Lohmann *et al.*, 1993; Long, 1997; Misra *et al.*, 2002).

Cadmium and Metallothionein/Glutathion

Exposure of cells as well as whole animals to cadmium results in the induction of expression of several stress response genes such as those encoding for metallothionein (MT) synthesis, genes involved in the synthesis of glutathione (GSH), genes involved in oxidative stress response, genes encoding for heat-shot proteins and related genes (Waisberg *et al.*, 2003). Genes encoding for MT are the most studied genes with respect to the potential of cadmium to induce gene expression (Waisberg *et al.*, 2003). Subtoxic concentrations of cadmium result in rapid and significant induction of MT in vitro and in vivo (Waisberg *et al.*, 2003). MT sequester cadmium with high affinity resulting in decreased ability of Cd^{2+} capable of interacting with cellular targets to elicit toxicity (Waisberg *et al.*, 2003). Lack of expression of MT protein has been regarded as one of the major underlying causes of tissue susceptibility to cadmium toxicity (Waisberg *et al.*, 2003). Induced GSH and other thiol containing proteins play a key role in cellular defense against cadmium toxicity. The GSH redox cycle, which includes GSH, glutathione peroxidase and glutathione reductase, was important in the detoxification of ROS that are generated by cadmium so as to protect cells from the potential toxicity (Meister *et al.*, 1983).

Cadmium and Cell Growth

About 1 μM Cadmium stimulates DNA synthesis and cell proliferation in various cell lines, whereas more elevated concentrations are inhibitory. It has been reported that Ca^{2+}-

dependent processes by cadmium (< 1 μM) stimulate proliferation in peritoneal macrophages (Misra *et al*., 2002). At higher concentration of cadmium, there are abundant evidences that cadmium inhibits cell cycle progression (Biagioli *et al.*, 2001; Chao *et al.*, 2001). About 40% of cadmium-treated cells were blocked in G0/G1 phase in NIH 3T3 (Biagioli et al., 2001). In case of CL3 human lung adenocarcinoma cells, cadmium induced G2/M arrest and cadmium-activated p38 MAPK was responsible for this mitotic arrest and subsequent apoptosis (Chao *et al.*, 2001). Cadmium did not alter the overall distribution of cells in any G1, S, and G2/M phase, but markedly increased the hypodiploid (sub-G1) fraction in MCF human breast cancer cells (Meplan *et al.*, 1999). These reports indicate that the influence of cadmium on cell cycle arrest varies depending on the cell type.

Cadmium and Apoptosis

Cadmium-induced cytotoxicity involves apoptosis as a major mode of elimination of damaged cells (Habeebu *et al.*, 1998). Up-regulation of intracellular signaling pathways leading to increased mitogenesis or apoptosis is thought to be a major mechanism for cadmium toxicity (Beyersmann et al., 1997). Cadmium does not seem to activate the endonucleases, which induce internucleosomal cleavage of DNA in vitro (Hamada *et al.*, 1997). However, cadmium itself was associated with apoptosis through indirect oxidative stress by inhibition of antioxidant enzymes (Hamada et al., 1997). Metallothionein induced by cadmium played an important role not only as a cadmium carrier by which cadmium accumulates in the nucleus, but also as an inhibitor of zinc finger protein, which include transcriptional factors related to apoptosis (Hamada et al., 1997). Cadmium-induced c-myc, p53 and c-jun expression was suggested as a prelude to apoptosis in normal human prostate epithelial cells RWPE-1 (Achanzar *et al.*, 2000). Cadmium

induced cytochrome c release from mitochondria in cell-free and cell systems and that cytochrome c release from mitochondria into cytosol was the trigger of cadmium-induced apoptosis in human leukemia HL-60 cells (Kondoh *et al.*, 2001). Stimulation of p38 mitogen-activated protein kinase (MAPK) was responsible for the cadmium-induced apoptosis in U-937 human promonocytic cells (Galan *et al.*, 2000). C-Jun NH_2-terminal kinase (JNK) and p38 MAPK cooperatively participated in apoptosis induced by cadmium and the decreased extracellular signal-related kinase (ERK) signal induced by low cadmium doses contributed to growth inhibition or apoptosis in a human non-small cell lung carcinoma cell line, CL3 (Chuang *et al.*, 2000a). These contradictory results indicate that cadmium may induce different apoptotic pathways in different cell types.

Cadmium has been also shown anti-apoptotic effect. Cadmium inhibited caspase-3 activation and DNA fragmentation induced by apoptotic agent (Yuan *et al.*, 2000). Cadmium has been characterized as a caspase–3 inhibitor with IC_{50} values of approximately 9 μM in intact chinese hamster ovary CHO cells and 30 μM in cell-free extract system (Yuan et al., 2000). Cadmium-treated MRC-5 human fetal lung fibroblasts underwent caspase-independent apoptosis through mitochondria-ROS pathway including mitochondrial membrane depolarization and translocation of apoptosis-inducing factor (AIF) from mitochondria into nucleus (Shih *et al.*, 2004). In porcine kidney LLC-PK_1 cells, caspase activity was not associated with cadmium-induced apoptosis because caspase inhibitors failed to rescue cells (Ishido *et al.*, 1999). Cadmium did not significantly increase caspase-3 activity in liver cells of mouse model (Harstad *et al.*, 2002).

Mitogen-Activated Protein Kinases (MAPKs)

MAPKs are a family of Ser/Thr protein kinases that transmit extracellular signals into the nucleus (Robinson *et al.*, 1997; Schaeffer *et al.*, 1999). In mammalian systems, five distinguishable MAPKs modules have been identified so far: extracellular signal-related kinase (ERK1/2), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK, ERK3, and ERK5 (Schaeffer *et al.*, 1999). MAPK cascades have been shown to participate in a diverse array of cellular functions such as cell growth, cell differentiation, and cell death (Robinson *et al.*, 1997; Schaeffer *et al.*, 1999).

Extracellular Signal-Related Kinase (ERK)

ERK is activated by a variety of growth factors, insulin, and oxidative stress, and leads to fundamentally different cellular responses, including proliferation, survival, and memory consolidation (Aikawa *et al.*, 1997; Bergmann *et al.*, 1998; Boulton *et al.*, 1991; Impey *et al.*, 1999; Talarmin *et al.*, 1999; Wang *et al.*, 1998). ERK pathway can transmit both anti-apoptotic and pro-apoptotic signals, probably depending on the nature of other co-existing signals. In rat neonatal cardiomyocytes, hydroxyl radicals specifically activated ERK through activation of the Src family of tyrosine kinases, Ras and Raf-1, and activated ERK played a role in protecting cells from apoptosis (Aikawa *et al.*, 1997). Suppression of ERK activation enhanced apoptosis in hydrogen peroxide-treated HeLa cells (Wang *et al.*, 1998) and in S-nitrosoglutathione-treated RAW 264.7 macrophages (Callsen *et al.*, 1999). Conversely, ERK pathway was responsible for the induction of apoptosis in cadmium-treated CCRF-CEM (Iryo *et al.*, 2000), and in hyperoxia-induced murine lung epithelium cells (Zhang *et al.*, 2003), suggesting a pro-apoptotic role for ERK activity.

In mammalian cell lines, ERK activation and localization in nucleus is required for mitogen-induced gene expression and cell cycle re-entry (Brunet *et al.*, 1999). ERK is an absolute requirement for triggering proliferate responses and inhibition of MAPK/ERK–activating kinases (MEKs) and ERKs induces G0/G1 cell cycle arrest (Wilkinson *et al.*, 2000). Activation/inactivation and localization of ERK is highly associated with normal mitotic progression (Shapiro *et al.*, 1998). Inhibition of ERK was correlated with induction of p27 leading to G0/G1 arrest in NSCLC cell lines (Brognard *et al.*, 2002) and inhibited cell proliferation by preventing cyclin D1 expression (Squires *et al.*, 2002).

c-Jun NH_2-Terminal Kinase (JNK) and p38 MAPK

JNK (stress-activated protein kinase 1, SAPK1) and p38 MAPK (stress-activated protein kinase 2, SAPK2) are mainly activated by cytotoxic insults including heavy metals and are often associated with growth arrest and cell death (Kyriakis *et al.*, 1996). Recent evidence suggests that JNK pathway may play an important role in triggering apoptosis and signaling with mitochondria. Activated JNK was involved in loss of mitochondrial membrane potential and as downstream of caspase-3 in Jurket leukemia T cells (Srivastava *et al.*, 1999). Deactivation of p38 MAPK has been shown to lead both anti-apoptotic and pro-apoptotic responses. Exogenous nerve growth factor induced dephosphorylation of p38, which prevents Bcl-2 phosphorylation and apoptotic response in lymphoblastoid CESS B cell line (Rosini *et al.*, 2004). However, a natural anticancer depsipeptide, FR901228, induced apoptosis of *ras*-transformed 10T1/2 cells through suppression of p38 pathways (Fecteau *et al.*, 2002).

Calcium, Oxidative Stress and MAPKs

Disruption of Ca^{2+} homeostasis seems to take a part in initiating activation of MAPKs. Rise in $[Ca^{2+}]_i$ by thapsigargin promoted nitric oxide (NO) generation and induction of JNK

activity and apoptosis including activation of caspase-2 and –9 in Jurkat T cells (Srivastava *et al.*, 1999). Calcium was mobilized from intracellular stores by tributylin that played an important role for the phosphorylation of JNK and p38 MAPK in CCRF-CEM human T-cell line (Yu *et al.*, 2000). Elevated $[Ca^{2+}]_i$ was involved in p38 MAPK-induced neuronal cell death by neumolysin (Stringaris *et al.*, 2002). Extensively suppressed mitochondrial Ca^{2+} uptake by ruthenium red-sensitive in HeLa cells were mediated by p38 MAPK (Montero *et al.*, 2002). P38 MAPK were activated by cadmium in primary macrophages and depletion of $[Ca^{2+}]_i$ with BAPTA-AM inhibited such activation (Misra *et al.*, 2002). Oxidative stress is also known to activate JNK, p38 MAPK and apoptosis signal-regulated protein kinase 1 (ASK1) identified as one of the MAPK kinase kinases that activates JNK and p38 MAPK (Ichijo *et al.*, 1997).

Cadmium and MAPKs

Cadmium induces mitotic arrest in CL3 cells, which is associated with sustained activation of JNK (Chuang *et al.*, 2000b) as well as p38 MAPK (Chao *et al.*, 2001). Cadmium-activated p38 MAPK is responsible for apoptosis, mitotic arrest, activation of heat shock factor 1 and induction of heat shock protein 70 (Chao *et al.*, 2001; Galan *et al.*, 2000; Hung *et al.*, 1998). The exposure to cadmium induces the phosphorylation of JNK and results in the accumulation of phosphorylated c-Jun protein in LLC-PK_1 porcine renal epithelial cells (Matsuoka *et al.*, 1998). Furthermore, cadmium has been reported to activate JNK in Rat-1 fibroblasts (Iordanov *et al.*, 1999), and ERK as well as JNK in rat mesangial cells (Ding *et al.*, 2000). On the other hand, in LMH chicken hepatoma cells (Elbirt *et al.*, 1998) and 9L rat brain tumor cells (Hung *et al.*, 1998), ERK and p38 MAPK but not JNK were activated in response to cadmium exposure, suggesting that the members of the MAPK family are differentially activated by cadmium exposure, depending on the cell type. $[Ca^{2+}]_i$ elevation were required for JNK activation by

cadmium in LLC-PK_1 cells (Matsuoka *et al.*, 1998) suggesting that role/activation of MAPKs may be interrelated with $[Ca^{2+}]_i$.

Cadmium Complexes

It has been suggested that if cadmium interacts with cell surface receptors affecting intracellular signals, free Cd^{2+} ion will not be required to enter cells in order to exert toxic effects (Beyersmann *et al.*, 1997). The induction of MT and GSH by cadmium binds and detoxifies cadmium ions and ROS, on the other hand, moves cadmium ion from the MT complex and increase the toxicity of cadmium (Nordberg, 1978). Toxic effect of Cd-MT complexes on mitochondria was stronger than that of inorganic cadmium salts in vivo experiments (Nordberg, 1978). Cd-MT not only are less protective against radiation-induced DNA damage than Zn-MT but also under certain conditions have the ability to increase DNA damage (Cai *et al.*, 2003). DNA damage caused by cadmium in a cell-free assay was not due to direct metal-DNA interaction but rather by the induction of oxidative stress (Valverde *et al.*, 2001). Pronounced toxic effect of Cd-MT or cadmium complexes than free Cd^{2+} has been shown in some other studies. The organic cadmium complexes affected respiration and perturbed ion permeability significantly stronger than free Cd^{2+}, probably due to Cd^{2+} incorporated into the complexes can easily penetrate to adjacent –SH groups of mitochondrial respiratory enzymes and other molecules of the inner mitochondrial membrane that regulate the mitochondrial ion permeability (Korotkov *et al.*, 1999). These studies are evidence that cadmium complexes may exert substantial cytotoxic effect on mitochondrial activity compared with free Cd^{2+}.

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CHAPTER 3

EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK)-SIGNALING-DEPENDENT G2/M ARREST AND CELL DEATH IN MURINE MACROPHAGES BY CADMIUM[1]

[1]Kim, J., S.H. Kim, V.J. Johnson, and R.P. Sharma. Submitted to *Toxicological Sciences*

Abstract

The effect of cadmium on cell cycle progression and cell death, and the involvement of reactive oxygen species (ROS) and extracellular signal-regulated kinase (ERK) signaling pathway in murine macrophages was investigated. Cadmium is immunotoxic and is a persistent environmental pollutant. Cell cycle regulation is an important factor that mediates cell death; however, cadmium-modulated cell cycle arrest leading to cell death in macrophages has not been investigated. Thymidine uptake into cells with 20 μM camium treatment was increased at 8 hr and then dramatically decreased at 24 hr. Same concentration of cadmium induced both apoptotic and necrotic death at 24 hr. Cadmium at 20 μM triggered re-entry of G0/G1 to next phase and increased the number of cells in G2/M phase at 24 hr. Exposure to cadmium dose-dependently induced phosphorylation of ERK at 16 and 24 hr. Phosphorylation of ERK correlated with $p21^{WAF1/CIP1}$ induction. Inhibition of ERK activation by PD98059 resulted in G0/G1 arrest, and partially released the cadmium-mediated G2/M arrest. Inhibition of ERK phosphorylation by PD98059 strongly attenuated cadmium-induced necrotic cell death, but did not prevent caspase-3 activation and DNA fragmentation. Necrosis rather than apoptosis was caused by cadmium-induced ERK signaling in J774A.1 cells. A scavenger of ROS, N-acetylcystein, decreased cadmium-induced ERK activation and necrotic cell death suggesting that ROS are involved in cadmium-mediated ERK activation, $p21^{WAF1/CIP1}$ induction and cell death. Our findings suggest that cadmium induces ROS-ERK-$p21^{WAF1/CIP1}$ signaling pathway leading to G2/M arrest and cell death. Furthermore, PD98059 causes G0/G1 arrest that may play a role in protecting cells against oxygen-induced damage and cell death. Enhanced re-entry of cells from G0/G1 to next phases by activated ERK is a plausible mechanism for cellular death in response to cadmium damage in murine macrophages.

Keyword: Cadmium, cell cycle arrest, cell death, ROS, ERK

Abbreviations used:

AP-1, activation protein 1; AV, annexin V; $[Ca^{2+}]_i$, the level of intracellular calcium; Cdk, cyclin-dependent kinases; ERK, extracellular signal-regulated kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK–activating kinase; MTT, 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide; NAC, N-acetylcystein; NO, nitric oxide; PDTC, 1-pyrrolidinecarbodithioic acid; PI, propidium iodide; PKC, protein kinase C; ROS, reactive oxygen species; TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling)

Introduction

Cadmium is a nonessential and toxic heavy metal commonly found in cigarette smoke and industrial effluent (Jarup, 2003). It has been reported that smokers have 4-5 times higher level of cadmium in blood than non-smokers (Jarup *et al.*, 1998). Inhalation of cadmium will directly effect on macrophages in respiratory system without first-pass elimination and be a factor of immunodepression of smokers. Cultured mammalian cells exposed to cadmium show genotoxicity and carcinogenicity such as mutation, DNA strand breaks, and chromosomal aberrations (Beyersmann *et al.*, 1997). Cadmium causes apoptotic cell death in CCRF-CEM human T-cell line (Iryo *et al.*, 2000), NIH 3T3 murine fibroblasts (Biagioli *et al.*, 2001), and rat testicular tissues (Xu *et al.*, 1996). It has been reported that cadmium interferes with major cellular signal transduction elements including cell surface receptors, cytosolic and nuclear calcium levels, phosphorylation of proteins such as protein kinase C (PKC), mitogen-activated protein kinases (MAPKs), and calmodulin–dependent protein kinase, transcription factors and

other regulatory proteins (Behra *et al.*, 1991; Beyersmann and Hechtenberg, 1997; Hanas *et al.*, 1996; Misra *et al.*, 2002). Up-regulation of intracellular signaling pathways leading to increase of mitogenesis or apoptosis is thought to be a major mechanism for cadmium toxicity (Beyersmann and Hechtenberg, 1997). It stimulates the expression of the proto-oncogenes such as c-*jun*, c-*myc* and c-*fos*, (Jin *et al.*, 1990; Wang *et al.*, 1998b), and the tumor suppressor gene *p53* (Zheng *et al.*, 1996).

Extracellular signal-regulated kinase (ERK) is in the center of multiple signal transduction pathways to accomplish a variety of functions. ERK is activated by a variety of growth factors, insulin, and oxidative stress, and leads to fundamentally different cellular responses, including proliferation, survival, and memory consolidation (Aikawa *et al.*, 1997; Bergmann *et al.*, 1998; Boulton *et al.*, 1991; Impey *et al.*, 1999; Talarmin *et al.*, 1999; Wang *et al.*, 1998a). In most cellular models ERK activation inversely correlates with apoptosis. In rat neonatal cardiomyocytes, hydroxyl radicals specifically activated ERK through activation of the Src family of tyrosine kinases, Ras and Raf-1, and activated ERK played a role in protecting cells from apoptosis (Aikawa *et al.*, 1997). Suppression of ERK activation by ERK kinase 1 inhibitor PD98059 enhanced apoptosis in hydrogen peroxide-treated HeLa cells (Wang *et al.*, 1998a) and in S-nitrosoglutathione-treated RAW 264.7 macrophages (Callsen *et al.*, 1999). Conversely, ERK pathway was responsible for the induction of apoptosis in cadmium-treated CCRF-CEM (Iryo *et al.*, 2000), and in hyperoxia-induced murine lung epithelium cells (Zhang *et al.*, 2003), suggesting a pro-apoptotic role for ERK activity. Previous studies suggest that the ERK pathway can transmit both anti-apoptotic and pro-apoptotic signals, probably depending on the nature of other co-existing signals.

About 40% of cadmium treated cells were blocked in G0/G1 phase in NIH 3T3 (Biagioli et al., 2001). In case of CL3 human lung adenocarcinoma cells, cadmium induced G2/M arrest and cadmium-activated p38 MAPK was responsible for this mitotic arrest and subsequent apoptosis (Chao *et al.*, 2001). Cadmium did not alter the overall distribution of cells in any G1, S, and G2/M phase, but markedly increased the hypodiploid (sub-G1) fraction in MCF human breast cancer cells (Meplan *et al.*, 1999). These reports indicate that the influence of cadmium on cell cycle arrest varies depending on the cell type. Exact mechanism responsible for altered cell cycle progression by cadmium is still somewhat uncertain. Cell cycle progression is regulated by a series of cyclin-dependent kinases (Cdks) that consist of catalytic subunits and activating subunits cyclins (Sherr, 1993). The Cdk inhibitor, $p21^{WAF1/CIP1}$ is up-regulated and plays a major role in arresting cells in either at G1 or G2/M phase in response to DNA damage (Bunz *et al.*, 1998; Deng *et al.*, 1995). The p27 is a $p21^{WAF1/CIP1}$ related protein and has been described as a negative regulator of G1 progression (Toyoshima *et al.*, 1994). Expression of $p21^{WAF1/CIP1}$ is previously shown to be regulated through MAPK signaling pathway (Liu *et al.*, 1996). ERK activation is known to be essential for cell cycle progression from G1 to S phase (Talarmin *et al.*, 1999). Activation/inactivation and localization of ERK is highly associated with normal mitotic progression (Shapiro *et al.*, 1998). Cadmium influence on cell cycle progression may be related with ERK signaling.

J774A.1 cells are a commonly used murine macrophage cell line possessing similarities to mature macrophages making them an alternative to primary cells (Yan *et al.*, 2004). J774A.1 cells originated from BALB/c mouse reticulum cell sarcoma, and due to the tumor-like property, these cells may be more resistant to cadmium toxicity. Cadmium exposure to J774A.1 exhibited increased oxidative stress induced by reactive oxygen species (ROS) and nitric oxide

(NO) production, following single strand breaks and apoptosis (Bagchi *et al.*, 1998; Hassoun *et al.*, 1996). Even though macrophages are considered terminally differentiated, studies using macrophages on cadmium-induced inhibition of cell cycle progression and subsequent cell death would be helpful to understand cadmium effect on the immune system. The objective of this study is to explore ERK activation in response to cadmium, and to outline the mechanisms and consequences of such activation in macrophages. We hypothesized that cadmium activates ROS-ERK signaling in J774A.1 murine macrophage cells. This process alters Cdk inhibitors mediating cell cycle progression and cell death by cadmium. To the best of our knowledge, this is the first report to indicate the functional importance of ERK on $p21^{WAF1/CIP1}$ induction, cell cycle arrest and cell death in response to cadmium damage.

Materials and Methods

Reagents

Cadmium ($CdCl_2$, Sigma Chemical Co., St. Louis, MO) was dissolved in water, sterilized with 0.22 μm filter, and added into cultures at the indicated time and concentrations. Cell culture reagents were procured from GIBCO Life Technology (Grand Island, NY). Antibodies specific for the total and phosphorylated forms of ERK (p44/42 MAPK) were obtained from Cell Signaling (Beverly, MA). Antibodies for specific $p21^{WAF1/CIP1}$ and p27 were procured from Santa Cruz Biotechnology (Santa Cruz, CA). The MAPK/ERK–activating kinase 1 (MEK1) inhibitor, PD98059, and 1-pyrrolidinecarbodithioic acid (PDTC), a potent antioxidant, were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). Hoechst 33258, propidium iodide (PI), N-acetylcystein (NAC) and all other chemicals used in this study were obtained from Sigma and were of cell culture grade.

Cell culture

Macrophage cell line, J774A.1 (American Type Culture Collection TIB-67), established from BALB/c mouse, was maintained in Dulbecco's Modified Eagle's Medium, supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and 10% non-heat-inactivated fetal bovine serum (Atlanta Biologics, Atlanta, GA) in 5% CO_2 atmosphere at 37 ºC. The J774A.1 cells were grown in 75 cm^2 culture flasks and subcultured when the cells reached 70 – 80% confluence (every 3 days). Cells were used during 3rd or 4th passage. Cultures were allowed to grow overnight (15 hr) prior to the treatment. PD98059 (20 μM), NAC (1 mM), and PDTC (100 μM) were added 30 min prior to cadmium treatment. The concentrations of PD98059, NAC and PDTC employed were not cytotoxic in preliminary trials.

Cytotoxicity assay

Trypan blue assay was performed to determine cytotoxicity. Cells were seeded at 2×10^6 cells/well in 6-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) and treated with the indicated concentrations of cadmium. An aliquot of the cell suspension was diluted 1:10 (v/v) with 0.4% Trypan blue, and the stained and the total cells were counted with a hemocytometer (Hausser Scientific, Horsham, PA).

The breakdown of cell membrane was assessed via determining release of lactate dehydrogenase (LDH) into the media. Cells were seeded at 8×10^4 cells/well in 96–well microplates (Falcon) and treated with the indicated concentrations of cadmium at 24 hr. Total media was added to a new 96-well plate to determine LDH release and same volume of 1% Triton X-100 was added to the original plate to lyse cells for determination of intracellular LDH. Samples were treated with 100 μl of 4.6 mM pyruvic acid in 0.1 M potassium phosphate buffer (PH 7.5) and then 100 μl of 0.4 mg/ml β-NADH in 0.1 M potassium phosphate buffer (PH 7.5)

was added to the wells. The kinetic change in absorbance at 240 nm was read for the duration of 1 min using a PowerWave^{TM} absorbance microplate reader (Bio-Tek Instruments, Winooski, VT). Results are expressed as percent over basal level of LDH/well.

MTT (3[4,5-dimethyl thiazolyl-2]2,5-diphenyl tetrazolium bromide, Sigma) assay was used to compare with proliferation as previously described (Johnson *et al.*, 2003). Cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with the indicated concentrations of cadmium.

DNA synthesis as an index of proliferation

The [methyl-^{3}H] thymidine incorporation assay was used as an index of proliferation as described previously (Johnson *et al.*, 2003). Cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with indicated concentrations of cadmium. At 16 hr prior to harvesting cells, each well was pulsed with 20 µl of [methyl-^{3}H] thymidine (25 µCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA) using a cell harvester (PHD, Cambridge Technology, Cambridge, MA). Proliferative response (uptake of [^{3}H] thymidine) in the harvested cells was counted in a liquid scintillation counter (Pharmacia, Turku, Finland) and expressed as percent of control proliferation.

Cell cycle analysis

Cells were treated with cadmium for 24 hr at 2×10^6 cells/well in 6-well plates. The cells were then scrapped and re-suspended in nuclear isolation medium (NIM, 50 µg/ml PI, 1 mg/ml RNase A, 0.1% Triton X-100). Nuclear fluorescence is proportional to DNA content. Analysis of DNA ploidy and discrimination of cells in G0/G1 versus S versus G2/M phases of the cell cycle was done by measuring cellular DNA content using fluorescent automated cell

sorting with a FACSCalibur™ (Becton Dickinson, San Jose, CA) flow cytometer. DNA histograms were analyzed using CellQuest™ (Becton Dickinson) analysis software.

Determination of early apoptosis and necrosis by flow cytometry

To further determine the extent of early apoptosis and necrosis, cell death was analyzed by staining the cells with annexin V-FITC (Molecular Probes, Eugene, OR) and PI. For staining, 2×10^6 cells/well in 6-well plates were treated with various concentrations of cadmium for 24 h following which 100,000 cells were washed with cold PBS, centrifuged, and suspended in a final volume of 100 µl binding solution (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 µl of annexin V and 5 µl of PI (final concentration 2.5 µg/ml) as provided by the manufacturer. The cells were incubated at room temperature for 15 min then 400 µl binding solution was added and the cells were analyzed using a FACSCalibur™ flow cytometer. Excitation was at 488 nm and the emitted green fluorescence of annexin V (FL1) and red fluorescence of PI (FL2) were collected using a 525 and 575 nm band pass filter, respectively. A total of at least 50,000 cells were analyzed per sample. The amount of live, early apoptosis and necrosis was determined by CellQuest™ flow analysis software, respectively, as the percent of AV-/PI-, AV+/PI- or PI+ cells.

Western blot analysis

The ERK, phospho-ERK, $p21^{WAF1/CIP1}$ and p27 were determined using specific antibodies as described previously (Johnson *et al.*, 2003). Cells were grown at 2×10^6 cells/well in 6-well microplates and treated with cadmium for indicated time and concentrations. Following treatment, cells were washed with phosphate buffered saline (PBS) and total cell lysates were prepared by scrapping in 100 µl of lysis buffer [20 mM Tris–HCl (pH 8.0), 1 mM sodium

orthovanadate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1% Triton X-100, 50 mM β-glycerolphosphate, and 10 μg/ml each aprotinin, leupeptin, and pepstatin]. Fifty micrograms of proteins for ERK and 10 μg of proteins for $p21^{WAF1/CIP1}$ and p27 determined by Bradford assay was electrophoretically separated using a 12% SDS-PAGE gel and transferred to nitrocellulose paper followed by antibody staining. Equal loading and transfer of total protein was verified with the reversible Ponceau red dye (Sigma) and also by detecting total ERK. Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

Determination of caspase-3 activation

Caspase-3 activity was determined using CaspACE™ fluorometric activity assay (Promega, Madison, WI) with modifications as follows. Briefly, cells were treated in 96-well microplates following which Triton X-100 was added and repeatedly pipetted to lyse the cells. The homogenates were centrifuged at 4,000×g for 10 min to remove cell debris. The supernatant was assayed for caspase-3 activities using CaspACE™ system according to the manufacture's instructions. The fluorescence signal was digitized and analyzed using SoftMax Pro™.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay

TUNEL assay was performed using the *in situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Cells were plated at 8×10^4 cells/well in 96-well microplates and allowed to attach overnight. Cells were then treated with cadmium for 24 hr, fixed with paraformaldehyde, and analyzed for stained nuclei according to the manufacturer's instructions.

The fluorescence signal was read by SpectraMax Gemini, digitized and analyzed using SoftMax Pro™. In addition, fluorescence microscopy was performed to examine fragmented DNA morphology using IX71 inverted microscope. Digital images were captured using MagnaFire SP® Olympus digital camera.

PI and Hoechst 33258 staining

Necrotic cell death was detected by staining with PI, a membrane impermeable dye excluded from viable cells. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stochiometry of one dye per 4-5 base pairs of DNA. Dead cells are PI-bright and live cells are PI-dim. Apoptotic morphological changes in the nuclear chromatin of cells were detected by staining with the DNA binding fluorochrome Hoechst 33258 (bis-benzimide). Hoechst 33258 exhibits fluorescence enhancement upon binding to A-T rich regions of double stranded DNA. Following 24 hr treatment with cadmium, 120 µl of supernatant was removed and 20 µl of PI (1 µg/ml) or Hoechst 33258 (2 µg/ml) was added. Fluorescence microscopy was performed to examine necrotic and apoptotic morphology at 535/617 nm or 350/450 nm (excitation/emission) for PI or Hoechst 33258, respectively, using IX71 inverted microscope (Olympus, Melville, NY). Digital images were captured using MagnaFire SP® Olympus digital camera.

Replication and statistical analysis

Experiments were repeated 3-4 times with consistent results. Data from representative experiments have been presented. All statistical analyses were performed using the SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A p value of < 0.05 was considered significant unless indicated otherwise.

Results

Viability and proliferation of cadmium-treated cells

Trypan blue exclusion is used to measure the membrane integrity of viable cells. Cadmium decreased Trypan blue-excluded viable cells in a concentration dependent manner at 24 hr (Fig. 1A). At 20 μM or greater, cadmium-damaged cells showed a significant LDH release (Fig. 1B). LDH release represents cadmium-induced necrotic (also late stage of apoptotic) cell death.

The decreased proliferation of cells by cadmium was compared with the loss of viability by cadmium via MTT assay at 24 hr (Fig. 1C). The [^{3}H]thymidine uptake was concentration-dependently decreased by cadmium. At ≥ 20 μM cadmium, cells showed a gap between viability and proliferation indicating the likely cell cycle arrest prior to cell death. At 20 μM cadmium, the viability was 70% of control; however, the cell proliferation was reduced to below 20% of the normal. Relative proliferation compared to viability was also examined in time-course study (Fig. 1D). Cadmium at 20 μM caused slight reduction of viability at every time point up to 24 hr. On the other hand, same concentration of cadmium increased thymidine uptake into cells at 8 hr and then dramatically inhibited its incorporation as time goes until 24 hr.

Cadmium-induced G2/M arrest

In cell cycle analysis, ~15% of the cells were arrested in the G2/M phase with 20 μM cadmium treatment at 24 hr (Fig. 2, Table 1), compared to 4% of the untreated cells. On the other hand, 59% of the cells remained in the G0/G1 phase compared to 78% of the untreated cells indicating that G0/G1 to next phases was significantly triggered by cadmium treatment. The G0/G1 and S phases of the normal cells progressed and remained at G2/M phase in response to cadmium damage. At 50 μM cadmium, percent of the cells arrested in G2/M phase

decreased down to control level with significant increase of sub-G1 phase. Accumulation of cells with hypodiploid (sub-G1) fraction is characteristic of cell death. Untreated cells had less than 2% of sub-G1 phase; however, cadmium at 20 and 50 μM for 24 hr increased sub-G1 phase to 16 and 31%, respectively.

Cadmium-induced necrotic and apoptotic cell death

To further examine the potential of cadmium to induce necrotic and apoptotic conditions of macrophages, annexin V-FITC vs. PI assay was conducted. Phosphatidylserine is located predominantly on the internal leaflet of the plasma membrane of intact cells, but in apoptotic cells phosphatidylserine translocates from the inner to the outer surface of the cell membrane, which allows to be detected by annexin V (Vermes *et al.*, 1995). PI is used to distinguish cells that are in the late stage of apoptosis or necrosis, which lose plasma membrane integrity and are permeable to PI. Cadmium induced both necrotic and apoptotic cell death. Percent of annexinV negative and PI negative live cells was significantly decreased at 20 and 50 μM cadmium at 24 hr (Fig. 3A). PI only treated control cells already showed ~20% of background PI staining (Fig. 3B, blank bar). Cadmium at 20 μM showed significant increase of necrosis and also apoptosis. Higher concentration of 50 μM cadmium showed necrosis rather than apoptosis mostly. Concentration-dependent cadmium-induced necrotic cell death was confirmed with fluorescence microscopy with PI staining (Fig. 9A). Apoptotic morphological change by 20 μM cadmium was also identified by Hoechst 33258 and TUNEL staining (Fig. 9B-C).

Cadmium-induced activation of ERK

ERK activation was investigated in cells treated with 20 μM cadmium for different time. Phosphorylation of ERK was observed at 16 and 24 hr (Fig. 4A). To examine the effect of

various cadmium concentrations, the level of ERK and phospho-ERK was measured after 16 hr cadmium exposure; ERK activation was elevated significantly at concentrations of 20 and 50 μM cadmium (Fig. 4B).

Role of activated ERK on cadmium-altered p21$^{WAF1/CIP1}$ and cell cycle progression

Addition of the MEK1 inhibitor, PD98059 (20 μM), abrogated cadmium-induced ERK activation at 16 hr, confirming the role of MEK1 in mediating cadmium-induced ERK activation (Fig. 5A). To determine whether the magnitude of ERK activation by cadmium-induced damage played a functional role in G2/M cell cycle arrest, cells were treated with cadmium (20 μM) with or without PD98059 for 24 hr (Table 1). PD98059 itself at 20 μM reduced proliferation (Fig. 5E) leading to increased G0/G1 phase of cell cycle (Table 1), which is consistent with the report that ERK activity facilitates G1 progression (Talarmin *et al*., 1999). Pretreatment of PD98059 in cadmium-treated cells also caused significant release from the G2/M arrest seen by cadmium-damage, suggesting that G2/M arrest by cadmium is regulated by ERK activation. To corroborate the results of G2/M arrest and G0/G1 transition obtained by FACS analysis, we analyzed Cdk inhibitor, p21$^{WAF1/CIP1}$ and p27 after cadmium and PD98059 treatments by western blot (Fig. 5B-D). Our results show that p21$^{WAF1/CIP1}$ was induced and p27 was down-regulated by cadmium treatment. We suggest that cadmium-activated ERK induces G2/M arrest via p21$^{WAF1/CIP1}$ regulation. The function of ERK activity on G0/G1, S and G2/M progression is indicated as altered proliferation by PD98059 and cadmium treatment (Fig. 5E). P27 induction showed very similar pattern with percent of cells in G0/G1 phase (Fig. B, D and Table 1). Cadmium at 20 μM down-regulated p27 and caused G0/G1 transition.

Role of activated ERK on cadmium-induced cell death

To examine whether activation of ERK played a functional role in cadmium–induced cell death, cells were treated with cadmium (20 μM) with or without PD98059 (20 μM) for 24 hr. ERK inhibition showed less cell death by cadmium (Fig. 6A) and the percent of PI-stained necrotic cells by cadmium was significantly decreased when ERK activation was inhibited (Fig. 6B). On the other hand, PD98059 was not able to reduce annexin V-positive apoptotic cell death (Fig. 6C).

The activation of caspase-3 is an integral step in the majority of apoptotic events. Cadmium at 20 μM showed more than 2-fold caspase-3 activation; however, ERK inhibition was unable to reduce cadmium-induced caspase-3 activation (Fig. 7A). DNA fragmentation by TUNEL confirmed that ERK inhibition was not able to change cadmium-induced apoptosis (Fig. 7B and 9C). Even at earlier time point of 16 hr PD98059 did not change the cadmium-induced caspase-3 activation and TUNEL staining (data not shown). No effect of PD98059 on cadmium-induced apoptosis was also confirmed by Hoechst 33258 staining (Fig. 9B).

Effect of antioxidant on cadmium-induced ERK activation, p21$^{WAF1/CIP1}$ and cell death

To determine whether ERK activation mediated by cadmium depends on ROS, cells were treated with NAC, a precursor of glutathione with antioxidant activity (Fig. 8A-B). NAC (1 mM) inhibited cadmium-induced phosphorylation of ERK, suggesting an involvement of ROS in cadmium-induced ERK activation. p21$^{WAF1/CIP1}$ induction was decreased as ERK activation was inhibited by NAC, but p27 was not related (Fig. 8C-E). Inhibition of ROS by NAC (1 mM) and PDTC (100 μM) dramatically decreased cadmium-induced necrotic cell death (Fig. 9A).

Confirmation of necrosis and apoptosis by fluorescence microscopy

Dying cells stained with PI are illustrated in Fig. 9A. Cadmium-induced necrotic cell death was shown in concentration-dependent manner. The intensity of PI fluorescence by cadmium-induced necrotic cell death was significantly decreased when activation of ERK was inhibited. Antioxidants NAC and PDTC completely inhibited cadmium-induce necrosis.

Apoptotic cells stained with Hoechst 33258 are pictured in Fig. 9B. The nuclei of cells exposed to cadmium showed the condensed and fragmented chromatin characteristics of apoptosis. Cadmium at 20 μM showed 3 and 4-fold increased apoptosis over control. To protect necrotic cells from being stained by Hoechst 33258, the fluorescence was measured after 15 min dye binding to DNA. Inhibition of ERK by PD98059 could not inhibit cadmium-induced Hoechst 33258 staining. TUNEL assay confirmed no effect of PD98059 on cadmium-induced DNA fragmentation (Fig. 9C).

Discussion

Results of current study indicate that cells treated with sublethal concentrations of cadmium were alive, but they have stopped proliferating. Cells were arrested by cadmium at some point of their cell cycle progression, possibly attempting to repair cadmium-induced damage. Because cadmium showed inhibition of proliferation, we performed cell cycle analysis to see whether cadmium inhibits cell cycle progression. Cadmium inhibits proliferation in murine macrophages via G2/M arrest and a gap between viability and proliferation was explained by inhibited cell cycle progression. Cell growth kinetics in a time-dependent fashion showed increased thymidine incorporation at 8 hr and then thymidine uptake was dramatically decreased at 24 hr (Fig. 1D). This result suggests that cadmium is

accelerating DNA content doubling by going through G0/G1 to S at earlier time point and then later arresting cells at G2/M phase. Arrested cells at G2/M phase by cadmium may not process to next G0/G1 and show totally inhibited proliferation at 24 hr. We observed elevated phosphorylation of ERK by cadmium at 16 and 24 hr. Inhibition of ERK by PD98059 resulted in G0/G1 arrest and partial release of the cadmium-mediated G2/M arrest. PD98059 also inhibited $p21^{WAF1/CIP1}$ expression suggesting ERK-$p21^{WAF1/CIP1}$–G2/M arrest signaling. PD98059 was able to decrease cadmium-increased percent of PI positively stained necrotic cells; however, did not change apoptotic characteristics. Regulation of cell death by ERK was more related with necrosis than apoptosis in J774A.1 cells. Because ROS is known to play a central role in the activation of MAPKs (Aikawa *et al*., 1997; Wang *et al*., 1998a), we examined whether cadmium-mediated cell death can be inhibited by antioxidants. It was apparent that ROS facilitate cadmium-induced ERK- $p21^{WAF1/CIP1}$ signaling pathway and cell death.

It has been reported that cadmium modulates the level of intracellular calcium ($[Ca^{2+}]_i$) and Ca^{2+}- dependent processes by cadmium (< 1 μM) stimulate proliferation in peritoneal macrophages (Misra *et al*., 2002). At higher concentration of cadmium, there are abundant evidences that cadmium inhibits cell cycle progression (Biagioli et al., 2001; Chao and Yang, 2001). Cadmium induced irreversible mitotic arrest and G2/M was the most effective phase to cytotoxicity, apoptosis, micronucleus, and p38 MAPK and ERK activities in CL3 cells (Chao and Yang, 2001). Cells synchronized at G2/M phase showed high increase of ERK activation with 40 and 80 μM cadmium for 2 hr in CL3 (Chao and Yang, 2001). Phosphorylation of ERK was essential for the cell progression from G2 to mitosis in NIH 3T3 (Wright *et al.*, 1999). Inhibition of ERK attenuated $p21^{WAF1/CIP1}$ induction, resulting in partial release of the G2/M cell cycle arrest induced by etoposide (Tang *et al.*, 2002). Intracellular peroxide elicited by

cadmium in CL3 played a critical role in the ERK signal transduction pathways (Chao and Yang, 2001). Hydrogen peroxide induced activation protein (AP-1) and its effect on $p21^{WAF1/CIP1}$ mediated G2/M arrest in p53-deficient human lung cancer cells (Chung *et al.*, 2002). In this system, inhibition of activated ERK diminished hydrogen peroxide-induced phosphorylation of c-*Jun* and DNA binding activity of AP-1, decreased expression of $p21^{WAF1/CIP1}$ and released the cells from G2/M arrest (Chung *et al*., 2002). These reports are consistent with our finding that activation of ERK by oxidative stress mediates cadmium-induced G2/M arrest and $p21^{WAF1/CIP1}$ is involved in ERK signaling on cadmium-altered cell cycle progression.

In mammalian cell lines, ERK activation and localization in nucleus is required for mitogen-induced gene expression and cell cycle re-entry (Brunet *et al.*, 1999). ERK is an absolute requirement for triggering proliferative responses and inhibition of MEKs and ERKs induces G0/G1 cell cycle arrest (Talarmin *et al*., 1999; Wilkinson *et al.*, 2000). Inhibition of ERK was correlated with induction of p27 leading to G0/G1 arrest in NSCLC cell lines (Brognard *et al.*, 2002) and inhibited cell proliferation by preventing cyclin D1 expression (Squires *et al.*, 2002). In our study, cadmium-treated cells triggered re-entry of G0/G1 to next phases and activated ERK, confirming the ERK pathway is rate limiting for cell cycle at about G0/G1 to next phases. Percent of cells at G0/G1 phase showed very similar pattern with p27 induction in our experiment (Fig. 5B, D and Table 1) suggesting that p27 was correlated with G1 progression in J774A.1 murine macrophages. It has been reported that growth arrest in G1 protects against oxygen-induced DNA damage and cell death (Rancourt *et al.*, 2002). Cell culture conditions, which restrict cells from exiting G1, can limit the adverse effects on cell viability and DNA integrity associated with hyperoxia exposure (Rancourt *et al*., 2002). Release of G2/M arrest by inhibition of ERK may suppress cadmium-induced cell death but also

G0/G1 arrest by inhibition of ERK may limit the adverse effect on cell viability and DNA integrity associated with cadmium exposure.

Cadmium is known to diminish activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase and to decrease antioxidant contents including glutathione (GSH) (del Carmen *et al.*, 2002). GSH regulates intracellular level of ROS by direct scavenging reaction or via GSH peroxidase/GSH system. Hampered protection by diminished antioxidant enzymes and antioxidants to the cells against oxidative damage by cadmium has been suggested to be the cause for increased lipid peroxidation in the cells. Cadmium has been previously shown to increase $[Ca^{2+}]_i$ in various cell lines (Beyersmann and Hechtenberg, 1997; Misra *et al*., 2002). Disturbance of calcium homeostasis can be another initiation to cause cadmium-induced oxidative stress. High $[Ca^{2+}]_i$ may disrupt mitochondrial Ca^{2+} equilibrium and stimulate electron flux along the electron transport chain resulting in ROS formation (Chacon *et al.*, 1991). The involvement of ROS as intracellular messengers to induce DNA single strand breaks and fragmentation in the toxicity of cadmium has been proposed in J774A.1 murine macrophage cells (Bagchi *et al*., 1998; Hassoun and Stohs, 1996). Hydrogen peroxide accumulation following apoptosis in response to cadmium-induced damage was found in U-937 (Galan *et al.*, 2000). Hyperoxia-induced cell death in murine lung epithelial cells is associated with cytochrome c release, subsequent caspase-9 and -3 activation, and poly (ADP-ribosyl) polymerase cleavage (Zhang *et al*., 2003). Cadmium-induced cytochrome c release from mitochondria was the trigger of apoptosis in human leukemia HL-60 cells (Kondoh *et al.*, 2001). These findings suggest that cadmium-induced apoptotic cell death may be a response of induced cytochrome c release and subsequent activation of caspases. It has been reported that among the MAPKs examined, the ERK pathway was important in cadmium-induced apoptosis in

CCRF-CEM (Iryo *et al*., 2000). In our experiments, PD98059 could not inhibit annexin V binding, caspase-3 activation and DNA fragmentation by cadmium indicating ERK activity was not relevant for apoptotic cell death by cadmium.

In summary, we hereby present the relationship among ROS, ERK activation, cell cycle arrest and cell death in cadmium-treated macrophages. Exposing J774A.1 cells to cadmium markedly induced $p21^{WAF1/CIP1}$ and G2/M arrest, triggered G0/G1 to next phases, and subsequently induced cell death. Activated ERK by cadmium was a major mediator for inhibition of proliferation, and G2/M arrest. The data presented here demonstrates that ERK activation by cadmium responses to cellular damage. Our results suggest that expression of ROS by cadmium play an important role in ERK- $p21^{WAF1/CIP1}$ signaling. Activated ERK was not related with apoptotic cell death but necrotic cell death. The fact that growth arrest in G1 protects against oxygen-induced cellular damage and cell death (Rancourt *et al*., 2002) and that inhibition of ERK caused G0/G1 arrest in our system, confirms that phosphorylated ERK plays a role for transition from G0/G1 to next phases, which will give a synergetic effect to ERK-mediated G2/M arrest leading to cell death.

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Table 3.1.

The Effect of inhibition of ERK activity on cadmium-altered cell cycle progression.

	G0/G1 (%)*	S (%)	G2/M (%)	Sub-G1 (%)
control	78.48 ± 0.18^{a}	15.58 ± 0.15^{a}	3.94 ± 0.11^{a}	2.00 ± 0.39^{a}
Cd 5	76.44 ± 0.37^{a}	14.18 ± 0.16^{a}	5.76 ± 0.25^{a}	3.62 ± 0.32^{a}
Cd 20	58.98 ± 0.94^{b}	8.94 ± 0.05^{b}	14.98 ± 0.76^{b}	17.10 ± 0.24^{b}
Cd 50	54.95 ± 2.81^{b}	8.26 ± 0.20^{b}	4.48 ± 0.06^{a}	32.31 ± 0.43^{c}
PD	83.71 ± 0.15^{c}	8.71 ± 0.18^{b}	4.01 ± 0.14^{a}	3.57 ± 0.22^{a}
PD + Cd 20	62.29 ± 0.68^{d}	8.47 ± 0.32^{b}	10.81 ± 0.17^{c}	18.43 ± 0.43^{b}

J774A.1 cells were exposed to $CdCl_2$ (μM) for 24 hr with or without pre-treated of PD98059 (20 μM) for 30 min

* Percent of cells in each phase was determined by PI staining with flow cytometry. Mean ± SE (n=3).

[a] Different letters on top of numbers indicate a significant difference ($p < 0.05$).

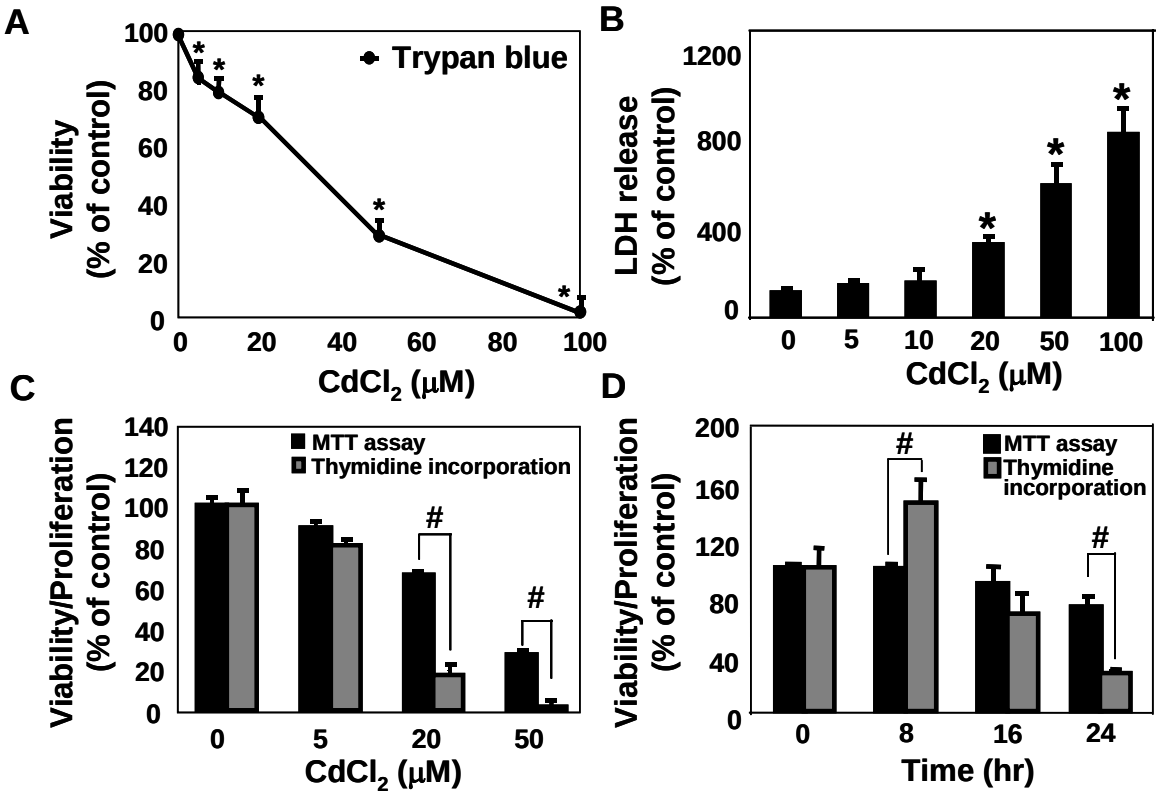

Fig. 3.1. Effect of $CdCl_2$ on cell viability and proliferation. J774A.1 cells were exposed to $CdCl_2$ at different concentrations for 24 hr (A-C). (A) Viability was measured by Trypan blue exclusion. Each point depicts percent of control viability. (B) Necrotic (also late apoptotic) cell death was measured by LDH release. (C) Proliferation by [^{3}H]thymidine incorporation was compared to viability measured by MTT assay in response to $CdCl_2$ damage. (D) J774A.1 cells were exposed to 20 μM $CdCl_2$ at different time. Proliferation by [^{3}H]thymidine incorporation was compared to viability measured by MTT assay in response to $CdCl_2$ damage. *Significantly different from the respective control at $p < 0.05$. #Distinct difference ($p < 0.05$) between viability and proliferation is indicated.

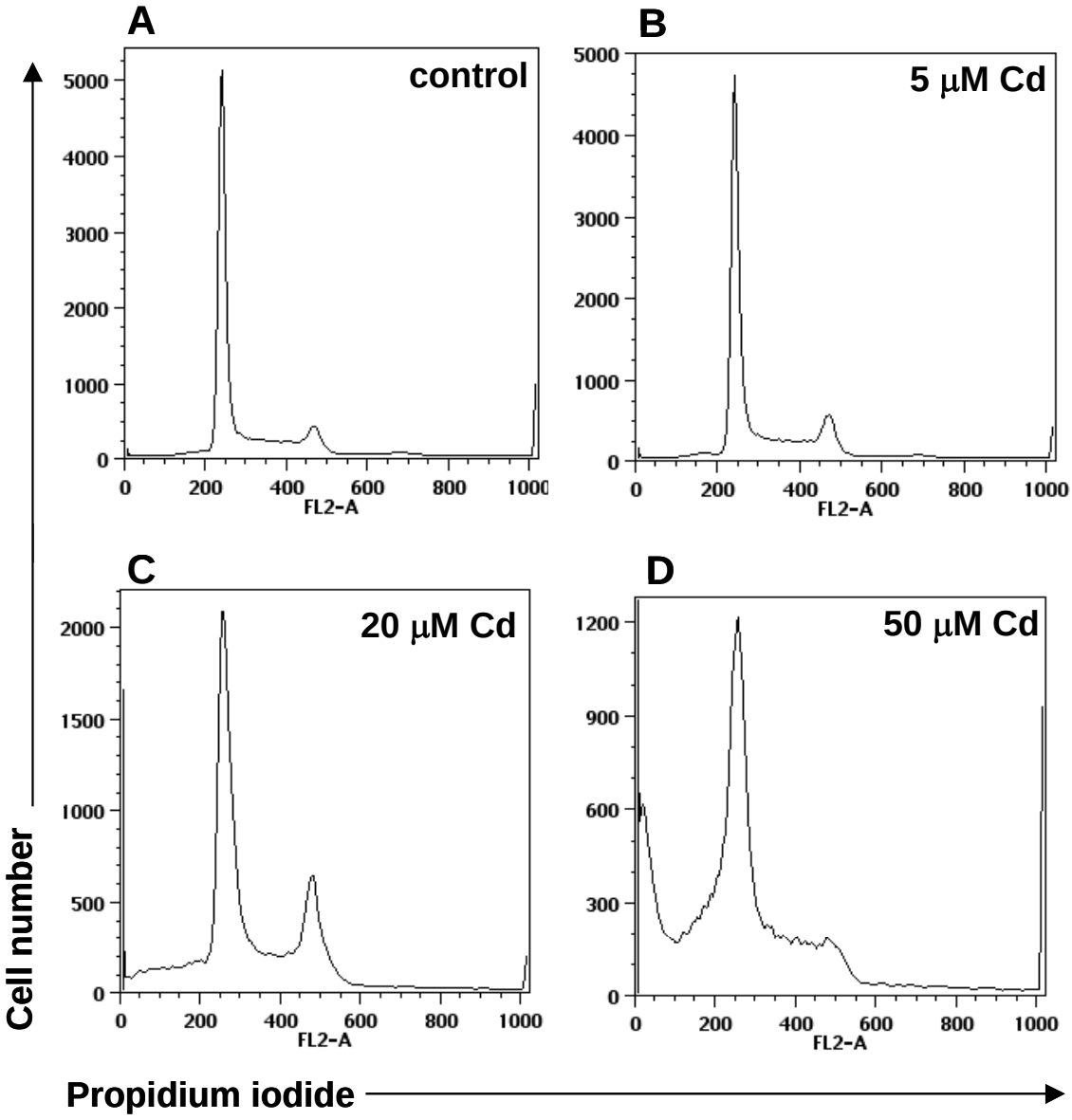

Fig. 3.2. Effect of $CdCl_2$ on cell cycle progression. J774A.1 cells were exposed to 0 (A), 5 (B), 20 (C), 50 (D) μM $CdCl_2$ for 24 hr. The percent of cells in each phase determined by PI staining by flow cytometry was represented on Table 1.

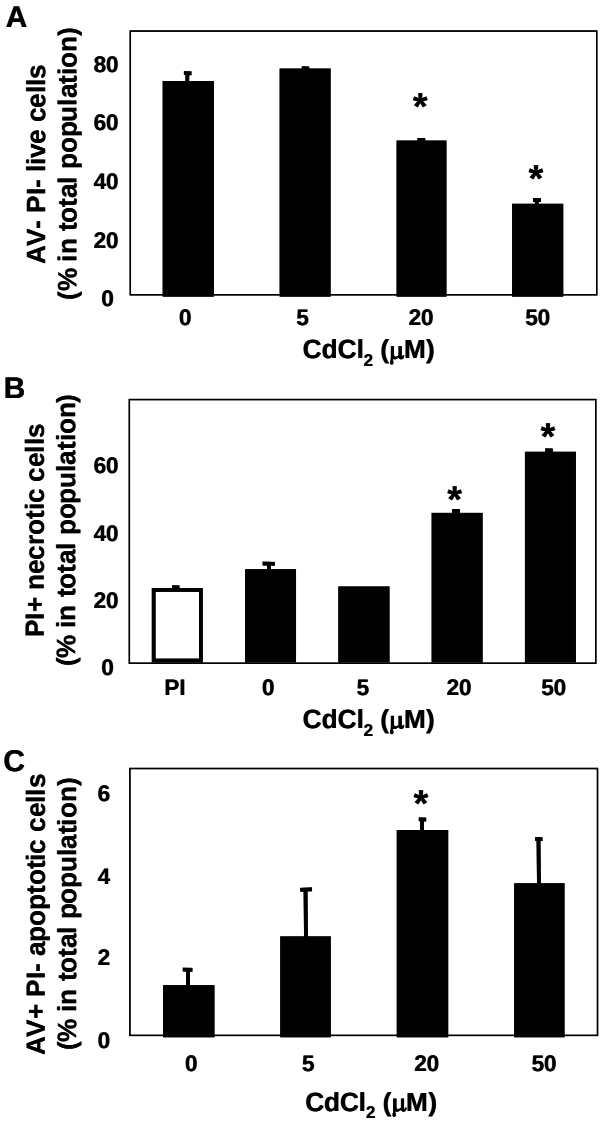

Fig. 3.3. Flow cytometric analysis of necrotic and apoptotic cell death. J774A.1 cells were exposed to $CdCl_2$ at indicated concentrations for 24 hr, then the cells were stained with annexin V-FITC (AV) and PI and analyzed by flow cytometry. (A) The percent of live cells with AV negative (-) and PI negative (-), (B) the percent of necrotic cells with PI positive (+), and (C) the percent of apoptotic cells with AV positive (+) and PI negative (-) were shown as mean + SE (n=3). * indicates significant difference from the control group at $p < 0.05$.

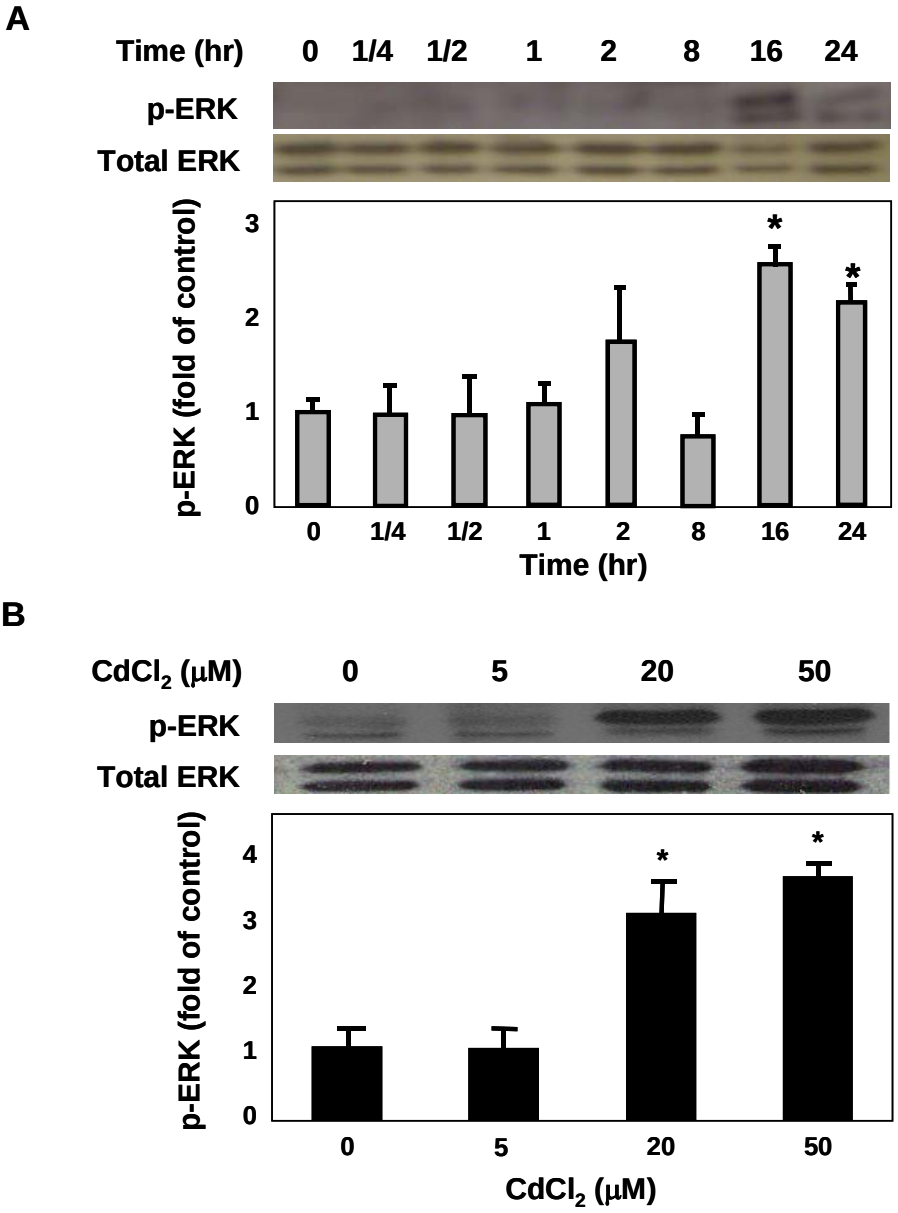

Fig. 3.4. Effect of $CdCl_2$ on phosphorylation of ERK. J774A.1 cells were exposed to $CdCl_2$ at indicated concentrations and time. The level of total and p-ERK was measured by western blot. (A) ERK activation with $CdCl_2$ 20 μM over time. (B) ERK activation after 16 hr with different $CdCl_2$ concentrations. * indicates significant difference from control group at $p < 0.05$. Results from a representative experiment are expressed as mean + SE (n=3).

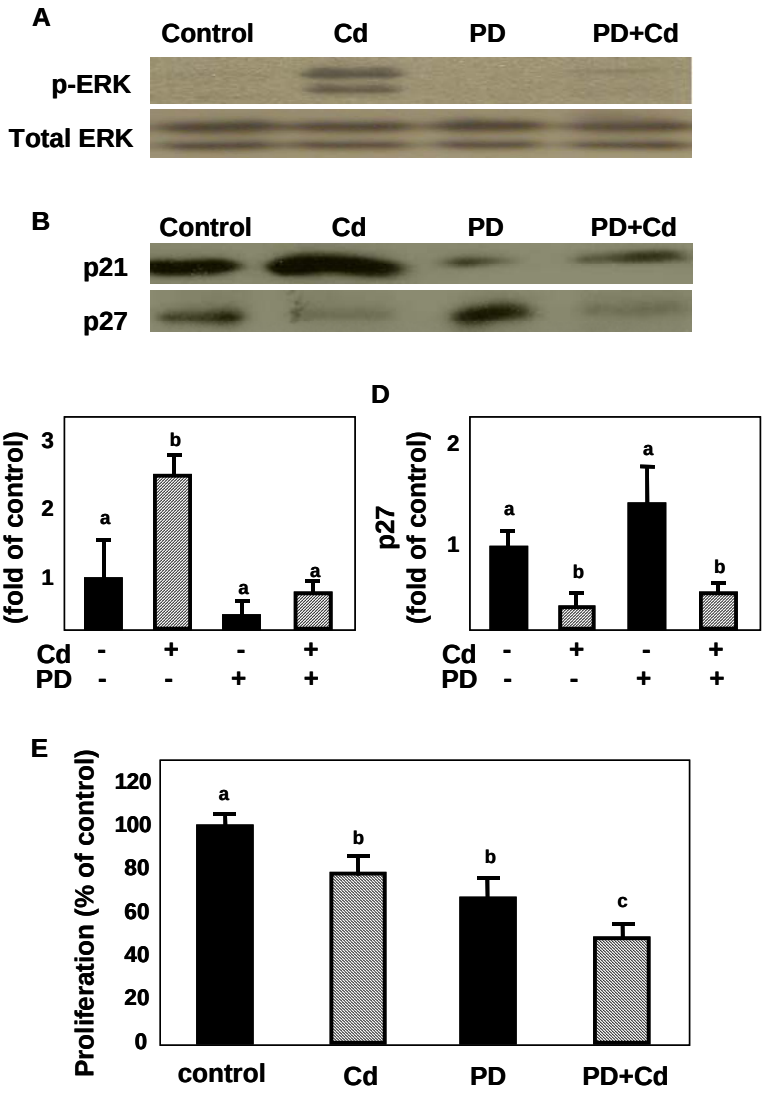

Fig. 3.5. Effect of PD98059 on $CdCl_2$-induced ERK activation, $p21^{WAF1/CIP1}$ induction, p27 down-regulation and inhibition of proliferation. J774A.1 cells were pretreated with PD98059 (20 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 hr. (A) Total and p-ERK, and (B) $p21^{WAF1/CIP1}$ and p27 were measured by western blot after 16 hr $CdCl_2$ exposure. Results shown are representative western blot and densitometric analysis of $p21^{WAF1/CIP1}$ and p27 (C-D). (E) Proliferation was measured by [^{3}H] thymidine incorporation after 16 hr $CdCl_2$ exposure. Different letters on top of bars indicate a significant difference ($p < 0.05$).

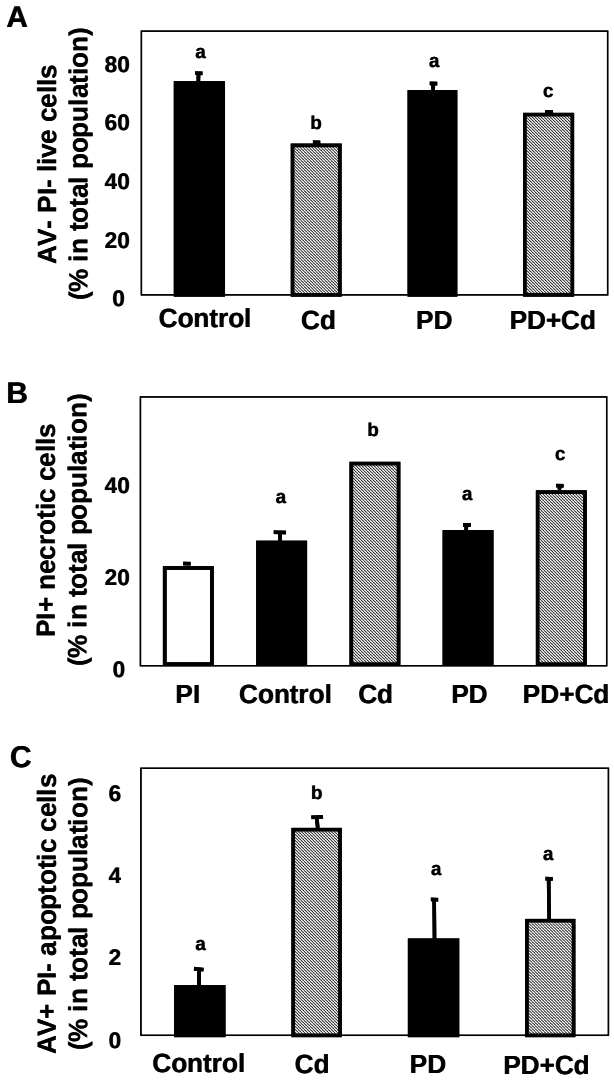

Fig. 3.6. Effect of PD98059 on $CdCl_2$-induced necrotic and apoptotic cell death. J774A.1 cells were pre-treated with PD98059 (20 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 hr. Cells were stained with annexin V (AV) and PI and analyzed by flow cytometry. (A) The percent of live cells with AV negative (-) and PI negative (-), (B) the percent of necrotic cells with PI positive (+), and (C) the percent of apoptotic cells with AV positive (+) and PI negative (-) were shown as mean + SE (n=3). Different letters on top of bars indicate a significant difference ($p < 0.05$).

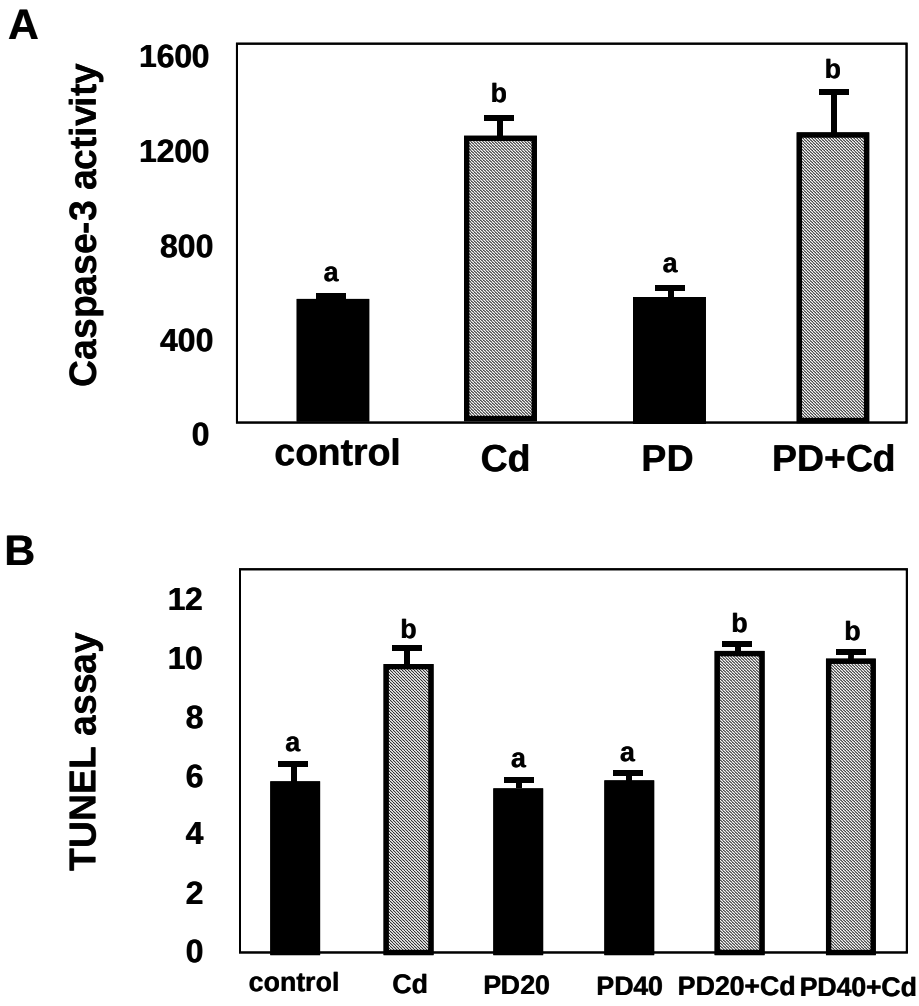

Fig. 3.7. Effect of PD98059 on $CdCl_2$-induced caspase-3 activation and DNA fragmentation. (A) J774A.1 cells were pre-treated with PD98059 (20 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 hr. Fluorometric caspase-3 enzyme activity was measured. (B) Cells were pre-treated with PD98059 (20 μM and 40 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 hr. Fluorescence of labeling DNA strand breaks by TUNEL-reaction after $CdCl_2$ exposure was read by microplate spectrofluorometer. Different letters on top of bars indicate a significant difference ($p < 0.05$). Results from a representative experiment (out of 3) are illustrated as mean + SE (n=3).

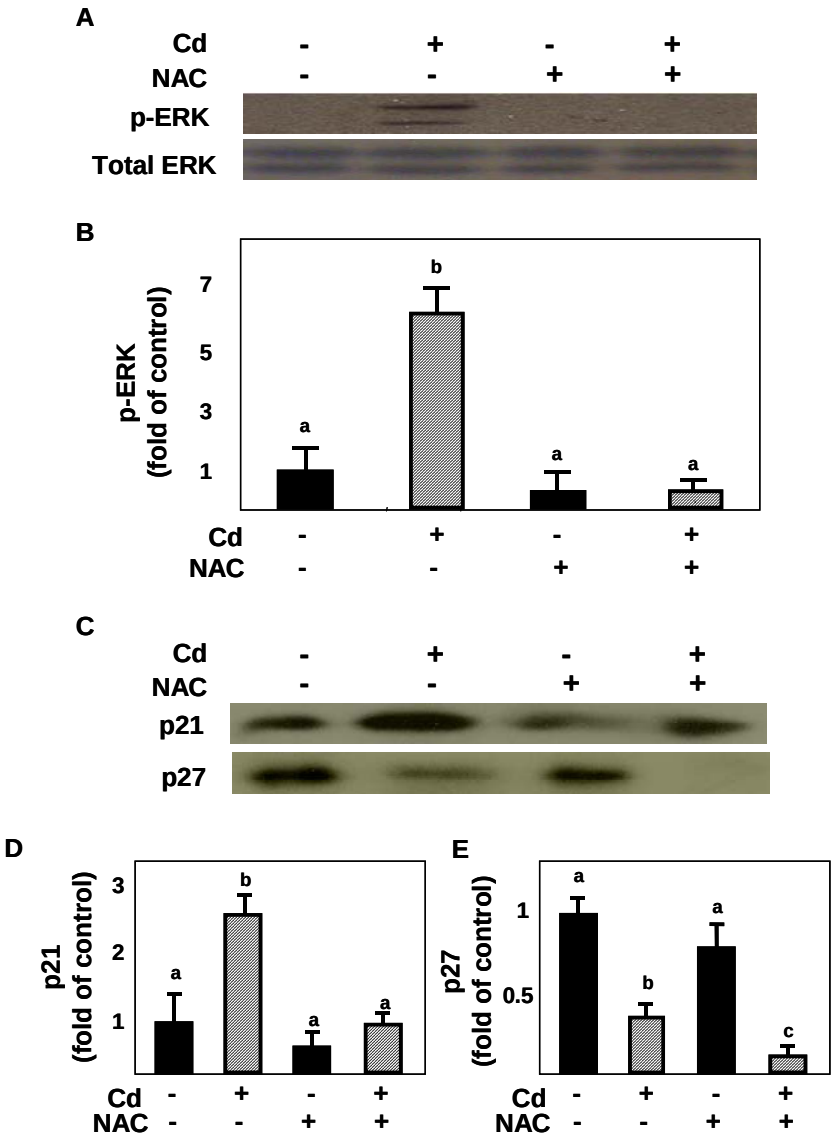

Fig. 3.8. Effect of antioxidants on $CdCl_2$-induced ERK activation, $p21^{WAF1/CIP1}$ induction, and p27 down-regulation. J774A.1 cells were pre-treated with NAC (1 mM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 hr. (A, C) NAC effect on $CdCl_2$-induced ERK activation, $21^{WAF1/CIP1}$ induction and p27 down-regulation was analyzed with western blot. Results shown are representative western blot and (B, D-E) densitometric analysis of p-ERK, $p21^{WAF1/CIP1}$ and p27. Different letters on top of bars indicate a significant difference ($p < 0.05$). Each value (mean + SE, n=3) represents the fold of control.

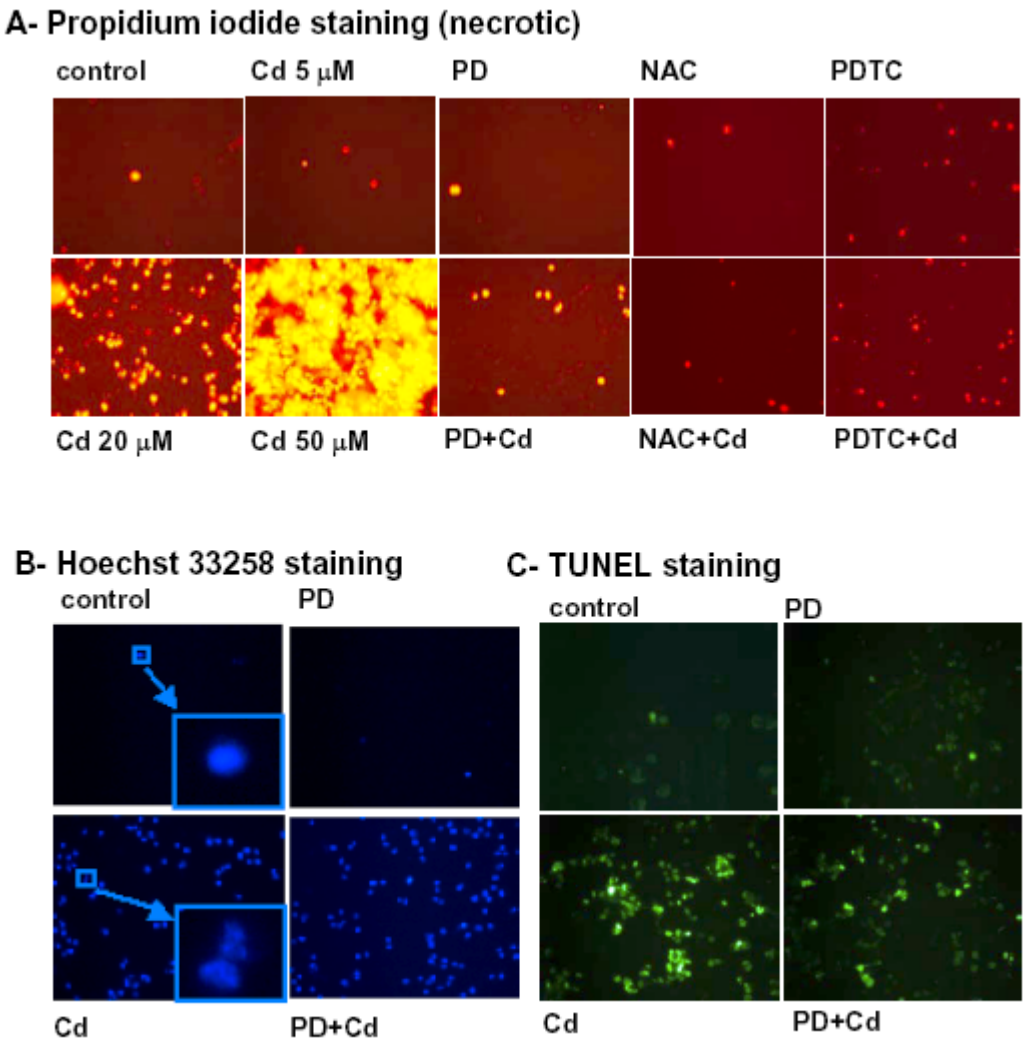

Fig. 3.9. Confirmation of necrosis and apoptosis by fluorescence microscopy. J774A.1 cells were pretreated with PD98059 (20 μM), NAC (1 mM) or PDTC (100 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 hr. (A) Fluorescence on PI stained necrotic cells was pictured by fluorescence microscopy. (B) Cell nuclei were visualized under fluorescence microscope with Hoechst 33258 staining. (C) Fluorescence on TUNEL positive nuclei was visualized under fluorescence microscope. The experiment was repeated three times with similar results and representative micrographs are shown.

CHAPTER 4

CALCIUM-MEDIATED ACTIVATION OF C-JUN NH_2-TERMINAL KINASE (JNK) AND APOPTOSIS IN RESPONSE TO CADMIUM IN MURINE MACROPHAGES[2]

[2]Kim, J. and R.P. Sharma. Accepted to *Toxicological Sciences*, 06/24/2004

Abstract

Cadmium is a well-known carcinogenic and immunotoxic metal commonly found in cigarette smoke and industrial effluent. Altered intracellular calcium ($[Ca^{2+}]_i$) level has been implicated in the pathophysiology of immune dysfunction. Present study was designed to determine the possible involvement of calcium (Ca^{2+}) and mitogen-activated protein kinases (MAPKs) signaling pathways on cadmium-induced cell death in J774A.1 murine macrophage cells. Cadmium caused a low amplitude $[Ca^{2+}]_i$ elevation at 20 μM and rapid and high amplitude $[Ca^{2+}]_i$ elevation at 500 μM. Exposure to cadmium dose-dependently induced phosphorylation of c-Jun NH_2-terminal kinase (JNK) and deactivated p38 MAPK. Use of selective JNK inhibitor, SP600125, suggested that activation of JNK is pro-apoptotic and pro-necrotic. Buffering of the calcium response with 1,2-bis-(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid tetrakis (acetoxy-methyl) ester (BAPTA-AM) and ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) completely blocked cadmium-induced apoptotic response. The pretreatment of cells with BAPTA-AM and EGTA suppressed the cadmium-induced cell injury including growth arrest, mitochondrial activity impairment and necrosis as well as recovered the cadmium-altered JNK and p38 MAPK activity. Chelating $[Ca^{2+}]_i$ also reversed cadmium-induced hydrogen peroxide generation suggesting that production of reactive oxygen species (ROS) is related to $[Ca^{2+}]_i$. The present study showed that cadmium induces ROS-$[Ca^{2+}]_i$-JNK-caspase-3 signaling pathway leading to apoptosis. Furthermore, cadmium-induced $[Ca^{2+}]_i$ regulates phosphorylation/dephosphorylation of JNK and p38, and modulates signal transduction pathways to proliferation, mitochondrial activity and necrosis.

Keyword: cadmium, calcium, ROS, MAPKs, growth arrest, apoptosis

Abbreviations used:

BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid tetrakis (acetoxy-methyl) ester; $[Ca^{2+}]_i$, intracellular calcium; CM-H_2DCFDA, 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; DPM, disintegrations per min; EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ERK, extracellular signal-related kinase; JNK, c-Jun NH_2-terminal kinase; MAPKs, mitogen-activated protein kinases; MTT, 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide; NF-κB, nuclear factor-κB; PI, propidium iodide; PKC, protein kinase C; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling

Introduction

Cadmium is a naturally occurring nonessential and toxic heavy metal commonly found in stabilizers in polyvinyl chloride products, color pigment, several alloys and, most commonly, in re-chargeable nickel-cadmium batteries (Jarup, 2003). Cigarette smoking may cause significant increases in blood cadmium level and it has been reported that smokers have 4-5 times higher level of cadmium in blood than non-smokers (Jarup et al., 1998). Inhalation of cadmium through tobacco smoking will directly effect the respiratory system without first-pass elimination and elevated blood level of cadmium may be a factor of immunodepression in smokers. Cadmium causes apoptotic cell death in NIH 3T3 murine fibroblasts (Biagioli et al., 2001), CCRF-CEM human T-cell line (Iryo et al., 2000), and rat testicular tissues (Xu et al., 1996). The International Agency for Research on Cancer (IARC) has classified cadmium as carcinogenic to humans

(Group 1), based on sufficient evidence for carcinogenicity in both human and animal studies (IARC, 1993).

Calcium ions are central to multiple signal transduction pathways to accomplish a variety of biological functions. The spatial and temporal regulation of intracellular calcium ($[Ca^{2+}]_i$) serves as a modulator of pathways involved in learning and memory, fertilization, proliferation and development (Berridge *et al.*, 2000). However, high $[Ca^{2+}]_i$ can cause disruption of mitochondrial Ca^{2+} equilibrium which results in reactive oxygen species (ROS) formation due to the stimulation of electron flux along the electron transport chain (Chacon *et al.*, 1991). Under oxidative stress, mitochondrial Ca^{2+} accumulation can switch from physiologically beneficial process to cell death signal (Ermak *et al.*, 2001). Ca^{2+}-dependent processes by cadmium (< 1 μM) activated $p21^{ras}$-dependent MAPK pathways, and nuclear factor-κB (NF-κB)-dependent gene expression, to stimulate proliferation in peritoneal macrophages (Misra *et al*., 2002). Besides the direct interaction of cadmium with intracellular molecules, altered $[Ca^{2+}]_i$ homeostasis has been considered as a target of toxic action by cadmium.

Cd^{2+} and Ca^{2+} are two closely related elements with similarity in many aspects, partially due to their similar ionic radii; the radius of a common form of free Cd^{2+} in the body and that of Ca^{2+} are 0.099 and 0.097 nm, respectively (Weast *et al.*, 1982). Cadmium is a potent Ca^{2+} channel blocker and inhibits Ca^{2+} cellular uptake (Thevenod *et al.*, 1992). Cadmium has a high affinity for and activates calmodulin, a Ca^{2+} binding protein that regulates a variety enzymes and cell progresses (Behra *et al.*, 1991). Moreover, a number of recent studies demonstrated that cadmium interacts with the function of Ca^{2+}-dependent enzymes such as endonuclease and regulatory proteins such as protein kinase C (PKC) and phospholipase C, thus interfering with the Ca^{2+}-signaling pathways (Lohmann *et al.*, 1993; Long, 1997; Misra *et al.*, 2002).

Mitogen activated protein kinases (MAPK) 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 NH_2-terminal kinase (JNK, also known as stress-activated protein kinase), p38 MAPK, and extracellular signal-related kinase (ERK) (Schaeffer *et al.*, 1999). These MAPKs are believed to be important biomolecules in cell differentiation, cell movement, cell division and cell death induced by extracellular stimuli (Schaeffer *et al.*, 1999). We recently reported ERK signaling-dependent G2/M arrest and cell death in murine macrophages by cadmium (Kim *et al*., 2003). However, it is not clear if cadmium-altered MAPKs activity is interrelated with Ca^{2+}. Intracellular calcium elevation by cadmium was required for JNK activation in LLC-PK_1 cells (Matsuoka *et al.*, 1998). Calcium-dependent PKC activation by serotonin contributed to ERK phosphorylation in the nudibranch mollusk *Hermissenda* (Crow *et al.*, 2001). Elevated $[Ca^{2+}]_i$ was involved in p38 MAPK-induced neuronal cell death by pneumolysin (Stringaris *et al.*, 2002). These findings suggest that the role/activation of ERK, JNK and p38 may be connected with $[Ca^{2+}]_i$.

Based on substantial evidence, mostly from in vivo animal models, cadmium is able to damage both the humoral immune response and cell mediated immunity (Descotes, 1992). J774A.1 cells are commonly used murine macrophage cell line which possesses similarities to mature macrophages, making them an alternative to primary cells (Yan *et al.*, 2004). J774A.1 cells originated from BALB/c mouse reticulum cell sarcoma, and due to their tumor-like property, these cells may be resistant to cadmium toxicity. Studies using macrophages on cadmium-induced inhibition of growth progression and subsequent cell death are helpful to understand cadmium effect on the immune system. There have been studies to show that elevated $[Ca^{2+}]_i$ by cadmium mediates several different signaling pathways depending on cell

types. However, the kinetics of $[Ca^{2+}]_i$ elevation and exact involvement of $[Ca^{2+}]_i$ on cadmium toxicity including cell death, growth arrest, mitochondrial activity and ROS generation, has not been clearly determined in macrophages. We have proved the role of intracellular and extracellular Ca^{2+} in cadmium-altered JNK and p38 MAPK and the importance of JNK activation on cadmium-induced activation of caspase-3 and apoptosis in murine macrophages.

Materials and Methods

Reagents

Cadmium ($CdCl_2$, Sigma Chemical Co., St. Louis, MO) was dissolved in water, sterilized with 0.22 μm filters, and added to cultures at the indicated time and concentrations. Cell culture reagents were procured from GIBCO Life Technology (Grand Island, NY). Antibodies specific for the total and phosphorylated forms of JNK (p54/46) and p38 MAPK were obtained from Cell Signaling (Beverly, MA). Specific JNK inhibitor SP600125 was purchased from Calbiochem (La Jolla, CA). Fluorescent probes Fluo-3/AM and propidium iodide (PI) were procured from Molecular probes (Eugene, OR). 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetra acetic acid tetrakis (acetoxy-methyl) ester (BAPTA-AM), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA), Hoechst 33258 and all other chemicals used in this study were obtained from Sigma and were of cell culture grade.

Cell culture

Macrophage cell line, J774A.1 (American Type Culture Collection TIB-67), established from BALB/c mouse, was maintained in Dulbecco's Modified Eagle's Medium, supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and 10% non-heat-inactivated fetal bovine serum (Atlanta Biologics, Atlanta, GA) in 5% CO_2 atmosphere at 37 ºC.

The J774A.1 cells were grown in 75 cm^2 culture flasks and subcultured when the cells reached 70–80% confluence (every 3 days). Cells were used during 3rd or 4th passages. Cultures were allowed to grow overnight (15 h) prior to the treatment. The concentrations used for various reagents, added 30 min prior to cadmium treatment, were 20 μM for SP 600125, 10 μM for BAPTA-AM and 1 mM for EGTA. The used concentrations of above agents were not cytotoxic.

Determination of intracellular Ca^{2+}

$[Ca^{2+}]_i$ levels were monitored by Fluo-3, which is a Ca^{2+}-sensitive fluorescent indicator. Cells were seeded at 8×10^4 cells/well in 96-well microplates (Falcon, Becton Dickinson, Franklin Lakes, NJ) and treated with indicated time and concentration of cadmium. Cells were loaded with Fluo-3/AM (10 μM) in dark at 37°C for 1 h and washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 12 mM $NaHCO_3$, and 5.5 mM Glucose). Cells were incubated in Tyrode's solution for another 30 min and the morphological fluorescence intensity of cells was determined using Olympus IX71 inverted microscope (Olympus America, Melville, NY). A 488 nm excitation wavelength was used to illuminate Fluo-3, and fluorescence was detected at emission wavelength of 510 nm. Digital images were acquired using the Magnafire SP (Olympus) digital camera.

Western blot analysis of phosphorylated JNK and p38 MAPK

The activation status (phosphorylation) of JNK and p38 MAPK was determined using phospho-specific antibodies as described previously (Johnson *et al.*, 2003). Cells were grown at 2×10^6 cells/well in 6-well microplates and treated with cadmium for indicated time and concentrations. Following treatment, cells were washed with phosphate buffered saline (PBS) and total cell lysates were prepared by scrapping in 100 μl of lysis buffer [20 mM Tris–HCl (pH

8.0), 1 mM sodium orthovanadate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetate (EDTA), 1% Triton X-100, 50 mM β-glycerolphosphate, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin]. Fifty micrograms of proteins determined by Bradford assay was electrophoretically separated using a 12% SDS-PAGE gel and transferred to nitrocellulose paper followed by antibody staining. Equal loading and transfer of total protein was verified with the reversible Ponceau S stain (Sigma) dye and also by detecting total JNK and p38 MAPK. Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

Determination of caspase-3 activation

Caspase-3 activity was determined using CaspACE^{TM} fluorometric activity assay (Promega, Madison, WI) with modifications as follows. Briefly, cells were treated in 96-well microplates following which Triton X-100 was added and repeatedly pipetted to lyse the cells. The homogenates were centrifuged at 4,000×g for 10 min to remove cell debris. The supernatant was assayed for caspase-3 activities using CaspACE^{TM} system according to the manufacture's instructions. The plates were read at 360/460 nm (excitation/emission) using a SpectraMax Gemini (Molecular Devices, Sunnyvale, CA). The fluorescence signal was digitized and analyzed using SoftMax Pro^{TM} (Molecular Devices, Irvine, CA).

Hoechst and PI 33258 staining

Apoptotic morphological changes in the nuclear chromatin of cells were detected by staining with the DNA binding fluorochrome Hoechst 33258 (bis-benzimide). Hoechst 33258 exhibits fluorescence enhancement upon binding to A-T rich regions of double stranded DNA. Necrotic cell death was detected by staining with PI, a membrane impermeable dye excluded

from viable cells. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4-5 base pairs of DNA. Dead cells are PI-bright and live cells are PI-dim. Following 24 h treatment with cadmium, 120 µl of supernatant was removed and 20 µl of Hoechst 33258 (2 µg/ml) or PI (1 µg/ml) was added. The plates were read at 350/450 nm or 535/617 nm (excitation/emission) for Hoechst 33258 or PI fluorescence, respectively, using a SpectraMax Gemini. The fluorescence signal was digitized and analyzed using SoftMax Pro™.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay

TUNEL assay was performed using the in situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Cells were plated at 8×10^4 cells/well in 96-well microplates and allowed to attach overnight. Cells were then treated with cadmium for 24 h, fixed with paraformaldehyde, and analyzed for stained nuclei according to the manufacturer's instructions. The fluorescence signal was read by SpectraMax Gemini, digitized and analyzed using SoftMax Pro™. In addition, fluorescence microscopy was performed to examine fragmented DNA morphology using IX71 inverted microscope. Digital images were captured using MagnaFire SP® Olympus digital camera.

Mitochondrial activity

MTT (3[4,5-dimethyl thiazolyl-2]2,5-diphenyl tetrazolium bromide, Sigma) assay was performed to investigate mitochondrial activity. Cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with the indicated concentration of cadmium for 24 h. The cells were incubated with addition of 20 µl MTT (5 mg/ml). After 4 h, 120 µl of MTT media was taken out

from each well and 100 µl of 0.02 N HCl-isopropanol (warm) added to dissolve formazan crystals. The absorbance of each cell was measure by UV spectrometer at 570 nm.

DNA synthesis as an index of proliferation

The [methyl-^{3}H]thymidine incorporation assay was used as an index of proliferation. Cells were seeded at 8×10^4 cells/well in 96-well microplates. At 16 h prior to harvesting cells, each well was pulsed with 20 µl of [methyl-^{3}H]thymidine (25 µCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA). Cells were harvested onto glass fiber filter paper (Cambridge Technology, Watertown, MA) using a cell harvester (PHD, Cambridge Technology). Proliferative response (uptake of [^{3}H]thymidine) in the harvested cells was counted in a liquid scintillation counter (Pharmacia, Turku, Finland) and expressed as net disintegrations per min (DPM).

Production of H_2O_2

The production of H_2O_2 was measured by detecting the fluorescent intensity of H_2O_2-sensitive probes after adding 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes). The cells were incubated in the presence of various concentration of cadmium and fluorescent intensity recorded using SpectraMax Gemini fluorescence plate reader. The CM-H_2DCFDA fluorescence was detected by excitation at 485 nm, and emission at 530 nm. The fluorescence readings were digitized using SoftMax Pro. The results were similar in three independent replications and data from a representative experiment (n=5 wells) have been illustrated.

Replication and statistical analysis

Experiments were repeated at least 3-4 times with consistent results. Means + SE from representative experiments have been presented. All statistical analyses were performed using

the SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A *p* value of < 0.05 was considered significant unless indicated otherwise in figure legends.

Results

Cadmium-induced $[Ca^{2+}]_i$

To examine whether the $[Ca^{2+}]_i$ change was involved in cytotoxicity by cadmium, we tested the $[Ca^{2+}]_i$ using the calcium indicator Fluo-3. After cells were loaded with Fluo-3/AM, the fluorescent intensity of Fluo-3 was detected by fluorescence microscope. Time-course study on elevated $[Ca^{2+}]_i$ level was performed for 24 h with various cadmium concentration (Fig. 1A). Cadmium at 20 and 500 μM showed distinct increase of $[Ca^{2+}]_i$ at 2 h compared with the stable baseline level of $[Ca^{2+}]_i$ in control cells. The changes in fluorescent intensity of peak calcium showed concentration-dependence. Cadmium at 20 μM slightly increased $[Ca^{2+}]_i$ at 2 h, persisted for the whole period until 18 h, and later this elevated $[Ca^{2+}]_i$ tended to go down after that. Higher concentration of cadmium at 100 and 500 μM showed much stronger fluorescence intensity at earlier time point and the fluorescence declined away faster than that in lower concentration cells.

Cadmium-altered activation of JNK and p38

We recently reported that cadmium induce activation of ERK in J774A.1 (Kim et al., 2003). To know whether cadmium alters activity of other MAPKs including JNK and p38, phosphorylated forms of JNK and p38 were examined by western blots. In J774A.1 cells treated with 20 μM cadmium, the levels of phosphorylated forms of p 54 (JNK2) and p46 (JNK1) increased clearly after 8 h (Fig. 2A). Phosphorylated form of JNK remained elevated even at 16

h and then declined at 24 h. In contrast, the levels of total (phosphorylation state-independent) JNK were not changed during the incubation period of 24 h. When cells were incubated with 1-50 μM cadmium for 16 h, the levels of phosphorylated JNK increased in a dose-dependent manner while those of total JNK were not changed (Fig. 2B). Besides activity of JNK changed by cadmium, altered p38 activity was also observed. After incubation with 20 μM cadmium for 16 h, the apparent bands of phosphorylated p38 started to decline (Fig. 3A). After 16 h of incubation, > 20 μM cadmium resulted in suppression of p 38 activity in a dose-dependent manner (Fig. 3B). However, the level of total p38 was not changed significantly indicating that the specific kinase activity of p38 was down-regulated in cadmium-treated cultures.

Role of activated JNK on cadmium-induced apoptosis and necrosis

To examine the relationship between cadmium-induced JNK activation and apoptosis, we employed JNK inhibitor, anthrapyrazolone (SP600125, Bennett *et al.*, 2001). J774A.1 cells were incubated with cadmium 20 μM in the absence or presence of 20 μM of SP 600125 and assayed by caspase-3 activity, Hoechst 33258 staining and TUNEL at 24 h. The levels of caspase-3 activity, Hoechst 33258 fluorescence and TUNEL staining were similar in SP600125-treated or control cultures, but it significantly decreased in cultures treated with cadmium and SP600125 than in cultures treated with cadmium-only (Fig. 4). PI is used to distinguish cells that are in the late stage of apoptosis or necrosis, which lose plasma membrane integrity and are permeable to PI (Fig. 5). Inhibition of JNK activity by 20 μM SP600125 decreased fluorescence of PI staining, which was increased by 20 μM cadmium at 24 h. We interpret the data to indicate that JNK activation is involved in cadmium-induced both apoptosis and necrosis.

Inhibitory effect of BAPTA and EGTA on cadmium-induced $[Ca^{2+}]_i$ elevation, growth arrest, mitochodrial activity and necrosis

To verify the role of $[Ca^{2+}]_i$ as a key second messenger, cells were pre-loaded with 10 μM BAPTA-AM and 1 mM EGTA for 30 min. BAPTA-AM is an effective membrane permeable intracellular Ca^{2+} chelator, trapped in the cells after cytoplasmic hydrolysis. As shown in Fig. 6A, chelating intracellular Ca^{2+} with BAPTA prevented the elevation of $[Ca^{2+}]_i$, demonstrating that the release of intracellular Ca^{2+} is essential for cadmium-induced $[Ca^{2+}]_i$ overloading. Extracellular Ca^{2+} removal by EGTA also diminished cadmium-induced $[Ca^{2+}]_i$ overloading but showed slight elevation of $[Ca^{2+}]_i$ suggesting that the extracellular Ca^{2+} is an important source for elevated $[Ca^{2+}]_i$ but other source from intracellular Ca^{2+} storage is also important. Results presented in Figure 6B-C demonstrate that BAPTA-AM and EGTA pretreatment suppressed cadmium-induced growth arrest and mitochondrial impairment. BAPTA-AM and EGTA itself were able to reduce mitochondrial activity indicating the importance of normal Ca^{2+} signaling on mitochondrial function. Inhibitory effect of $[Ca^{2+}]_i$ chelation on cadmium-induced necrotic cell death examined by PI staining is shown in Fig. 6D.

Inhibitory effect of BAPTA and EGTA on cadmium-induced apoptosis

To determine the role of calcium in the regulation of cadmium-induced apoptosis, J774A.1 cells were incubated with 20 μM cadmium in the absence or presence of BAPTA-AM or EGTA. Treatment of cultures with cadmium resulted in activation of caspase-3 at 24 h. Chelating intracellular and extracelluar calcium totally inhibited cadmiun-induced caspase-3 activation (Fig 7A). The elevation of Hoechst 33258 fluorescence by cadmium was also abolished (Fig 7B). The TUNEL staining confirmed that over-loaded $[Ca^{2+}]_i$ is an important mediator for cadmium-induced apoptotic cell death (Fig. 7C).

Inhibitory effect of BAPTA and EGTA on cadmium-altered MAPKs activity

To delineate the further signaling pathways of elevated $[Ca^{2+}]_i$ by cadmium, we examined the phosphorylation of MAPKs (JNK and p38) in J774A.1 cells. The immunoblot with phosphorylated form of JNK specific antibody revealed that BAPTA and EGTA significantly, but not completely, inhibited cadmium-induced JNK activation (Fig 8A). Down regulated p38 activity by cadmium was recovered up to control level in the presence of 10 μM BAPTA-AM or EGTA (Fig. 8B). Higher concentration of BAPTA-AM (50 μM) was not able to prevent p38 activity down-regulated by cadmium.

Relationship between $[Ca^{2+}]_i$ and ROS

H_2DCFDA is a dye specifically binding to H_2O_2. Analysis of cells stained with H_2DCFDA revealed that 20 μM cadmium treatment caused significantly high cellular level of H_2O_2 (Fig. 9). Chelating intracellular- and extracellular-calcium by BAPTA-AM and EGTA dramatically prevented cadmium-induced H_2O_2 generation, suggesting that cadmium-induced ROS generation may be critical for $[Ca^{2+}]_i$ elevation.

Discussion

Results presented here suggest that cadmium activated JNK, which plays a critical role in the apoptotic suicide of cells. Cadmium strongly stimulated JNK activity after 8 h exposure of J774A.1 murine macrophage cells and this stimulation persisted until 16 h. The sustained JNK activation was Ca^{2+}-dependent and served as a death signal in cadmium-induced apoptosis. Chelation of $[Ca^{2+}]_i$ by BAPTA-AM and EGTA prevented the cadmium-induced H_2O_2 generation, hampered mitochondrial activity, JNK, caspase-3 activation and apoptosis, confirming the early mediating role of Ca^{2+} during cadmium-induced apoptosis. We also present

evidence that cadmium down-regulates activation of p38 MAPK. Cadmium-mediated modulation of JNK and p38 MAPK activity was tightly correlated with elevated $[Ca^{2+}]_i$. Chelating $[Ca^{2+}]_i$ reduced H_2O_2 production indicating that ROS act concert with $[Ca^{2+}]_i$ signaling.

The inhibitory effect of cadmium on intracellular mechanisms of Ca^{2+} regulation has been reported earlier. Cadmium inhibited Ca^{2+} extruding Ca^{2+}-ATPase pump in both endoplasmic reticulum and plasma membrane (Benters *et al.*, 1996; 1997; Zhang *et al.*, 1990). In isolated bovine liver nuclei, cadmium resulted in inhibition of ATP-dependent nuclear Ca^{2+} uptake (Hechtenberg *et al.*, 1994). Besides its inhibitory effect on Ca^{2+}-ATPase pump for sequestration of $[Ca^{2+}]_i$, cadmium is also known to disturb Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular stores. Cadmium at 20 μM evoked a transient rise in cellular IP_3 and the perturbation of IP_3/Ca^{2+} messenger system was suggested as an early and discrete effect of cadmium in E367 neuroblastoma cells (Benters *et al.*, 1997). A specific cell surface metal ion receptor was suggested as an interacting site with cadmium in *xenopus* oocyte to activate IP_3-mediated Ca^{2+} release (Hague *et al.*, 2000). Cadmium may interact with cell surface membrane proteins coupled to a pertussis toxin-sensitive G protein, which drives IP_3 induction and Ca^{2+} release in primary murine macrophages (Misra *et al.*, 2002). Inhibition of Ca^{2+} influx by chelating extracellular Ca^{2+} showed significantly reduced $[Ca^{2+}]_i$ in current study, but it was not totally abolished. This suggests that extracellular Ca^{2+} is required in $[Ca^{2+}]_i$ elevation by cadmium; however, there are other sources from intracellular Ca^{2+} storage in response to cadmium damage.

We recently showed that cadmium induced activation of ERK in J774A.1 (Kim *et al.*, 2003). In the present study, cadmium activated JNK and deactivated p38 MAPK in a concentration-dependent manner. In contrast to our finding of down-regulation of 38 MAPK,

there have been reports showing that the cadmium-activated p38 MAPK is responsible for apoptosis, mitotic arrest, activation of heat shock factor 1 and induction of heat shock protein 70 (Chao *et al.*, 2001; Galan *et al.*, 2000; Hung *et al.*, 1998). Deactivation of p38 MAPK has been shown to lead both anti-apoptotic and pro-apoptotic responses. Exogenous nerve growth factor induced dephosphorylation of p38, which prevents Bcl-2 phosphorylation and apoptotic response in lymphoblastoid CESS B cell line (Rosini *et al.*, 2004). However, a natural anticancer depsipeptide, FR901228, induced apoptosis of *ras*-transformed 10T1/2 cells through suppression of p38 pathways (Fecteau *et al.*, 2002). Our study indicates that a potential value of cadmium may involve aberrant regulation of Ras through suppression of p38 MAPK pathway leading to apoptosis in macrophages.

Disruption of Ca^{2+} homeostasis seems to take a part in initiating activation of MAPKs. Calcium was mobilized from intracellular stores by tributylin that played an important role for the phosphorylation of JNK and p38 MAPK in CCRF-CEM human T-cell line (Yu *et al.*, 2000). P38 MAPK were activated by cadmium in primary macrophages and depletion of $[Ca^{2+}]_i$ with BAPTA-AM inhibited such activation (Misra *et al.*, 2002). We found the opposite phenomenon of down-regulation on p38 MAPK, however, it is consistent with other studies in terms of that Ca^{2+} is an important regulator for activity of p38 MAPK. Recent evidence suggests that JNK pathway may play an important role in triggering apoptosis and signaling with mitochondria. Activated JNK by thapsigargin was involved in loss of mitochondrial membrane potential and as downstream of caspase-3 in Jurket leukemia T cells (Srivastava *et al.*, 1999). Rise in $[Ca^{2+}]_i$ by thapsigargin promoted nitric oxide (NO) generation and induction of JNK activity and apoptosis including activation of caspase-2 and –9 in Jurkat T cells (Srivastava *et al.*, 1999). This is consistent with our data showing that elevated-$[Ca^{2+}]_i$ by cadmium mediated generation of H_2O_2

and activation of JNK leading to caspase-3 activation and DNA fragmentation. Ca^{2+} and JNK must be an important regulator of immune cell death.

Cadmium exposure to J774A.1 exhibited increased oxidative stress induced by ROS and NO production, following single strand breaks and apoptosis (Hassoun *et al.*, 1996). In general, inducing synthesis of protective sulfhydryl compounds including metallothionein and glutathione preceded lipid peroxidation and DNA damage in cadmium-treated cells and contributed to protection of cells from injury by cadmium (Beyersmann *et al.*, 1997). In the present study, cadmium still was able to induce level of H_2O_2 at 6 h so protective antioxidation system may be interrupted by cadmium damage. We have shown that the increase in H_2O_2 generation by cadmium was also detected in response to elevation of $[Ca^{2+}]_i$, suggesting a close relationship between Ca^{2+} and ROS in signal transduction pathways. Cadmium stimulates the proliferation of cultured mammalian cells only when applied at and below micromolar concentrations, whereas elevated concentrations and prolonged exposure induce inhibition (Beyersmann *et al.*, 1997). We have shown that 20 μM cadmium inhibits the proliferation of macrophage at 16 h and buffering $[Ca^{2+}]_i$ level with BAPTA-AM and EGTA was able to recover growth arrest (Fig. 6B). An earlier in vivo study found that cadmium exposure significantly decreased the number of thymocytes in S-phase, indicating the inhibitory effect of cadmium on DNA synthesis in these cells (Morselt *et al.*, 1988). We also reported inhibited proliferation of J774A.1 macrophages by cadmium via G2/M arrest (Kim *et al*., 2003). However, the exact signaling pathways from $[Ca^{2+}]_i$ elevation to growth arrest are largely unknown. Intracellular calcium elevation leading to oxidative stress and alterations in mitochondrial and nuclear function, is thought to be major event in cadmium-mediated growth arrest in our study. Impairment of nuclear Ca^{2+} regulation caused by cadmium is suggested to provoke alterations in nuclear events related to gene

expression and cell proliferation (Hechtenberg *et al.*, 1994). Interaction of cadmium with Ca^{2+} binding protein calmodulin was also suggested as a key factor to inhibit cell proliferation (Powlin *et al.*, 1997).

In summary, we have demonstrated here that cadmium-elevated $[Ca^{2+}]_i$ level primarily from extracellular space, activated JNK and down-regulated p38. Caspase-3 activation was involved in apoptosis by cadmium. JNK was involved for cadmium-induced caspase-3 activation and apoptosis. Hydrogen peroxide generation was mediated by cadmium-induced $[Ca^{2+}]_i$. Rising $[Ca^{2+}]_i$ concentration and ROS may cause Ca^{2+} influx into mitochondria and disrupt normal metabolism of mitochondria leading to apoptosis and growth arrest. Chelating $[Ca^{2+}]_i$ recovered growth arrest, mitochondria impairment and subsequently necrosis. Taken together, the current study indicates that cadmium-induced $[Ca^{2+}]_i$ elevation triggers growth arrest, regulates phosphorylation/dephosphorylation of protein kinases and modulates signal transduction pathways. JNK and caspase-3 activation by cadmium is interrelated with $[Ca^{2+}]_i$ to modulate cadmium toxicity.

Acknowledgement

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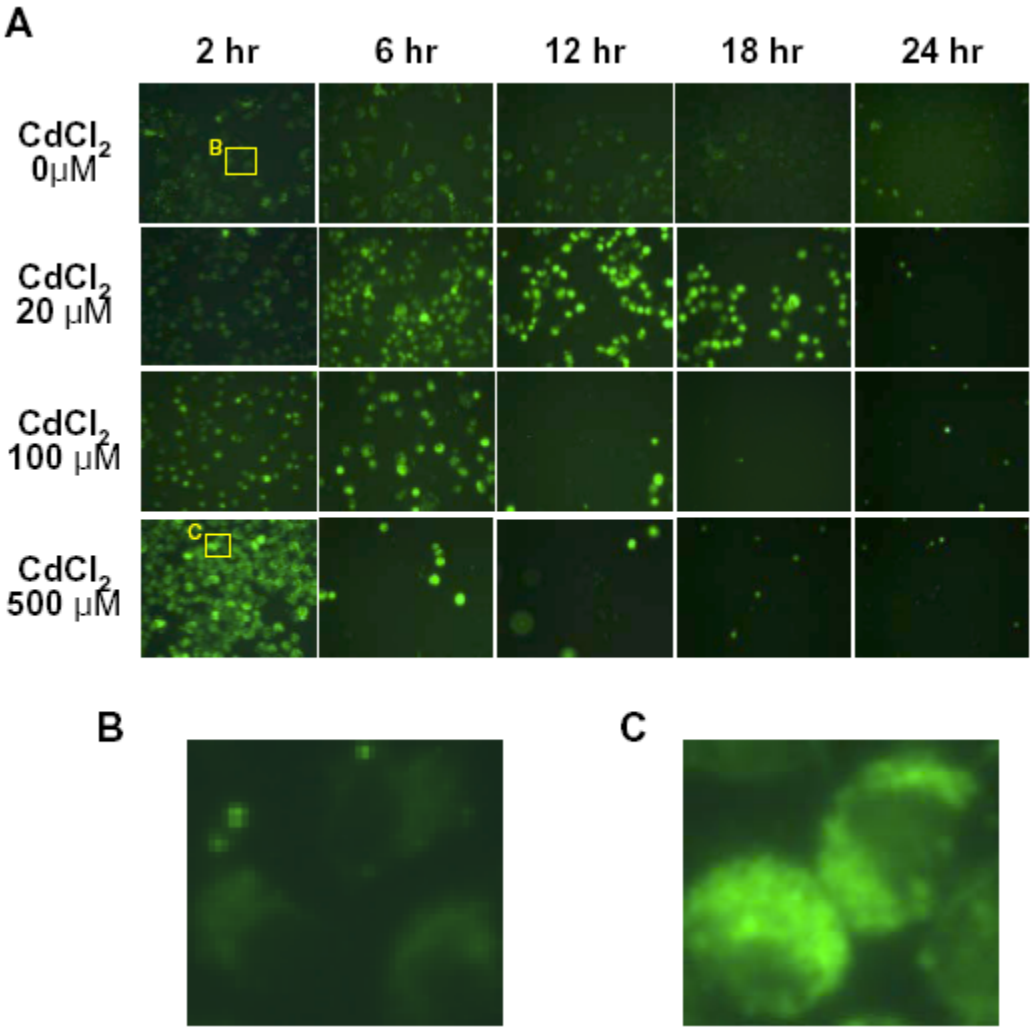

Fig. 4.1. Effect of $CdCl_2$ on $[Ca^{2+}]_i$ level observed by fluorescence microscopy. (A) Cells were treated with 0, 20, 100 and 500 μM $CdCl_2$ for 24 h. Altered $[Ca^{2+}]_i$ levels were examined by Fluo-3, Ca^{2+}-sensitive fluorescent indicator. Morphological fluorescence intensity of cell was visualized under a fluorescence microscope. For the closer look for altered $[Ca^{2+}]_i$ levels, 2-3 cells are selected and magnified (B) Treated with 0 μM $CdCl_2$ at 2 h and (C) Treated with 500 μM $CdCl_2$ at 2 h. The experiment was repeated three times with similar results and representative micrographs are shown.

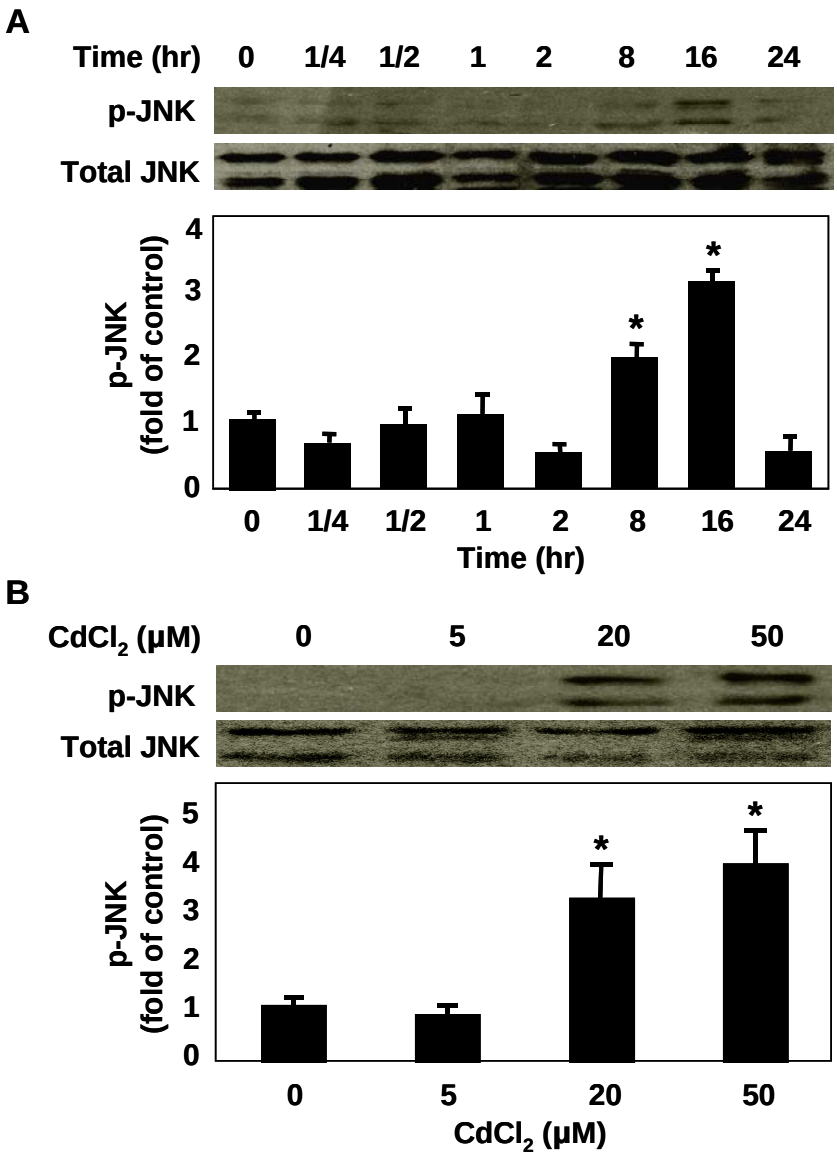

Fig. 4.2. Effect of $CdCl_2$ on phosphorylation of JNK. J774A.1 cells were exposed to $CdCl_2$ at indicated concentrations and time. Cell extracts were analyzed by western blot to detect the p-JNK1 (p46) and p-JNK2 (p54) using a phosphospecific JNK antibody. P-JNK represents activated JNK whereas total JNK indicates total protein loading. (A) JNK activation with $CdCl_2$ 20 μM over time. (B) JNK activation after 16 h with different $CdCl_2$ concentrations. Mean + SE (n=3). * indicates significant difference compared to control group at $p < 0.05$ analyzed with one-way ANOVA followed by Duncan's multiple range test.

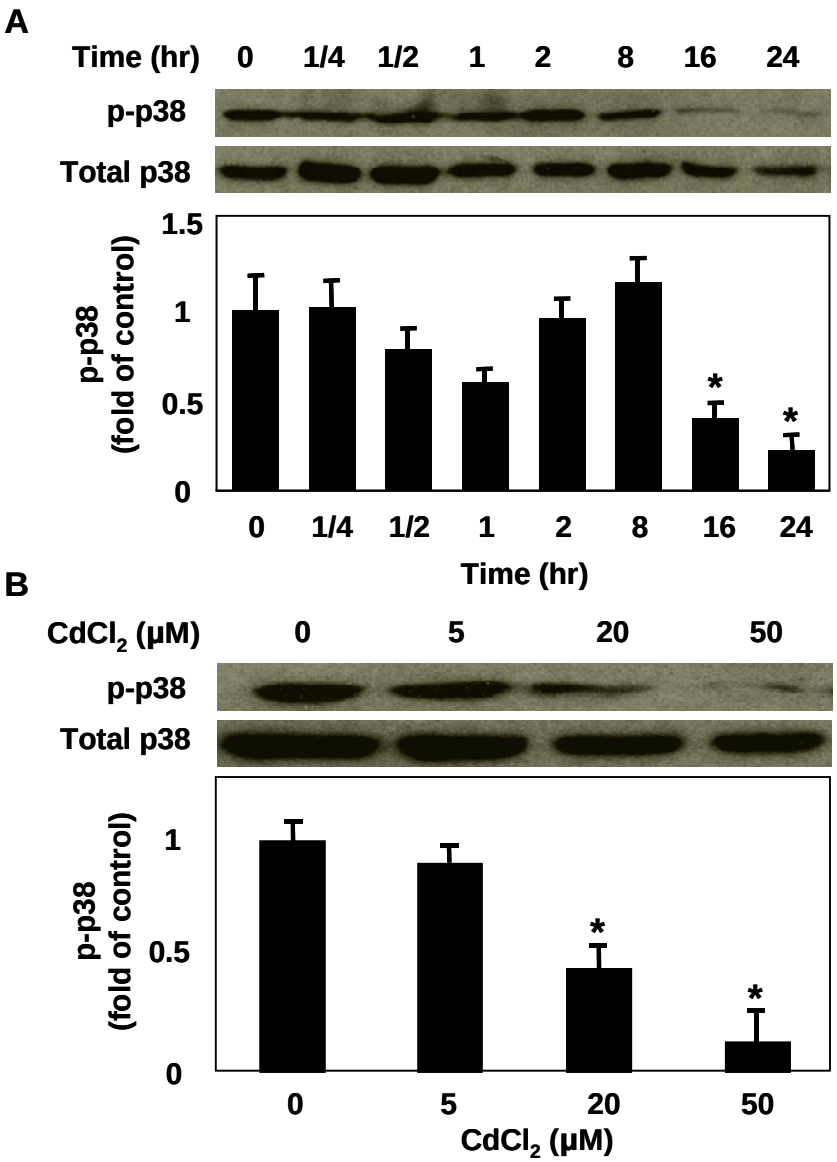

Fig. 4.3. Effect of $CdCl_2$ on phosphorylation of p38 MAPK. J774A.1 cells were exposed to $CdCl_2$ at indicated concentrations and time. The level of total and p-p38 MAPK was measured by western blot. P-p38 represents activated p38 whereas total p38 indicates total protein loading. (A) p38 activation with $CdCl_2$ 20 μM over time. (B) p38 activation after 16 h with different $CdCl_2$ concentrations. Mean + SE (n=3). * indicates significant difference compared to control group at $p < 0.05$ analyzed with one-way ANOVA followed by Duncan's multiple range test.

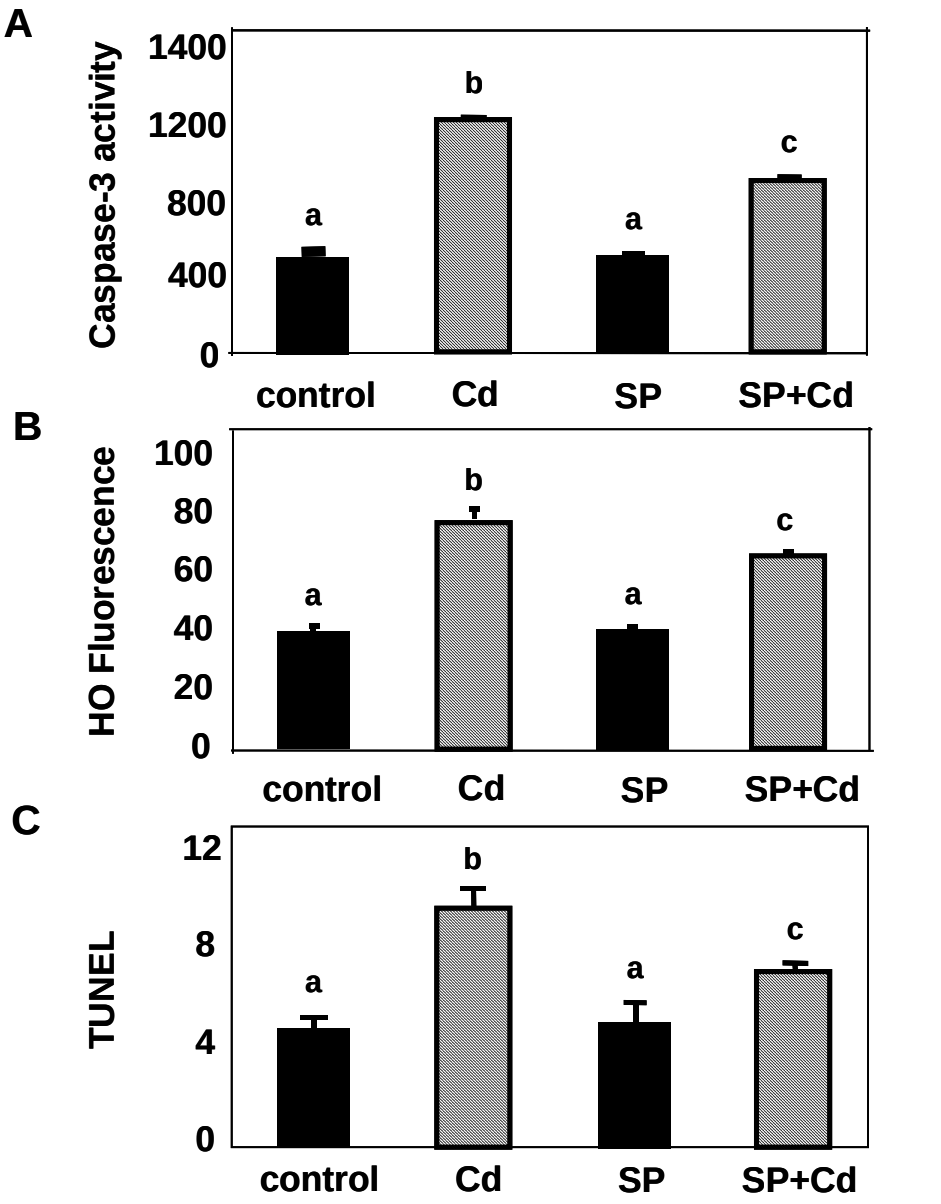
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Fig. 4.4. Effect of SP600125 on $CdCl_2$-induced caspase-3 activation and DNA fragmentation. J774A.1 cells were pre-treated with 20 μM SP600125 for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 h. (A) The intensity of fluorescence on caspase-3 enzyme activity was measured. (B) The intensity of fluorescence on apoptotic nuclei stained by Hoechst 33258 was read by microplate spectrofluorometer. (C) The intensity of fluorescence on labeled DNA strand breaks by TUNEL-reaction after $CdCl_2$ exposure was read by microplate spectrofluorometer. (D) Fluorescence on TUNEL positive nuclei was visualized under fluorescence microscope. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed with ANOVA followed by Duncan's multiple range test.

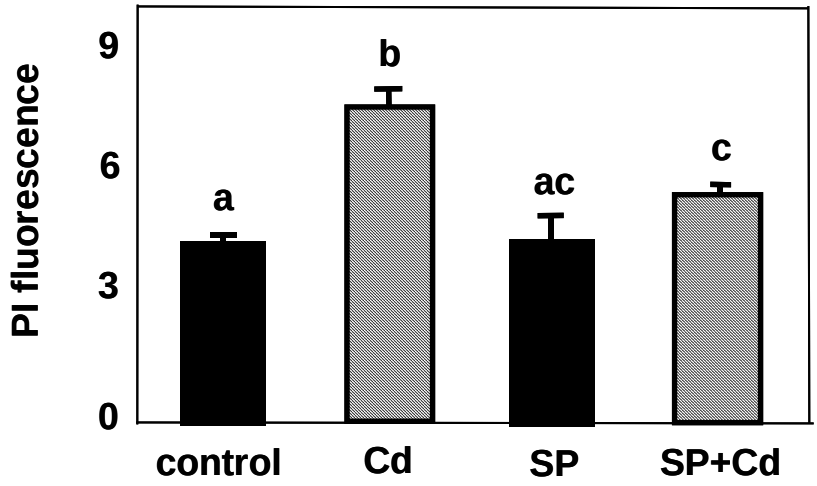
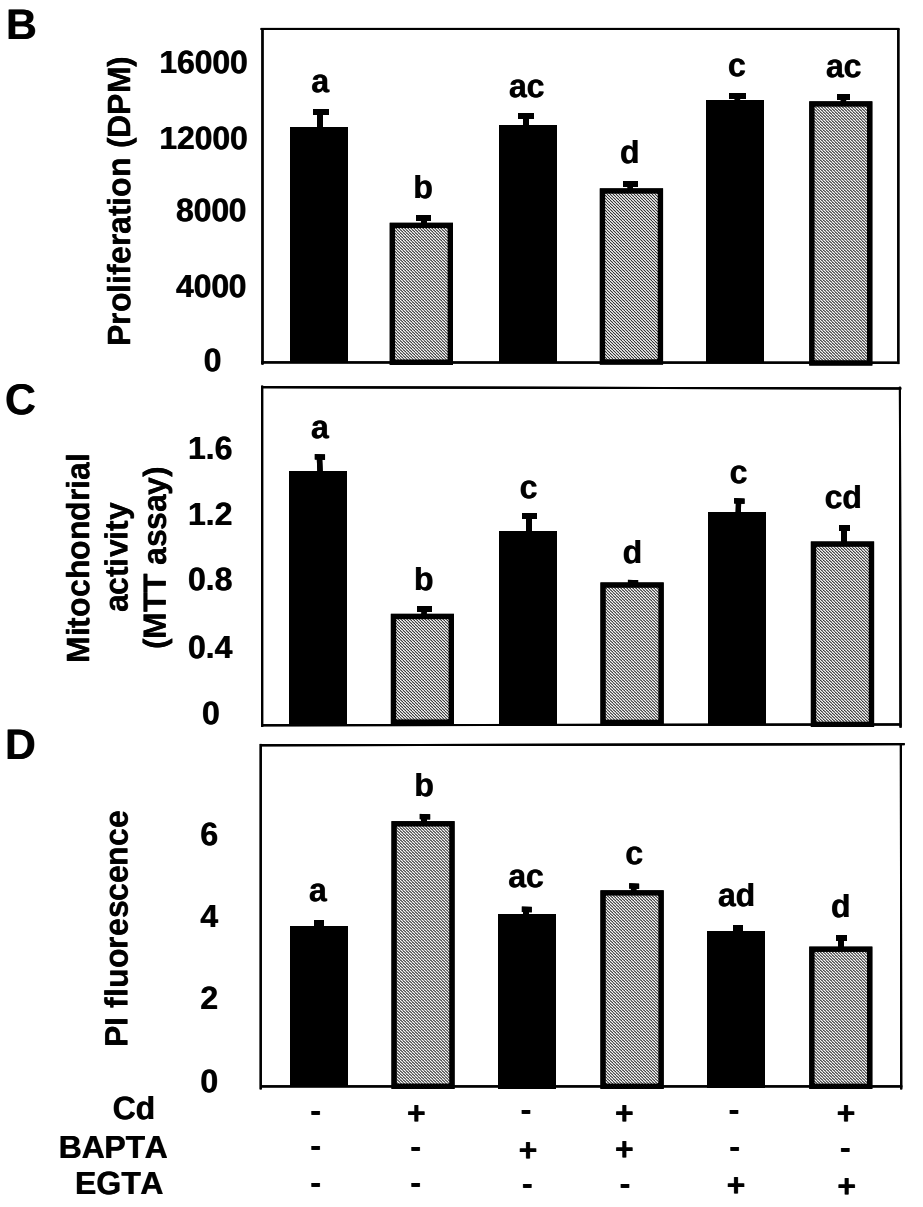


Fig. 4.5. Effect of SP600125 on $CdCl_2$-induced necrosis. J774A.1 cells were pre-treated with 20 μM SP600125 for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 h. The intensity of PI fluorescence after $CdCl_2$ exposure was read by microplate spectrofluorometer. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed with ANOVA followed by Duncan's multiple range test.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Mitochondrial activity (MTT assay)
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BAPTA
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- + - + - +
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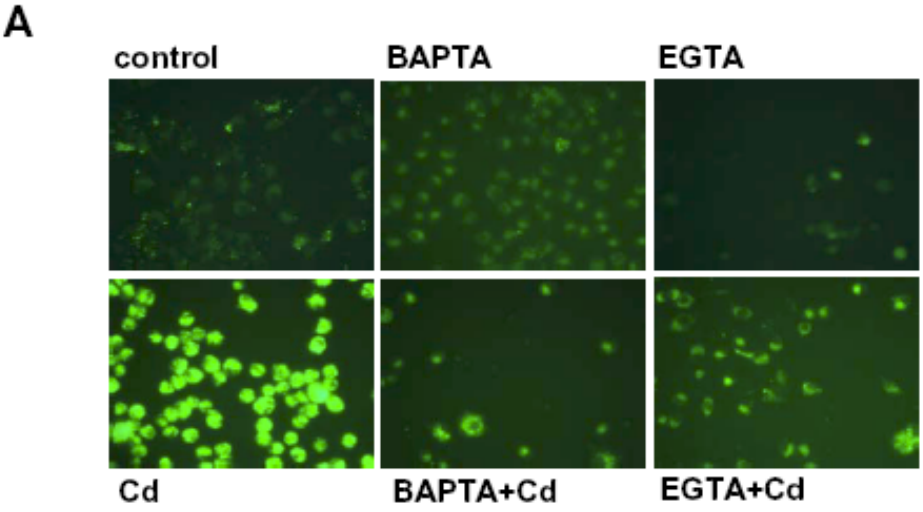
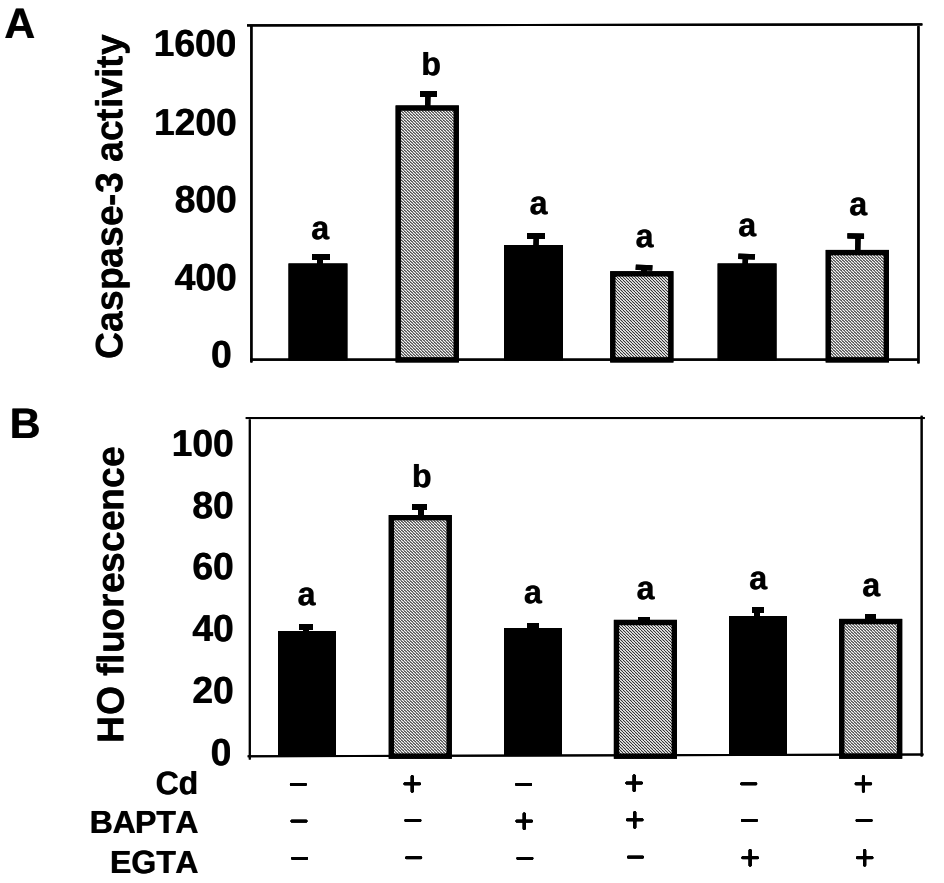

A
control
BAPTA
EGTA
Cd
BAPTA+Cd
EGTA+Cd

Fig. 4.6. Effect of BAPTA-AM and EGTA on $CdCl_2$–induced $[Ca^{2+}]_i$ elevation, growth arrest, mitochondrial activity impairment and necrosis. J774A.1 cells were pre-treated with BAPTA-AM (10 μM) or EGTA (1 mM) for 30 min and then exposed to $CdCl_2$ (20 μM). (A) Morphological fluorescence intensity of altered $[Ca^{2+}]_i$ level was visualized under fluorescence microscope using Fluo-3 after 6 h $CdCl_2$ exposure. Representative pictures from experiments that were replicated a minimum of three times are shown. (B) Proliferation was measured by [^{3}H]thymidine incorporation after 18 h $CdCl_2$ exposure. (C) Mitochondrial activity was measured by MTT assay after 24 h $CdCl_2$ exposure. (D) The intensity of PI fluorescence after 24 h $CdCl_2$ exposure was read by microplate spectrofluorometer. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed with ANOVA followed by Duncan's multiple range test.


A
Caspase-3 activity
1600
1200
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400
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a
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B
HO fluorescence
100
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20
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a
b
a
a
a
a
Cd
BAPTA
EGTA
C
control
BAPTA
EGTA
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BAPTA+Cd
EGTA+Cd

Fig. 4.7. Effect of BAPTA and EGTA on $CdCl_2$-induced caspase-3 activation and DNA fragmentation. J774A.1 cells were pre-treated with BAPTA-AM (10 μM) or EGTA (1 mM) for 30 min and then exposed to $CdCl_2$ (20 μM). (A) The intensity of fluorescence on caspase-3 enzyme activity was measured after treatment of cells with cadmium. (B) The intensity of fluorescence on apoptotic nuclei stained by Hoechst 33258 (HO) - by microplate spectrofluorometer. (C) The intensity of fluorescence on TUNEL positive nuclei - visualized under fluorescence microscope. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed with ANOVA followed by Duncan's multiple range test.

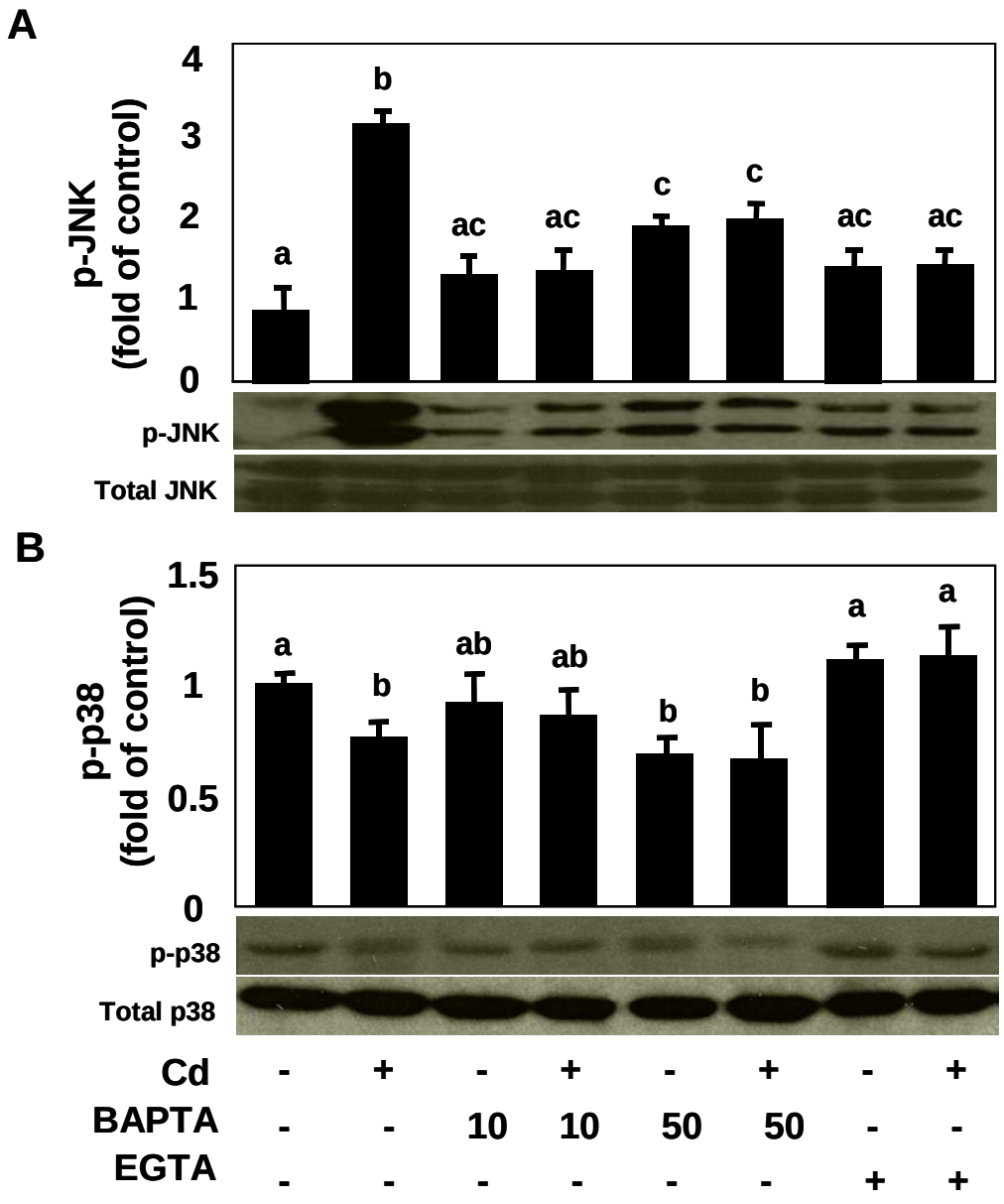

Fig. 4.8. Effect of BAPTA and EGTA on $CdCl_2$-induced JNK activation and down-regulation of p38. J774A.1 cells were pretreated with BAPTA-AM (10 and 50 μM) or EGTA (1 mM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 h. (A) Total and p-JNK (B) Total and p-p38 were measured by western blot after 16 h $CdCl_2$ exposure. Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed with ANOVA followed by Duncan's multiple range test.

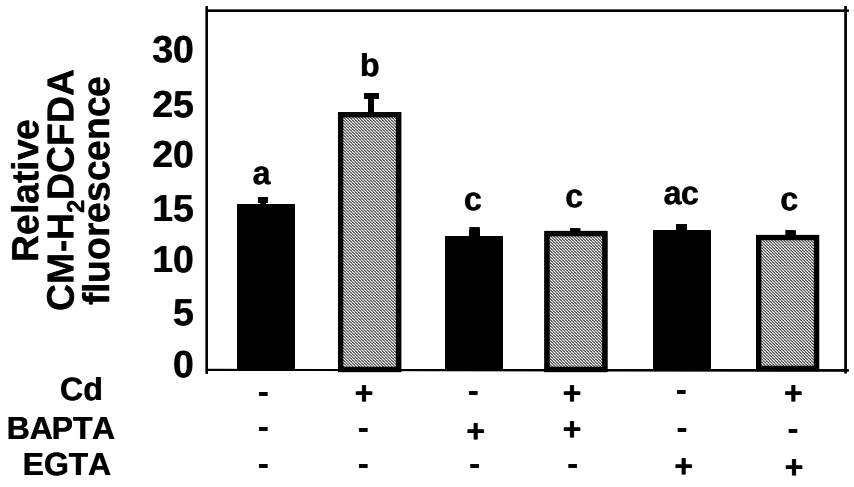

Fig. 4.9. Interrelationship of ROS and $[Ca^{2+}]_i$ elevation. Effect of BAPTA and EGTA on $CdCl_2$-induced H_2O_2. J774A.1 cells were pretreated with BAPTA-AM (10 μM) or EGTA (1 mM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 h. Fluorescence stained with H_2DCFDA was read by microplate spectrofluorometer. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$ analyzed with ANOVA followed by Duncan's multiple range test.

CHAPTER 5

CADMIUM-INDUCED APOPTOSIS IN MURINE MACROPHAGES: ROLE OF REACTIVE OXYGEN SPECIES AND CASPASE-3 ACTIVATION[3]

[3]Kim, J. and R.P. Sharma. To be submitted to *Toxicological Sciences*

Abstract

Cadmium is a toxic heavy metal accumulated in the environment, and commonly found in cigarette smoke and industrial effluent. Present study was designed to determine the time frame of hydrogen peroxide generation and apoptosis induced by cadmium and to investigate the role of oxidative stress and caspase-3 activation on cadmium-mediated cell signaling in J774A.1 murine macrophage cells. Cadmium-generated hydrogen peroxide was observed from 6 h and reverted to control level at 16 and 24 h. The hydrogen peroxide production was dose-related between 20-500 μM cadmium. Activation of caspase-3 was observed at 8 h and DNA fragmentation at 16 h by 20 μM cadmium, suggesting that caspase-3 activation is a prior step to DNA fragmentation in cadmium-induced apoptosis. Inhibitors of caspase-3, -8, -9 and general caspases suppressed cadmium-induced caspase-3 activation and apoptosis indicating the importance of caspase-3 on cadmium toxicity. Buffering against the oxidative stress with *N*-acetylcysteine (NAC) and silymarin (an antioxidant flavonoid) completely blocked cadmium-induced apoptotic response. The pretreatment of cells with NAC and silymarin recovered the cadmium-induced cell injury including growth arrest, mitochondrial impairment and necrosis as well as reduced the cadmium-elevated intracellular calcium ($[Ca^{2+}]_i$) indicating oxidative stress is a source of increased $[Ca^{2+}]_i$. NAC inhibited cadmium-induced activation of c-Jun NH_2-terminal protein kinase (JNK) and extracellular signal-related kinase (ERK) in a dose-dependent manner. However, silymarin showed partial inhibition of JNK activation and only the low concentration of silymarin totally inhibited ERK activation in response to cadmium. Inhibition of caspase-3 protected oxidative stress by cadmium, suggesting that the activation of caspase-3 can also modulate generation of reactive oxygen species.

Keyword: cadmium, ROS, MAPKs, caspase-3, apoptosis, cytotoxicity

Abbreviations used:

$[Ca^{2+}]_i$, intracellular calcium; CM-H_2DCFDA, 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; DPM, disintegrations per min; ERK, extracellular signal-related kinase; JNK, c-Jun NH_2-terminal kinase; MAPKs, mitogen-activated protein kinases; MTT, 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide; NAC, *N*-acetylcysteine; PI, propidium iodide; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling

Introduction

Cadmium is a naturally occurring nonessential and toxic heavy metal, which belongs to transition metal group IIB of the periodic table. Cadmium is found in plastic stabilizers, color pigments, several alloys and, now most commonly, in re-chargeable nickel-cadmium batteries (Jarup, 2003). Cigarette smoking and consumption of cadmium-contaminated water, air, and food are major sources of cadmium exposure in public health (Jarup, 2003). Cadmium and zinc belong to the same group of transition metals and appear to influence each other in the intracellular environment (Hamada et al., 1997). Besides zinc, cadmium interferes with the utilization of essential metals e.g. calcium, selenium, chromium and iron, and abolishes their function in biological system (Nath et al., 1984). Chronic obstructive kidney injuries were found in occupationally exposed workers, and epidemic cadmium exposure in Japan has been reported to result from a hazardous incident in which patients suffered from multiple bone fractures including osteomalacia and osteoporosis (Jarup, 2003). The International Agency for Research

on Cancer (IARC) has classified cadmium as carcinogenic to humans (Group 1), based on sufficient evidence for carcinogenicity in both human and animal studies (IARC, 1993).

Cadmium is able to damage both the humoral immune response and cell-mediated immune response based on substantial evidence, mostly from in vivo animal models (Descotes, 1992). J774A.1 cells are originated from BALB/c mouse reticulum cell sarcoma and commonly used as an alternative to primary macrophage because of similarities to mature macrophages (Yan et al., 2004). Studies using macrophages on cadmium-mediated cell signaling and subsequent cell death are valuable to understand cadmium effect on the immune system.

Cadmium-induced cytotoxicity involves apoptosis as a major mode of elimination of damaged cells (Habeebu et al., 1998). Up-regulation of intracellular signaling pathways leading to increased mitogenesis or apoptosis is thought to be a major mechanism for cadmium toxicity (Beyersmann et al., 1997). Cadmium does not seem to activate the endonucleases, which induce internucleosomal cleavage of DNA in vitro (Hamada et al., 1997). However, cadmium itself was associated with apoptosis through indirect oxidative stress by inhibition of antioxidant enzymes (Hamada et al., 1997). Metallothionein induced by cadmium played an important role not only as a cadmium carrier by which cadmium accumulates in the nucleus, but also as an inhibitor of zinc finger protein, which include transcriptional factors related to apoptosis (Hamada et al., 1997). Cadmium-induced c-myc, p53 and c-jun expression was suggested as a prelude to apoptosis in normal human prostate epithelial cells RWPE-1 (Achanzar et al., 2000). Stimulation of p38 mitogen-activated protein kinase (MAPK) was responsible for the cadmium-induced apoptosis in U-937 human promonocytic cells (Galan et al., 2000). C-Jun NH_2-terminal kinase (JNK) and p38 MAPK cooperatively participated in apoptosis induced by cadmium and the decreased extracellular signal-related kinase (ERK) signal induced by low cadmium doses

contributed to growth inhibition or apoptosis in a human non-small cell lung carcinoma cell line, CL3 (Chuang et al., 2000b). These contradictory results indicate that cadmium may induce different apoptotic pathways in different cell types. Therefore, the intracellular signaling pathway responsible for cadmium-induced apoptosis requires further characterization.

Cadmium has been also shown anti-apoptotic effect. Cadmium inhibited caspase-3 activation and DNA fragmentation induced by apoptotic agent (Yuan et al., 2000). Cadmium has been characterized as a caspase–3 inhibitor with IC_{50} values of approximately 9 μM in intact chinese hamster ovary CHO cells and 30 μM in cell-free extract system (Yuan et al., 2000). Cadmium-treated MRC-5 human fetal lung fibroblasts underwent caspase-independent apoptosis through mitochondria-reactive oxygen sepecies (ROS) pathway including mitochondrial membrane depolarization and translocation of apoptosis-inducing factor (AIF) from mitochondria into nucleus (Shih et al., 2004). In porcine kidney LLC-PK1 cells, caspase activity was not associated with cadmium-induced apoptosis because caspase inhibitors failed to rescue cells (Ishido et al., 1999). Cadmium did not significantly increase caspase-3 activity in liver cells of mouse model (Harstad et al., 2002).

In the present study, changes of H_2O_2 by cadmium were monitored as an upstream event prior to caspase-3 and MAPKs activation in J774A.1 murine macrophage cells. The objective of this study was to explore the time frame of H_2O_2 generation and apoptosis in response to cadmium, and to outline the mechanisms and consequences of such elevation in murine macrophages.

Materials and Methods

Reagents

Cell culture reagents were obtained from GIBCO Life Technology (Grand Island, NY). Cadmium ($CdCl_2$, Sigma Chemical Co., St. Louis, MO) was dissolved in water, sterilized with 0.22 μm filter, and added to cultures at the indicated time and concentrations. A set of caspase inhibitors, MAPK/ERK-activating kinase 1 (MEK1) inhibitor PD98059 and specific JNK inhibitor SP600125 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Antibodies specific for the total and phosphorylated forms of JNK (p54/46) and ERK (p44/42 MAPK) were procured from Cell Signaling (Beverly, MA). Fluorescent probes Fluo-3/AM and propidium iodide (PI) were purchased from Molecular probes (Eugene, OR). *N*-acetylcysteine (NAC), silymarin (product number: 254924), Hoechst 33258 and all other chemicals used in this study were obtained from Sigma and were of cell culture grade.

Cell culture

J774A.1 (American Type Culture Collection TIB-67), macrophage cell line was preserved in Dulbecco's Modified Eagle's Medium, added with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and 10% non-heat-inactivated fetal bovine serum (Atlanta Biologics, Atlanta, GA) in 5% CO_2 atmosphere at 37 ºC. The J774A.1 cells were grown in 75 cm^2 culture flasks and subcultured when the cells reached 70–80% confluence (every 3 days). Cells were used during 3rd or 4th passages. Cultures were allowed to grow overnight (15 h) as a monolayer prior to the treatment. The concentrations used for various reagents, added 30 min prior to cadmium treatment, were 20 μM for caspase inhibitors, 1 mM for NAC, 50 μM for silymarin, 20 μM for SP600125 and PD98059. The used concentrations of above agents were not cytotoxic to these cells.

ROS production determination

The production of H_2O_2 was measured by detecting the fluorescent intensity of oxidant-sensitive probe after adding 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes). The cells were incubated in the presence of various concentration of cadmium and fluorescent intensity recorded using SpectraMax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA). The CM-H_2DCFDA fluorescence, which specifically detects H_2O_2, was measured by excitation at 485 nm, and emission at 530 nm. The fluorescence readings were digitized using SoftMax Pro^{TM} (Molecular Devices, Irvine, CA).

Determination of caspase-3 activation

Caspase-3 activity was investigated using CaspACE^{TM} fluorometric activity assay (Promega, Madison, WI) with adjustments as follows. Briefly, cells were treated in 96-well microplates (Falcon, Becton Dickinson, Franklin Lakes, NJ) following which Triton X-100 was added and repeatedly pipetted to lyse the cells. The homogenates were centrifuged at 4,000×g for 10 min to remove cell debris. The supernatant was assayed for caspase-3 activities using CaspACE^{TM} system according to the manufacture's protocol. The plates were read at 360/460 nm (excitation/emission) using a SpectraMax Gemini. The fluorescence signal was digitized and analyzed using SoftMax Pro^{TM}.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay

TUNEL assay was carried through using the in situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Cells were plated at 8×10^4 cells/well in 96-well microplates

and allowed to attach overnight. Cells were then treated with cadmium for 24 h, fixed with paraformaldehyde, and analyzed for stained nuclei according to the manufacturer's instructions. The fluorescence signal was read by SpectraMax Gemini, digitized and analyzed using SoftMax Pro™. In addition, fluorescence microscopy was performed to examine fragmented DNA morphology using IX71 inverted microscope (Olympus America, Melville, NY). Digital images were captured using MagnaFire SP® Olympus digital camera.

Hoechst and PI 33258 staining

Apoptotic morphological transform in the nuclear chromatin of cells were revealed by staining with the DNA binding fluorochrome Hoechst 33258 (bis-benzimide). PI is a membrane impermeable dye, which binds to DNA by intercalating between the bases with little or no sequence preference and with a stochiometry of one dye per 4-5 base pairs of DNA. Dead cells are PI-bright and live cells are PI-dim. Following 24 h treatment with cadmium, 120 µl of supernatant from 200 µl total medium was removed and 20 µl of Hoechst 33258 (2 µg/ml) or PI (1 µg/ml) was added. The plates were read at 350/450 nm or 535/617 nm (excitation/emission) for Hoechst 33258 or PI fluorescence, respectively, using a SpectraMax Gemini. The fluorescence signal was digitized and analyzed using SoftMax Pro™.

DNA synthesis as an index of proliferation

The [methyl-^{3}H]thymidine incorporation assay was employed as an indicator of proliferation. Cells were seeded at 8×10^4 cells/well in 96-well microplates. At 16 h prior to harvesting cells, each well was pulsed with 20 µl of [methyl-^{3}H]thymidine (25 µCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA). Cells were harvested onto glass fiber filter paper (Cambridge Technology, Watertown, MA) using a cell harvester (PHD, Cambridge Technology). Proliferative response (uptake of [^{3}H]thymidine) in the harvested cells was

counted in a liquid scintillation counter (Pharmacia, Turku, Finland) and expressed as net disintegrations per min (DPM).

Mitochondrial activity

MTT (3[4,5-dimethyl thiazolyl-2]2,5-diphenyl tetrazolium bromide, Sigma) assay was performed to observe mitochondrial activity. Cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with the indicated concentration of cadmium for 24 h. The cells were incubated with addition of 20 µl MTT (5 mg/ml). After 4 h, 120 µl of MTT media was taken out from each well and 100 µl of 0.02 N HCl-isopropanol (warm) added to dissolve formazan crystals. The absorbance of each well was measure by UV spectrometer at 570 nm.

Determination of intracellular Ca^{2+}

Intracellular Ca^{2+} levels were checked by Fluo-3, which is a Ca^{2+}-sensitive fluorescent indicator. Cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with indicated time and concentration of cadmium. Cells were loaded with Fluo-3/AM (10 µM) in dark at 37°C for 1 h and washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 12 mM $NaHCO_3$, and 5.5 mM Glucose). Cells were incubated in Tyrode's solution for another 30 min and the morphological fluorescence intensity of cells was determined using Olympus IX71 inverted microscope. A 488 nm excitation wavelength was used to illuminate Fluo-3, and fluorescence was detected at emission wavelength of 510 nm. Digital images were acquired using the Magnafire SP (Olympus) digital camera.

Western blot analysis of phosphorylated JNK and ERK

The phosphorylated form (activation status) of JNK and ERK was measured using phospho-specific antibodies as described previously (Johnson *et al.*, 2003). Cells were grown at

2×10^6 cells/well in 6-well microplates and treated with cadmium for indicated time and concentrations. Following treatment, cells were washed with phosphate buffered saline (PBS) and total cell lysates were prepared by scrapping in 100 µl of lysis buffer [20 mM Tris–HCl (pH 8.0), 1 mM sodium orthovanadate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetate (EDTA), 1% Triton X-100, 50 mM β-glycerolphosphate, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin]. Fifty micrograms of proteins determined by Bradford assay was electrophoretically separated using a 12% SDS-PAGE gel and transferred to nitrocellulose paper followed by antibody staining. Equal loading and transfer of total protein were verified with the reversible Ponceau S stain (Sigma) dye and also by detecting total JNK and ERK. Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

Replication and statistical analysis

Means ± SD from representative experiments have been presented. Experiments were repeated at least 3-4 times with consistent results. All statistical analyses were performed using the SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A p value of < 0.05 was considered significant unless indicated otherwise.

Result

Cadmium-induced ROS generation

To examine the time frame of ROS generation by cadmium, we measured the level of H_2O_2 using the indicator CM-H_2DCFDA. After cells were loaded with CM-H_2DCFDA, the fluorescent intensity of CM-H_2DCFDA was detected by fluorescence plate reader. Time-course

study on elevated H_2O_2 level was performed for 24 h with various cadmium concentrations (Fig. 1A). Cadmium at 20 μM slightly increased H_2O_2 at 6 h, peaked at 10 h and this elevated H_2O_2 tended to decrease to basal level after that time. Higher concentrations of cadmium at 100 and 500 μM showed much stronger fluorescence intensity than that at the lower concentration; the intensity also peaked earlier. The level of H_2O_2 generated by 5-500 μM cadmium was compared with the baseline level of H_2O_2 in control cells at 6 h (Fig. 1B). As a positive control 1 mM Fe^{2+} was used. The changes in fluorescent intensity of H_2O_2 showed concentration-dependence at 6 h.

Cadmium-induced caspase-3 activation and apoptosis

Cadmium-induced apoptosis was studied using caspase-3 activation and TUNEL assay (Fig. 2). In the time-course study, it was observed that a significant increase of caspase-3 activation of apoptotic cells started at 8 h, peaked at 16 h and tended to decrease after that (Fig. 2A). DNA fragmentation determined by TUNEL assay also significantly increased at 16 h and later decreased (fig. 2C). The concentration-response of cadmium-induced apoptosis was evaluated at 16 h (Fig. 2B and D). Cadmium treatment at 20 and 50 μM showed 2 and 3-fold increased caspase-3 activation and TUNEL response over control. The micrographs of TUNEL staining confirmed that cadmium-induced apoptosis is concentration-dependent (Fig. 2E).

Caspase inhibitors on inhibition of cadmium-induced apoptosis

To determine which kinds of caspases are involved in apoptosis induced by cadmium, a series of inhibitors, caspase-3 inhibitor (Z-DEVD-FMK), caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK), and general caspase inhibitor (Z-VAD-FMK) at 20 μM were used. Caspase-3 was activated by 20 μM cadmium at 16 hr. Pretreatment with caspase-3, -8, -9 inhibitors and general caspase inhibitor totally inhibited cadmium-induced caspase-3 activation (Fig. 3A). Fragmented DNA was measured by staining nucleus with Hoechst 33258

(Fig. 3B). Similarly, caspase-3, -8, -9 inhibitor and general caspase inhibitor were significantly able to suppress cadmium-induced DNA fragmentation indicating the important role of caspase-3 activation and involvement of sequential activation of caspase-8 and caspase-9 on cadmium-induced apoptosis.

Role of ROS on cadmium-induced caspase-3 activation and apoptosis

To determine whether cadmium-induced caspase-3 activation and apoptosis depends on ROS generation, cells were treated with two different types of antioxidants (Fig. 4). NAC is a precursor of glutathione with antioxidant activity and silymarin is a mixture of natural flavonoids, which has been shown to scavenge free radicals and to inhibit lipid peroxidation (Middleton E Jr et al., 2000). J774A.1 cells were incubated with cadmium 20 μM in the absence or presence of 1 mM NAC or 50 μM silymarin. Both NAC and silymarin showed high inhibition of H_2O_2 generation by cadmium (Fig. 4A). Apoptotic effects were assayed by caspase-3 activity and TUNEL at 16 h. The levels of caspase-3 activity and TUNEL staining were similar in NAC or silymarin-treated or control cultures, but it was significantly decreased in cultures treated with cadmium and NAC or silymarin than in cultures treated with cadmium-only (Fig. 4B-D). This suggested that ROS generation is involved in cadmium-induced caspase-3 activation and apoptosis.

Role of ROS on cadmium-induced growth arrest, mitochodrial activity, necrosis and $[Ca^{2+}]_i$ elevation

To verify the role of oxidative stress generated by cadmium, cells were pre-loaded with 1 mM NAC or 50 μM silymarin for 30 min. Results presented in Figure 5A-B demonstrate that NAC or silymarin pretreatment suppressed cadmium-induced growth arrest and mitochondrial impairment. NAC or silymarin was able to recover mitochondrial activity indicating the

importance of oxidative stress on mitochondrial function. Inhibitory effect of antioxidants on cadmium-induced necrotic cell death examined by PI staining is shown in Fig. 5C. Our previous study reported that cadmium-induced intracellular calcium ($[Ca^{2+}]_i$) regulates growth arrest and cell death to modulate cadmium toxicity (Kim et al., 2004). We examined whether generated ROS by cadmium was related to elevated $[Ca^{2+}]_i$ using the calcium indicator of Fluo-3. After cells were loaded with Fluo-3/AM, the fluorescent intensity of Fluo-3 was detected by fluorescence microscope. As shown in Fig. 5D, antioxidant NAC and silymarin prevented $[Ca^{2+}]_i$ elevation, demonstrating that ROS generation is essential for cadmium-induced $[Ca^{2+}]_i$ overloading.

Inhibitory effect of antioxidants on cadmium-induced MAPKs activity

To delineate the further signaling pathways of elevated ROS by cadmium, we examined the phosphorylation of MAPKs (JNK and ERK) in J774A.1 cells. In J774A.1 cells treated with 20 μM cadmium, the levels of phosphorylated forms of p54 (JNK2) and p46 (JNK1) increased clearly at 16 h. Western blot analyses revealed that the activation of JNK was blocked by NAC in a concentration-dependent manner (Fig 6A). The immunoblot with phosphorylated form of JNK specific antibody revealed that silymarin significantly, but not completely, inhibited cadmium-induced JNK activation (Fig 6B). The levels of total (phosphorylation state-independent) JNK were not changed after cadmium or antioxidant treatments. Besides the activity of JNK inhibited by antioxidants, activated ERK was also observed with 1 mM NAC or 50 μM silymarin treatment. After incubation with 20 μM cadmium for 16 h, the apparent two bands of phosphorylated ERK (p44 and p42) appeared. Cadmium-induced ERK activation was also inhibited by NAC in a dose-dependent manner. Silymarin was significantly able to decrease cadmium-induced activation of ERK (Fig. 7).

Role of activated caspase-3 and MAPKs by cadmium on ROS generation

To examine the relationship between cadmium-induced activation of caspase-3/JNK/ERK and ROS generation, we employed caspase-3 inhibitor (Z-DEVD-FMK), specific JNK inhibitor anthrapyrazolone (SP600125, (Bennett et al., 2001) and MEK1 inhibitor PD98059. J774A.1 cells were incubated with cadmium 20 μM in the absence or presence of 20 μM of inhibitors and assayed using the indicator CM-H_2DCFDA. The levels of H_2O_2 were similar in caspase-3 inhibitor (Z-DEVD-FMK)-treated or control cultures, but it significantly decreased in cultures treated with cadmium and Z-DEVD-FMK than in cultures treated with cadmium-only (Fig. 8A). Inhibition of cadmium-induced activation of JNK and ERK was not able to reduce oxidative stress (Fig. 8B), suggesting that these MAPKs are involved later in apoptosis but do not influence the H_2O_2 generation. The activation of caspase-3 somehow appeared to regulate H_2O_2 generation in these cells.

Discussion

Results presented here indicate that cadmium generated ROS in a dose-dependent manner and elevated oxidative stress played a critical role in the apoptotic suicide of cells. Cadmium strongly stimulated H_2O_2 after 6 h exposure to J774A.1 murine macrophage cells and this stimulation persisted until 10 h. In our previous studies, cadmium at 20 μM has shown the induction of $[Ca^{2+}]_i$ from 6 h, activation of JNK and ERK at 16 h, and G2/M cell cycle arrest and apoptosis at 24 h (Chapter 1 and 2). The level of H_2O_2 produced by cadmium started to decrease after 10 h and became similar to control at 16 and 24 h, suggesting that the production of H_2O_2 is an initial step of cadmium-mediated cellular signaling. The time-frame of activation of apoptosis-inducing mediator caspase-3 and DNA fragmentation by cadmium was observed.

Antioxidation by NAC or silymarin prevented the cadmium-induced H_2O_2 generation, hampered mitochondrial activity, caspase-3 activation and apoptosis, confirming that oxidative stress is an early signal to cadmium-induced cellular damage. The antioxidant flavonoid silymarin inhibited cadmium-elevated $[Ca^{2+}]_i$ and chelating $[Ca^{2+}]_i$ reduced H_2O_2 generation in previous study, indicating that ROS act in concert with $[Ca^{2+}]_i$ signaling. The activation of JNK and ERK was ROS dependent and may serve as a death signal in cadmium-induced apoptosis. N-acetylcysteine, a precursor of glutathione, dose-dependently inhibited JNK and ERK. High concentration of NAC completely inhibited both MAPKs. On the other hand, silymarin, a scavenger of free radicals, showed partial inhibition of JNK activation and only low concentration of silymarin showed total inhibition of ERK, suggesting the activation of JNK may be less influenced by free radicals.

Cadmium is known to induce the production of hydroxyl radicals (O'Brien et al., 1998), superoxide anions, nitric oxide and hydrogen peroxide (Galan et al., 2001; Stohs et al., 2001). In this study, cadmium at 20 μM showed reduction on the amount of H_2O_2 production at the very early time point of 2 h. The level of cellular glutahione and metallothionein increases after treatment of toxic concentration of cadmium (Beyersmann et al., 1997; Son et al., 2001). A compensatory defense mechanism by antioxidation system may be involved in the reduction of H_2O_2 by cadmium as an initial response. However, after 6 h cadmium enormously increased the level of H_2O_2 in a dose-dependent manner, suggesting that protective antioxidation mechanism is compromised by cadmium damage at later time points.

Generation of H_2O_2, activation of caspase-3 and DNA fragmentation by cadmium were serially observed in J774A.1 macrophages. Inhibitors of caspase-3, -8, -9 and general caspases were completely able to inhibit cadmium-induced apoptosis suggesting that both caspase-8 and

caspase-9 are involved as initiator caspases to converge in the activation of caspase-3 and to execute apoptotic death in macrophages. The importance of activation of caspase-8 and –9 on cadmium-induced apoptosis has been reported in few studies. Caspase-8 and –3 inhibitors were able to inhibit cadmium-induced apoptosis in human histiocytic lymphoma cell line U937 (Li et al., 2000). Caspase-8 inhibitor was more effective than caspase-3 inhibitor in this system and inhibitor of caspase-9 failed to inhibit cadmium-induced apoptosis, suggesting that caspase-8 is the most apical caspase in cadmium-induced apoptosis in this system (Li et al., 2000). Caspase-9 is known to be intermediated with mitochondria and cytochrome c (Nunez et al., 1998). Caspase-9 activation was partially involved in cadmium-induced apoptosis in HL-60 human leukemia cells (Kondoh et al., 2002) and C6 rat glioma cells (Watjen et al., 2002). If caspase-8 and caspase-9 execute independently to lead activation of caspase-3, inhibition effect should be partial on caspase-3 activation. Caspase-8, known as a receptor-mediated caspase (Nunez et al., 1998), not only directly cleaves and activates caspase-3 (Muzio et al., 1998) but also indirectly activates caspase-3 by inducing cytochrome c release (Bossy-Wetzel et al., 1999). Indirect mechanism of caspase-8 leading to cytochrome c release may be a factor to induce caspase-9 activation in cadmium-insulted macrophage cell line.

Antioxidants, NAC and silymarin were very effective repressors to reduce the amount of H_2O_2 and cell death produced by cadmium in J774A.1 cells. These antioxidants were able to prevent inhibition of proliferation and mitochondrial activity. Oxidative stress, altered redox homeostasis, and injuries to organelles were suggested as the mechanism of cadmium-induced toxicity (Son et al., 2001). Major targets of oxidative stress are nucleus and mitochondria, resulting in damage to membrane lipids, protein enzymes and deletion or modification of DNA (Richter et al., 1998; Sauer et al., 2001). ROS may involve direct interaction with specific

receptors and signaling pathways such as protein kinases, protein phosphatases and transcription factors from the plasma membrane to the cell nucleus during cell growth and differentiation (Sauer et al., 2001). The exact signaling pathways from ROS elevation to growth arrest are largely unknown. However, oxidative stress leading to $[Ca^{2+}]_i$ elevation and alterations in mitochondrial and nuclear function, is thought to be a major event in cadmium-mediated growth arrest.

Antioxidant flavonoid silymarin could inhibit cadmium-elevated $[Ca^{2+}]_i$ in our system in murine macophages. Hydrogen peroxide-induced $[Ca^{2+}]_i$ release from intracellular stores such as mitochondria or nucleus was also observed in Chinese hamster V79 cells (Inanami et al., 1999). On the other hand, high $[Ca^{2+}]_i$ can cause disruption of mitochondrial Ca^{2+} equilibrium which results in ROS formation due to the stimulation of electron flux along the electron transport chain (Chacon et al., 1991) suggesting a close relationship between Ca^{2+} and ROS in signal transduction pathways. Besides the direct interaction of cadmium with intracellular molecules, altered redox system or $[Ca^{2+}]_i$ homeostasis is considered as a target of toxic action by cadmium. Because calcium homeostasis was important to cadmium-mediated cell signaling including activity of MAPKs and cell death in previous study, interacting oxidative stress must be also a crucial factor for phosphorylation of MAPKs and apoptosis in J774A.1 macrophages.

MAPKs are believed to be important biomolecules in cell differentiation, cell movement, cell division and cell death induced by extracellular stimuli (Schaeffer et al., 1999). We recently reported that Ca^{2+} elevation by cadmium triggers JNK and caspase-3 activation (Kim et al., 2004) and cadmium induced ERK signaling-dependent G2/M arrest and cell death in murine macrophages by cadmium (Kim et al., 2003). JNK activity can be induced by either antioxidants in Jurkat T lymphocytes (Gomez et al., 1996) or H_2O_2 in HeLa (Wang et al., 1998) and CL3 cells

(Chuang et al., 2000a), suggesting that JNK signaling is sensitive to a decreased or increased oxidative environment. Recent evidence suggests that ROS-JNK pathway may play an important role in triggering apoptosis and signaling related to mitochondria. JNK was activated via ROS by alkaloid ascididemin in Jurkat T cells where JNK-specific inhibitor SP600125 inhibited caspase-2 and –9 processing as well as cytochrome c release and DNA fragmentaion (Dirsch et al., 2004). β-adrenergic receptors-stimulated apoptosis in adult rat ventricular myocytes involved ROS-JNK dependent activation of the mitochondrial death pathway (Remondino et al., 2003). ERK played critical role in cell survival following oxidative injury (Guyton et al., 1996). Hydrogen peroxide induced apoptosis of chondrocytes and involved calcium ion and ERK (Asada et al., 2001) suggesting that oxidative stress induced ERK activation has both pro-survival and pro-apoptotic effect.

The production of ROS has been thought to adversely affect the physiology and survival of cadmium-insulted cells. Now there is a growing body of evidence to suggest that cadmium-generated ROS can influence the growth as well as death of murine macrophages in vitro through the activation of multiple signaling pathways that influence the cytotoxicity observed in affected cells including MAPKs and caspases. The amount of H_2O_2 produced by cadmium was affected by the presence of caspase-3 inhibitor, but not by inhibitors of MAPKs, indicating that the activation of caspase-3 is an important source for cadmium-elevated ROS generation and MAPKs are activated as downstream of oxidative stress. The modulation of macrophage function by cadmium via generation of ROS is a likely mechanism of immunotoxicity by this metal.

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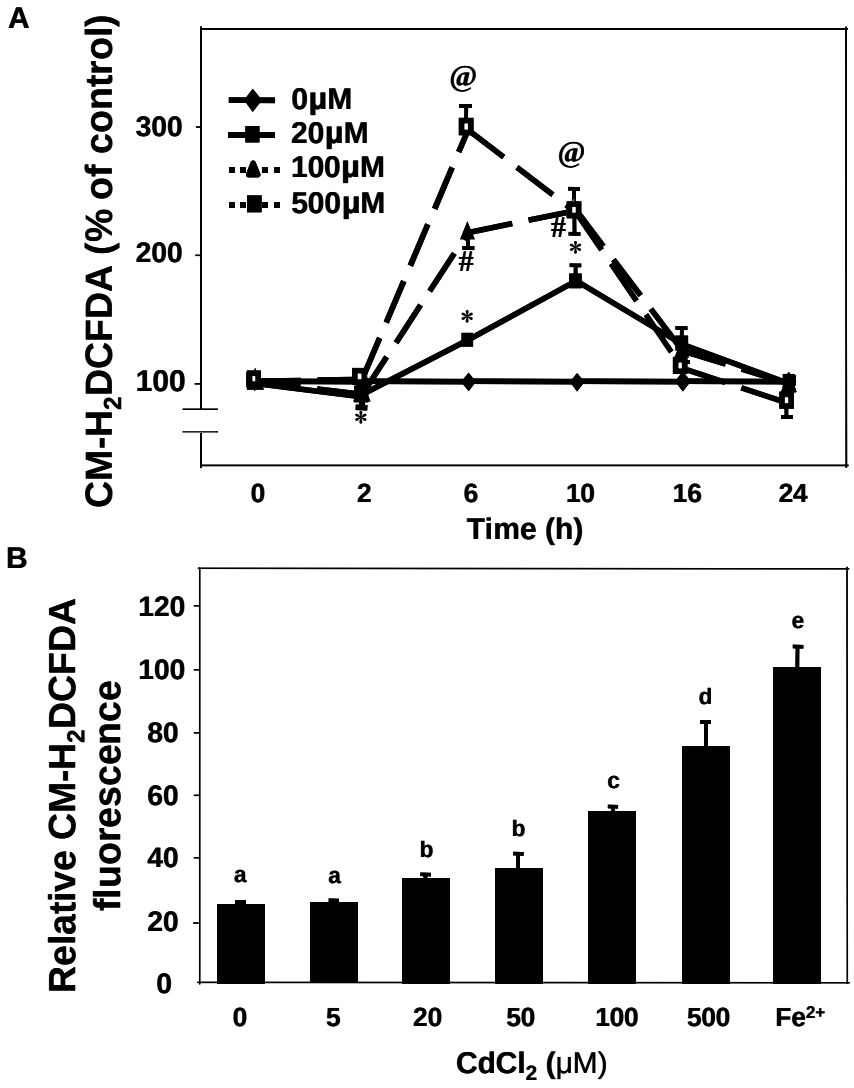


Fig. 5.1. Effect of $CdCl_2$ on H_2O_2 generation. The production of H_2O_2 was measured by detecting the fluorescent intensity of oxidant-sensitive probes CM-H_2DCFDA. J774A.1 cells were exposed to $CdCl_2$ at 0, 20, 100 and 500 µM $CdCl_2$ up to 24 hr (A) or at several concentrations for 6 hr (B). Iron ($FeSO_4$, 1 mM) was used as a positive control. Fluorescence by CM-H_2DCFDA after $CdCl_2$ exposure was read by microplate spectrofluorometer. *, # and @ indicate significant difference from control group at $p < 0.05$. Different letters on top of bars indicate significant difference at $p < 0.05$. Results are expressed as mean + or - SE (n=3).

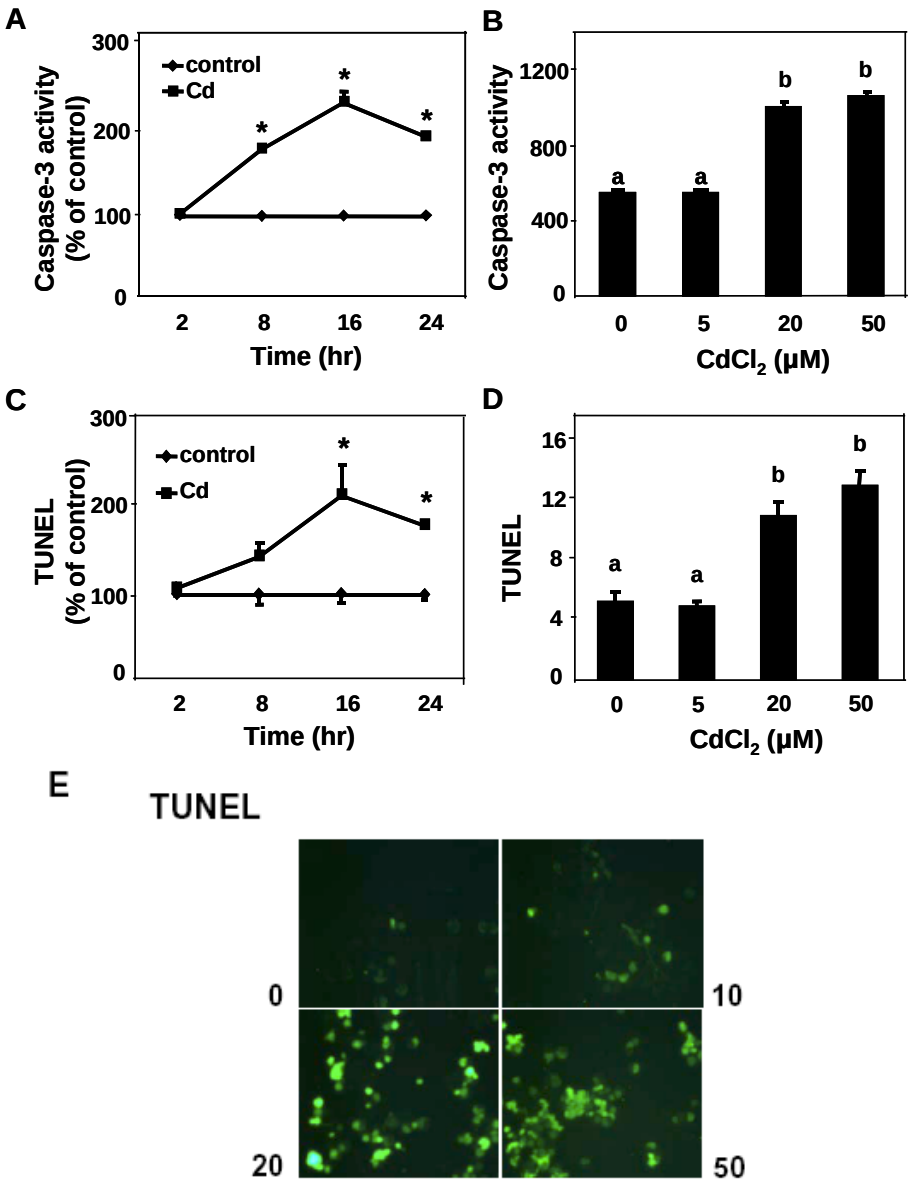


Fig. 5.2. Effect of $CdCl_2$ on caspase-3 activation and DNA fragmentation by TUNEL. J774A.1 cells were exposed to $CdCl_2$ at 20 μM for different time points (A, C) or at several concentrations for 16 hr (B, D). Fluorescence by caspase-3 activation (A-B) and TUNEL (C-D) after $CdCl_2$ exposure was read by microplate spectrofluorometer. (E) Fluorescence on TUNEL positive nuclei was visualized under fluorescence microscope. * indicates that significant difference from control group at $p < 0.05$. Different letters on top of bars indicate significant difference at $p < 0.05$. Results are expressed as mean + SE (n=3).

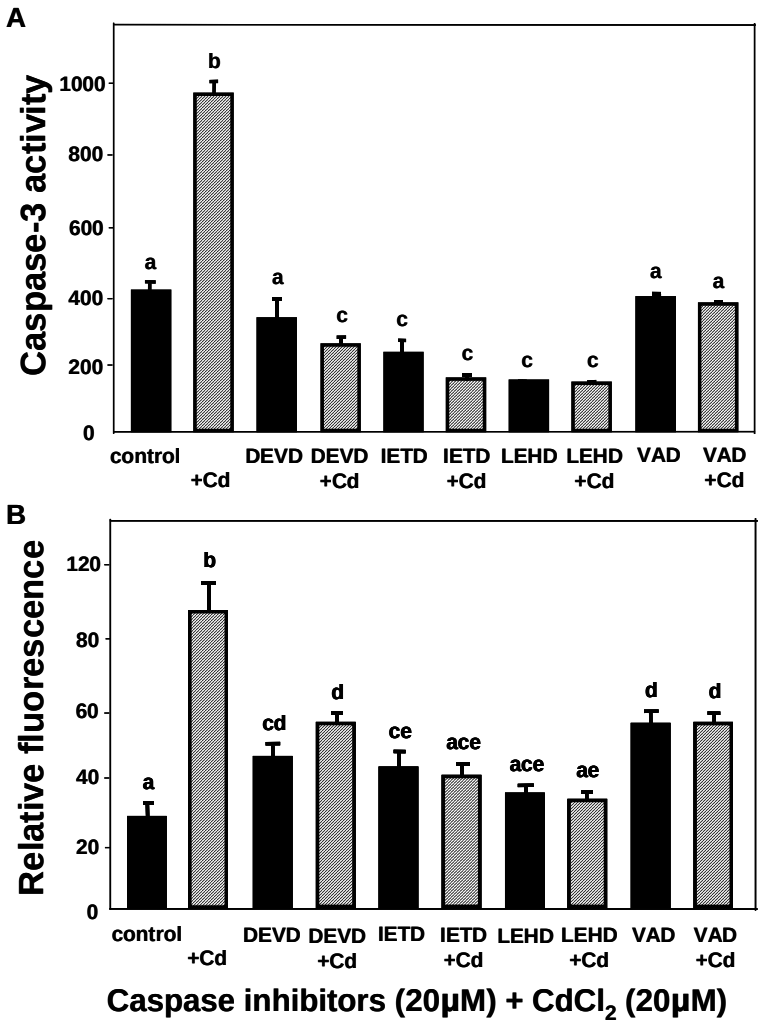
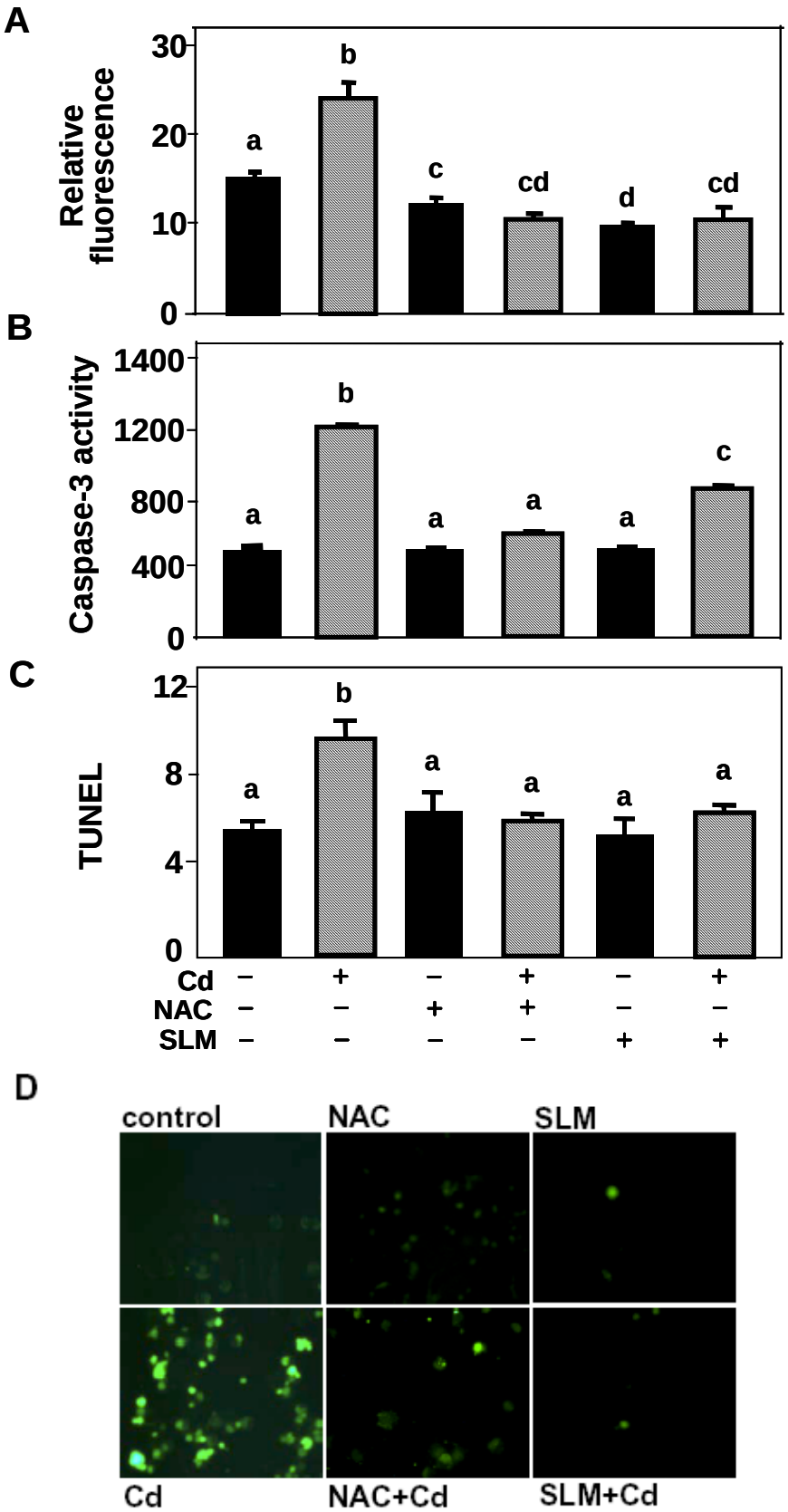


Fig. 5.3. Inhibition of caspases on $CdCl_2$-induced caspase-3 activation and apoptosis. J774A.1 cells were pre-treated with various caspase inhibitors (20 μM) for 30 min and then exposed to $CdCl_2$ at 20 μM for 16 hr. Abbreviation DEVD is used for caspase-3 inhibitor, IETD for caspase-8 inhibitor, LEHD for caspase-9 inhibitor, and VAD for general caspases inhibitor. (A) Fluorescence by caspase-3 activation after $CdCl_2$ exposure was read by microplate spectrofluorometer. (B) Fluorescence on apoptotic cells measured by microplate spectrofluorometer with Hoechst 33258 staining at 346/460nm. Different letters on top of bars indicate significant difference at $p < 0.05$. Results are expressed as mean ± SE (n=3).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Caspase-3 activity
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TUNEL
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NAC – – + + – –
SLM – – – – + +
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control
NAC
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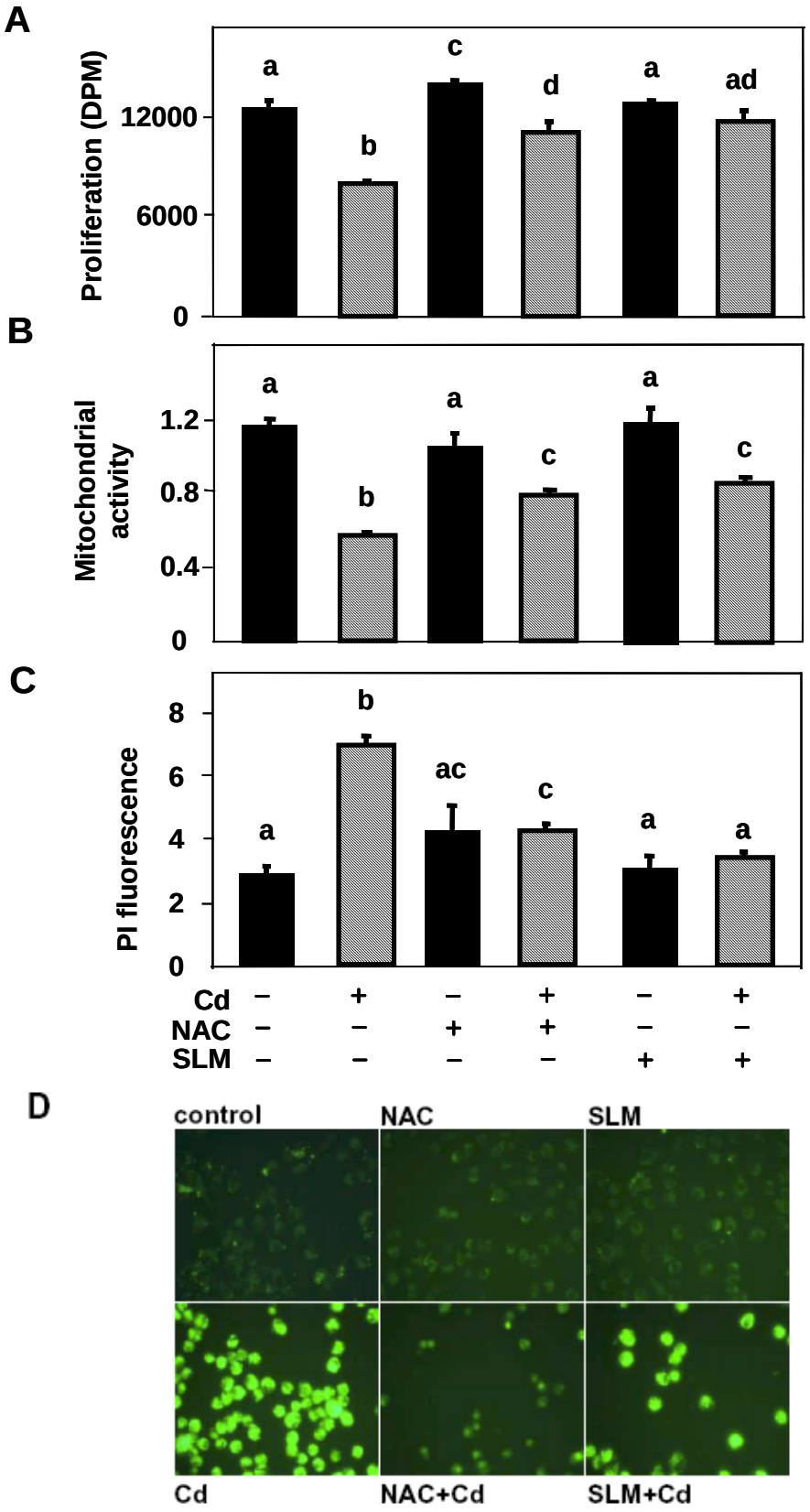
Cd
NAC+Cd
SLM+Cd

Fig. 5.4. Effect of antioxidatns on $CdCl_2$-induced caspase-3 activation and DNA fragmentation. J774A.1 cells were pre-treated with 1 mM NAC or 50 μM silymarin (SLM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 h. (A) The production of H_2O_2 was measured by detecting the fluorescent intensity of oxidant-sensitive probes CM-H_2DCFDA. (B) Fluorometric caspase-3 enzyme activity was measured. (C) Relative fluorescence of labeling DNA strand breaks by TUNEL-reaction after $CdCl_2$ exposure was read by microplate spectrofluorometer. (D) Fluorescence on TUNEL positive nuclei was visualized under fluorescence microscope. Mean + SE (n=3). Different letters on top of bars indicate a significant difference ($p < 0.05$).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Mitochondrial activity
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C
PI fluorescence
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6
4
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a
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c
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Cd − + − + − +
NAC − − + + − −
SLM − − − − + +
D
control
NAC
SLM
Cd
NAC+Cd
SLM+Cd

Fig. 5.5. Effect of antioxidants on $CdCl_2$–induced growth arrest, mitochondrial activity impairment, necrosis and $[Ca^{2+}]_i$ elevation. J774A.1 cells were pre-treated with 1 mM NAC or 50 μM silymarin (SLM) for 30 min and then exposed to $CdCl_2$ (20 μM). (A) Proliferation was measured by [^{3}H]thymidine incorporation after 18 h $CdCl_2$ exposure. (B) Mitochondrial activity was measured by MTT assay after 24 h $CdCl_2$ exposure. (C) Fluorescence by PI staining after 24 h $CdCl_2$ exposure was read by microplate spectrofluorometer. (D) Morphological fluorescence intensity of altered $[Ca^{2+}]_i$ level was visualized under fluorescence microscope using Fluo-3 after 6 h $CdCl_2$ exposure. Representative pictures from experiments that were replicated a minimum of three times are shown. Mean + SE (n=3). Different letters on top of bars indicate significant difference at $p < 0.05$.

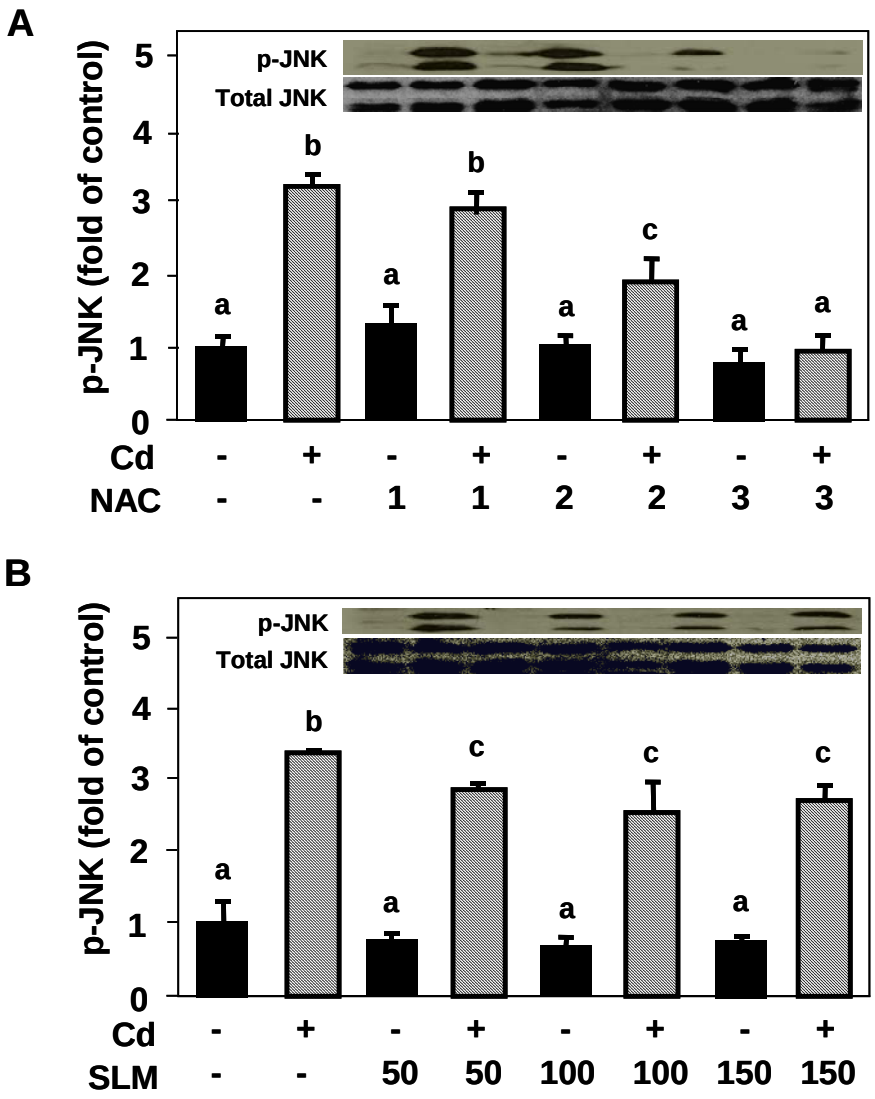

Fig. 5.6. Concentration-dependent effect of antioxidants on $CdCl_2$-induced JNK activation. J774A.1 cells were pretreated with (A) NAC (1-3 mM) or (B) silymarin (SLM, 50-150 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 h. Cell extracts were analyzed by western blot to detect the p-JNK1 (p46) and p-JNK2 (p54) using a phospho-specific JNK antibody. P-JNK represents activated JNK whereas total JNK indicates total protein loading. Mean + SE (n=3). Different letters on top of bars indicate significant difference at $p < 0.05$.

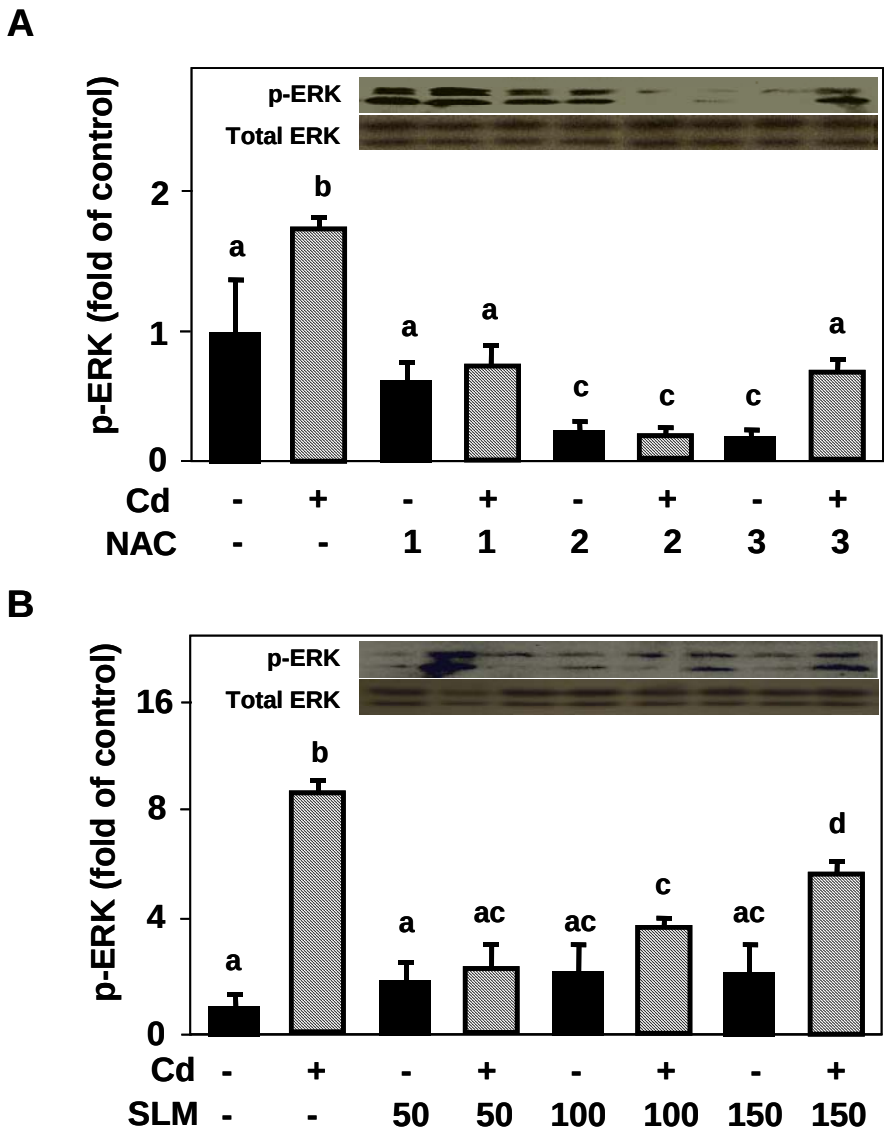

Fig. 5.7. Concentration-dependent effect of antioxidants on $CdCl_2$-induced ERK activation. J774A.1 cells were pretreated with (A) NAC (1-3 mM) or (B) silymarin (SLM, 50-150 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 h. The level of total and p-ERK was measured to detect the p-ERK1 (p42) and p-ERK2 (p44) by western blot. P-ERK represents activated ERK whereas total ERK indicates total protein loading. Mean + SE (n=3). Different letters on top of bars indicate significant difference at $p < 0.05$.

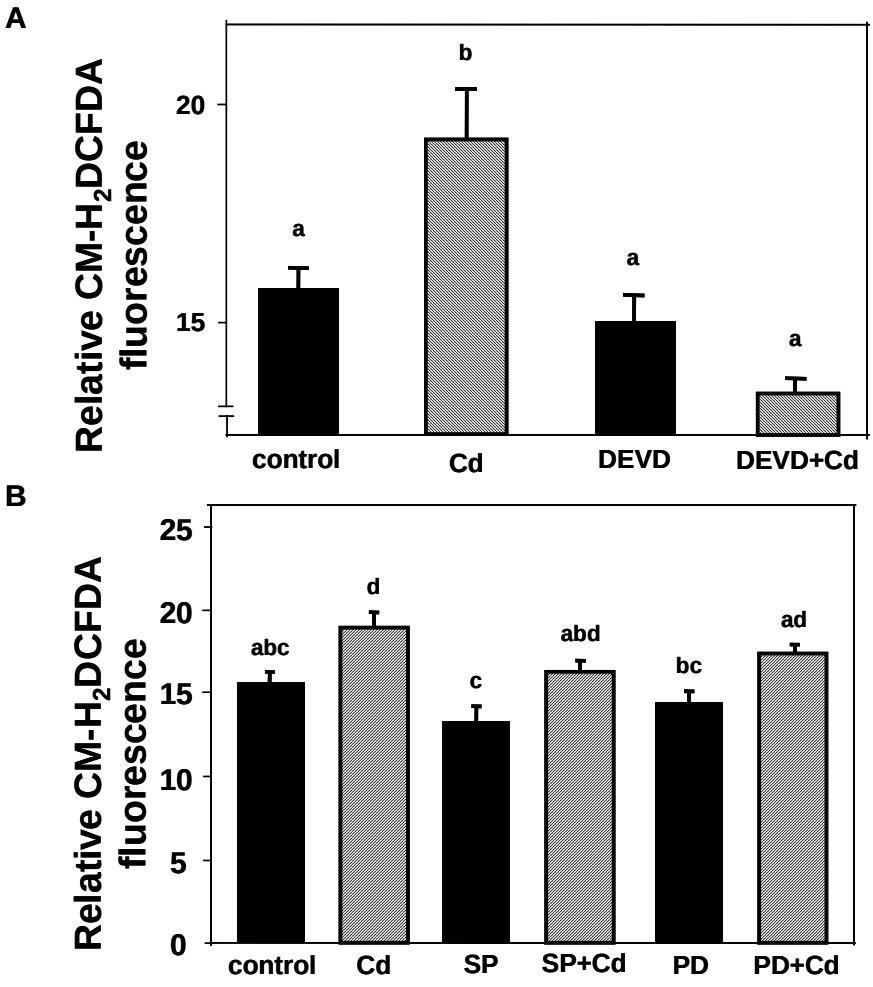

Fig. 5.8. Relation to caspase-3 and MAPKs activation on $CdCl_2$–induced H_2O_2 generation. J774A.1 cells were pre-treated with (A) Z-DEVD-FMK (DEVD, caspase-3 inhibitor, 20 μM) and (B) SP600125 (SP, JNK inhibitor, 20 μM) or PD98059 (PD, ERK inhibitor, 20 μM) for 30 min and then exposed to $CdCl_2$ (20 μM). Production of H_2O_2 by the fluorescent intensity of CM-H_2DCFDA after 6 hr $CdCl_2$ exposure was read by microplate spectrofluorometer. Different letters on top of bars indicate significant difference at $p < 0.05$. Results are expressed as mean ± SE (n=3).

CHAPTER 6

FREE VERSUS CHELATED CADMIUM

IN CADMIUM-INDUCED CYTOTOXICITY IN MURINE MACROPHAGES[4]

[4]Kim, J. and R.P. Sharma. To be submitted to *Toxicological Sciences*

Abstract

Cadmium (Cd) is a member of group IIb in the periodic table of elements, along with zinc and mercury. It is a well-known carcinogenic and immunotoxic heavy metal, commonly found in cigarette smoke and industrial effluents. Cadmium may exist in environment as a free Cd^{2+} or a complex of Cd^{2+} and molecule with thiol binding group. However, if free Cd^{2+} is responsible for cadmium toxicity is not exactly known. Cadmium as Cd^{2+} ion caused necrotic cell death at 24 h and N,N,N',N'-tetrakis(2-pyridylmethyl)ethylene diamine (TPEN), a cell permeable intracellular Cd^{2+} chelator, inhibited cadmium-induced necrosis. However, TPEN was not able to influence apoptotic response, including caspase-3 activation and DNA fragmentation by cadmium. Chelating Cd^{2+} by TPEN also failed to recover Cd-inhibited mitochondrial activity and proliferation suggesting that free Cd^{2+} is not a factor to induce apoptosis, mitochondrial impairment and growth arrest in cadmium toxicity. Chelating Cd^{2+} was still able to elevate $[Ca^{2+}]_i$ and hydrogen peroxide generation at 6 h. Exposure to cadmium induced phosphorylation of extracellular signal-related kinase (ERK) and c-Jun NH_2-terminal kinase (JNK), and deactivated p38 mitogen-activated protein kinase (MAPK). Use of TPEN indicated that only activation of ERK depends on free Cd^{2+}, while JNK and p38 MAPK were not dependent. In a previous study, we observed that ERK activation by cadmium was responsible for necrosis in J774A.1 cells. The inhibitory effect of TPEN on cadmium-activated ERK and necrosis demonstrates that free Cd^{2+} plays an important role in ERK-necrosis signaling. Altered $[Ca^{2+}]_i$ level or redox system by Cd complexes is capable of initiating toxic action leading to apoptosis, mitochondrial impairment and growth arrest.

Keyword: cadmium, chelation, necrosis, caspase-3, apoptosis, MAPKs

Abbreviations used:

$[Ca^{2+}]_i$, intracellular calcium; CM-H_2DCFDA, 5-chloromethyl-2',7'-dichlorodihydro fluorescein diacetate; DPM, disintegrations per min; ERK, extracellular signal-related kinase; GSH, glutathione; JNK, c-Jun NH_2-terminal kinase; MAPKs, mitogen-activated protein kinases; MT, metallothionein; MTT, 3(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide; PI, propidium iodide; ROS, reactive oxygen species; TPEN, N,N,N',N'- tetrakis(2-pyridylmethyl)ethylene diamine; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling

Introduction

Cadmium is a naturally occurring nonessential and toxic heavy metal, which belongs to transition metal group IIB of the periodic table. Cadmium and zinc belong to the same group and influence effect of each other in the intracellular environment (Hamada *et al.*, 1997). Besides zinc, cadmium interferes with the utilization of essential metals e.g. calcium, selenium, chromium and iron, and abolishes their function in biological system (Nath *et al.*, 1984). Cadmium is found in plastic stabilizers, color pigments, several alloys and, now most commonly, in rechargeable nickel-cadmium batteries (Jarup, 2003). There have been numerous studies regarding cadmium toxicity suggesting the interference with major cellular signal transduction elements such as cell surface receptors, cytosolic and nuclear calcium levels and phosphorylation of proteins including protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) as mechanisms for cadmium toxicity (Hechtenberg *et al.*, 1994; Hechtenberg *et al.*, 1996; Long, 1997; Misra *et al.*, 2002; Smith *et al.*, 1989).

The major cellular uptake of cadmium is thought to be through Ca^{2+} channels. Cadmium uptake occurs by an active transport rather than passive diffusion mechanism in Chinese Hamster Ovary (CHO) cells and hepatocytes (Planas-Bohne *et al.*, 1988). Free Cd^{2+} was taken up primarily through voltage-gated Ca^{2+} channels in cultured pituitary cells (Hinkle *et al.*, 1987). About a third of the cadmium was taken up into the cell through the receptor-operated Ca^{2+} channels in primary cultures of rat hepatocytes (Blazka *et al.*, 1991). The marked reduction of cadmium uptake by thiol binding agents indicated the involvement of –SH groups in the transport of Cd^{2+} ions (Klug *et al.*, 1988).

Cadmium in culture or tissues exists in several different forms. The level of cellular glutathione (GSH) and metallothionein (MT) is increased after treatment of cells or animals with toxic concentration of cadmium (Beyersmann *et al.*, 1997; Son *et al.*, 2001). Cd^{2+}-GSH, Cd^{2+}-MT and free Cd^{2+} are possible forms of cadmium in cell culture. However, which type of cadmium is responsible for cadmium toxicity and the role of free Cd^{2+} has not been determined. It has been suggested that if cadmium interacts with cell surface receptors affecting intracellular signals, free Cd^{2+} ion will not be required to enter cells in order to exert toxic effects (Beyersmann *et al.*, 1997). Further a direct interaction of cadmium with intracellular molecules, altered redox system or $[Ca^{2+}]_i$ homeostasis is considered to initiate toxic action by cadmium.

Effects of cadmium on immune functions and cells of immune system has been discussed (Descotes, 1992). It has been reported that smokers have 4-5 times higher level of cadmium in blood than non-smokers (Jarup *et al.*, 1998). Inhalation of cadmium will directly affect macrophages in the respiratory system without first-pass elimination and be a factor of immunodepression in smokers. J774A.1 cells possess similarities to mature macrophages, making them an alternative to primary cells (Yan *et al.*, 2004). Due to the tumor-like property,

these cells however may be more resistant to cadmium toxicity. Studies using macrophages on cadmium-mediated cell signaling and subsequent cell death are valuable to understand cadmium effect on the immune system. The objective of this study was to explore the role of free Cd^{2+} and to outline the mechanism of toxicity by free Cd^{2+} in cadmium-treated macrophages. Using N,N,N',N'-tetrakis(2-pyridylmethyl)ethylene diamine (TPEN), a cell permeable chelator of cadmium, we investigated whether intracellular free Cd^{2+} is required for cadmium-induced necrosis, apoptosis, inhibition of mitochondrial activity, growth arrest and altered MAPK activity. Because TPEN has higher affinity for Cd^{2+} (log K_{Cd} = 16.3) than other metals (Fe^{2+}; log K_{Fe} = 14.6, Ca^{2+}; log K_{Ca} = 4.4) (Anderegg G *et al.*, 1997; Arslan *et al.*, 1985), it has been used as a specific intracellular Cd^{2+} chelator in several studies (Iryo *et al.*, 2000; Matsuoka *et al.*, 1998). The results from this study showed that intracellular Cd^{2+} is capable of inducing activation of ERK and necrosis, but not generating elevated $[Ca^{2+}]_i$ or reactive oxygen species (ROS) in cadmium-induced apoptosis and growth arrest.

Materials and Methods

Reagents

Cadmium chloride ($CdCl_2$, Sigma Chemical Co., St. Louis, MO) was dissolved in water, sterilized with 0.22 μm filter, and added to cultures at the indicated time and concentrations. Cell culture reagents were procured from GIBCO Life Technology (Grand Island, NY). Antibodies specific for the total and phosphorylated forms of extracellular signal-related kinase (ERK, p44/42 MAPK), c-Jun NH_2-terminal kinase (JNK, p54/46 MAPK) and p38 MAPK were obtained from Cell Signaling (Beverly, MA). Fluorescent probes Fluo-3/AM and propidium

iodide (PI) were purchased from Molecular probes (Eugene, OR). TPEN, Hoechst 33258 and other chemicals used in this study were obtained from Sigma and were of cell culture grade.

Cell culture

Macrophage cell line, J774A.1 (American Type Culture Collection TIB-67), originated from BALB/c mouse reticulum cell sarcoma, was maintained in Dulbecco's Modified Eagle's Medium, supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin and 10% non-heat-inactivated fetal bovine serum (Atlanta Biologics, Atlanta, GA) in 5% CO_2 atmosphere at 37 ºC. The J774A.1 cells were grown in 75 cm^2 culture flasks and subcultured when the cells reached 70 – 80% confluence (every 3 days). Cells were used for experiments during 3rd or 4th passage. Cultures were allowed to grow overnight (15 hr) prior to the treatment. TPEN (1 or 10 µM) was added 30 min prior to cadmium treatment. The lower concentration of TPEN employed was not cytotoxic in preliminary trials.

PI and Hoechst 33258 staining

Necrotic cell death was detected by staining with PI, a membrane impermeant dye excluded from viable cells. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stochiometry of one dye per 4-5 base pairs of DNA. Apoptotic morphological changes in the nuclear chromatin of cells were detected by staining with the DNA binding fluorochrome Hoechst 33258 (bis-benzimide). Hoechst 33258 exhibits fluorescence enhancement upon binding to A-T rich regions of double stranded DNA. J774A.1 cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with 20 µM cadmium for 200 µl culture at each well. Following 24 h treatment with cadmium, 120 µl of supernatant was removed and 20 µl of PI (1 µg/ml) or Hoechst 33258 (2 µg/ml) was added. To protect necrotic cells from being stained by Hoechst 33258, the fluorescence was measured after 15 min dye

binding to DNA. The plates were read at 535/617 nm or 350/450 nm (excitation/emission) for PI or Hoechst 33258 fluorescence, respectively, using a SpectraMax Gemini (Molecular Devices, Sunnyvale, CA). The fluorescence signal was digitized and analyzed using SoftMax Pro™ (Molecular Devices). Fluorescence microscopy was performed to examine the apoptotic morphology for Hoechst 33258, using IX71 inverted microscope (Olympus, Melville, NY). Digital images were captured using MagnaFire SP® Olympus digital camera.

Determination of caspase-3 activation

Caspase-3 activity was determined using CaspACE™ fluorometric activity assay (Promega, Madison, WI) with modifications as follows. Briefly, cells were treated in 96-well microplates following which Triton X-100 was added and repeatedly pipetted to lyse the cells. The homogenates were centrifuged at 4,000×g for 10 min to remove cell debris. The supernatant was assayed for caspase-3 activity using CaspACE™ system according to the manufacture's instructions. The fluorescence signal was digitized and analyzed using SoftMax Pro™.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay

TUNEL assay was performed using the in situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). Cells were plated at 8×10^4 cells/well in 96-well microplates and allowed to attach overnight. Cells were then treated with cadmium for 16 hr, fixed with paraformaldehyde, and analyzed for stained nuclei according to the manufacturer's instructions. The fluorescence signal was read by SpectraMax Gemini, digitized and analyzed using SoftMax Pro™ (Molecular Devices, Irvine, CA). In addition, fluorescence microscopy was performed to

examine fragmented DNA morphology using IX71 inverted microscope. Digital images were captured using MagnaFire SP® Olympus digital camera.

Mitochondrial activity

MTT (3[4,5-dimethyl thiazolyl-2]2,5-diphenyl tetrazolium bromide, Sigma) was used to investigate mitochondrial activity. Cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with 20 µM cadmium for 24 h. The cells were incubated with addition of 20 µl MTT (5 mg/ml). After 4 h, 120 µl of MTT media was taken out from each well and 100 µl of 0.02 N HCl-isopropanol (warm) added to dissolve formazan crystals. The absorbance of each cell was measure by UV spectrometer at 570 nm.

DNA synthesis as an index of proliferation

The [methyl-^{3}H]thymidine incorporation was used as an index of proliferation. Cells were seeded at 8×10^4 cells/well in 96-well microplates. At 16 h prior to harvesting cells, each well was pulsed with 20 µl of [methyl-^{3}H]thymidine (25 µCi/ml, 6.7 Ci/mmol, DuPont NEN Products, Boston, MA). Cells were harvested onto glass fiber filter paper (Cambridge Technology, Watertown, MA) using a cell harvester (PHD, Cambridge Technology). Proliferative response (uptake of [^{3}H]thymidine) in the harvested cells was counted in a liquid scintillation counter (Pharmacia, Turku, Finland) and expressed as disintegrations per min (DPM).

Western blot analysis of phosphorylated ERK, JNK and p38 MAPK

The activation status (phosphorylation) of ERK, JNK and p38 MAPK was determined using phospho-specific antibodies as described previously (Johnson *et al.*, 2003). Cells were grown at 2×10^6 cells/well in 6-well microplates. J774A.1 cells were preincubated with 1 µM TPEN for 30 min and then treated with 20 µM cadmium for 16 h. To know the effect of

extracellular complex formation of TPEN and cadmium on MAPKs phosphorylation, medium containing 0 or 20 μM cadmium was incubated with 1 μM TPEN for 30 min in the absence of cells, J774A.1 cells were then incubated with these media for 16 h. Following treatment, cells were washed with phosphate buffered saline (PBS) and total cell lysates were prepared by scrapping in 100 μl of lysis buffer [20 mM Tris–HCl (pH 8.0), 1 mM sodium orthovanadate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetate (EDTA), 1% Triton X-100, 50 mM β-glycerolphosphate, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin]. Fifty micrograms of protein determined by Bradford assay was electrophoretically separated using a 12% SDS-PAGE gel and transferred to nitrocellulose paper followed by antibody staining. Equal loading and transfer of total protein was verified with the reversible Ponceau S stain (Sigma) dye and also by detecting total ERK, JNK and p38 MAPK. Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia, Piscataway, NJ).

Determination of hydrogen peroxide

The production of hydrogen peroxide was studied using by H_2O_2-sensitive probe 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes). The cells were incubated in the presence of various concentration of cadmium and fluorescent intensity recorded using SpectraMax Gemini fluorescence plate reader. The CM-H_2DCFDA fluorescence was detected by excitation at 485 nm, and emission at 530 nm. The fluorescence readings were digitized using SoftMax Pro^{TM}.

Determination of intracellular Ca^{2+}

Intracellular Ca^{2+} levels were checked by Fluo-3/AM, a Ca^{2+}-sensitive fluorescent indicator. Cells were seeded at 8×10^4 cells/well in 96-well microplates and treated with 20 μM

cadmium for 6 h. Cells were loaded with Fluo-3/AM (10 μM) in dark at 37°C for 1 h and washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.2 mM NaH_2PO_4, 12 mM $NaHCO_3$, and 5.5 mM Glucose). Cells were incubated in Tyrode's solution for another 30 min and the morphological fluorescence intensity of cells was determined using Olympus IX71 inverted microscope. A 488 nm excitation wavelength was used to illuminate Fluo-3/AM, and fluorescence was detected at emission wavelength of 510 nm. Digital images were acquired using the Magnafire SP digital camera.

Replication and statistical analysis

Experiments were repeated at least 3-4 times with consistent results. Means + SE from representative experiments have been presented. All statistical analyses were performed using the SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A p value of < 0.05 was considered significant unless indicated otherwise.

Results

Inhibitory effect of chelating Cd^{2+} on cadmium-induced necrosis

To examine the role of free Cd^{2+} on cadmium-induced necrosis, we measured the level of necrotic cell death using PI staining (Fig. 1). Cells treated with cadmium showed 3-times higher level of necrotic cell death than control. TPEN, a cell permeable Cd^{2+} chelator at 1 μM, did not show any cytotoxicity. Pretreatment of cells with 1 and 10 μM TPEN was able to reduce cadmium-induced necrotic cell death. The inhibitory effect in cadmium-induced necrosis was same between 1 and 10 μM TPEN. These observations indicate that intracellular free Cd^{2+} was partially required for cadmium-induced necrosis.

Effect of chelating Cd^{2+} on cadmium-induced caspase-3 activation and apoptosis

To determine whether cadmium-induced caspase-3 activation and apoptosis depend on intracellular free Cd^{2+}, J774A.1 cells were treated with 20 μM cadmium in the absence or presence of 1 or 10 μM TPEN. Cadmium-induced apoptotic effects were observed by caspase-3 activity, TUNEL and Hoechst staining at 16 h. TPEN at 1 μM caused no effect on caspase-3 activity induced by cadmium. Even higher concentration of 10 μM TPEN was not able to reduce cadmium-induced apoptotic response (Fig. 2A). A lack effect of free Cd^{2+} on cadmium-induced apoptosis was confirmed by Hoechst 33258 staining (Fig. 2B). Chelating Cd^{2+} by 1 or 10 μM TPEN did not reduce cadmium-increased Hoechst staining. DNA fragmentation by TUNEL confirmed that chelating intracellular Cd^{2+} was not able to reduce cadmium-induced apoptosis (Fig. 2C). Apoptotic cells stained with Hoechst 33258 are presented in Fig. 2D. The nuclei of cells exposed to cadmium showed the condensed and fragmented chromatin characteristics of apoptosis. These results suggest that intracellular free Cd^{2+} is not required in cadmium-induced caspase-3 activation and apoptosis.

Effect of chelating Cd^{2+} on the cadmium-inhibited mitochondrial activity and proliferation

Because chelating Cd^{2+} was only related with necrosis not caspase-3 activation and apoptosis, the effect of free Cd^{2+} on cadmium-inhibited mitochondrial activity and proliferation was analyzed. J774A.1 cells were incubated with 20 μM cadmium with or without 1 or 10 μM TPEN. Cadmium reduced the mitochondrial activity by half at 24 h. However, chelating Cd^{2+} with 1 or 10 μM TPEN was not able to recover cadmium-inhibited mitochondrial activity (Fig. 3A). Similarly, pretreatment with 1 or 10 μM TPEN and cadmium did not provide any recovery effect from cadmium-induced growth arrest (Fig. 3B). Results suggested that intracellular free Cd^{2+} was not required for the cadmium-inhibited mitochondrial function and cell proliferation.

Role of free Cd^{2+} on the cadmium-altered MAPKs activity

To delineate the role of free intracellular Cd^{2+} on the cadmium-induced cytotoxicity, we examined the phosphorylation of MAPKs (ERK, JNK and p38 MAPK) in J774A.1 cells. Western blot analyses revealed that 20 μM cadmium was able to activate ERK and JNK but deactivate p38 MAPK at 16 h (Fig. 4). TPEN (1 μM) pretreatment 30 min prior to cadmium added to J774A.1 cells or medium containing 20 μM cadmium incubated with 1 μM TPEN for 30 min in the absence of cells, then incubated with J774A.1 cells to chelate Cd^{2+} extracellularly showed identical effects. The activation of ERK by cadmium was significantly, but not completely, blocked by chelating intracellular and extracellular Cd^{2+} in J774A.1 cells (Fig 4A). The immunoblot with phosphorylated form of JNK specific antibody revealed that cotreatment with TPEN failed to inhibit cadmium-induced JNK activation (Fig. 4B). Down-regulated p38 MAPK activity by cadmium also did not recover to control level by the treatment with TPEN (Fig. 4C). Results suggested that free $Cd^{2+}$is partially related with ERK activation rather than JNK or p38 MAPK activity.

Effect of chelating Cd^{2+} on cadmium-induced H_2O_2 generation and $[Ca2+]_i$ elevation

Analysis of cells stained with H_2DCFDA revealed that 20 μM cadmium treatment generated significantly high cellular levels of H_2O_2 (Fig. 5A). Chelating intracellular Cd^{2+} by TPEN was not able to prevent cadmium-induced H_2O_2 generation suggesting that TPEN-Cd^{2+} complex still was able to induce H_2O_2 generation. To determine whether increase in $[Ca^{2+}]_i$ mediated by cadmium depends on free Cd^{2+}, cells were treated with TPEN (Fig. 5B). As unexpected, TPEN (1 μM) did not influence cadmium-induced $[Ca^{2+}]_i$ elevation.

Discussion

We recently reported that cadmium-induced Ca^{2+} elevation triggers JNK and caspase-3 activation (Kim *et al*., 2004). Cadmium also induced ERK signaling-dependent G2/M arrest and cell death in J774A.1 murine macrophages (Kim *et al*., 2003). In our previous studies, we observed that chelating intracellular and extracellular Ca^{2+} was able to prevent cadmium-inhibited proliferation and reduced mitochondrial activity in J774A.1 cells (Kim *et al*., 2004). We also found that oxidative stress is important in inhibition of proliferation and mitochondrial activity by cadmium (Chapter 5). Results presented here further support that cadmium increases $[Ca^{2+}]_i$ and H_2O_2 production at 6 h (Fig. 5), activates JNK and ERK but deactivates p38 MAPK at 16 h. Chelated intracellular Cd^{2+} was still able to generate H_2O_2 and to elevate $[Ca^{2+}]_i$. Presence of free Cd^{2+} was not a requirement for cadmium-induced JNK and caspase-3 activation and DNA fragmentation in macrophages. Free Cd^{2+} was responsible for cadmium-activated ERK and necrosis rather than apoptotic signaling pathways in cadmium toxicity.

There have been studies that suggest that elevated $[Ca^{2+}]_i$ is important in activation of MAPKs by cadmium toxicity and intracellular free Cd^{2+} is not required for the regulation of MAPKs activity. Intracellular free Cd^{2+} was not needed for the cadmium-induced activation of ERK, JNK and p38 MAPK, however, $[Ca^{2+}]_i$ chelation was able to inhibit these activation of MAPKs in CCRF-CEM human T cell line (Iryo *et al.*, 2000). Similar effect on cadmium-induced JNK activation was observed in porcine renal epithelial LLC-PK_1 cell line (Matsuoka *et al.*, 1998). TPEN at 10 and 50 μM was not able to inhibit elevated level of phosphorylated JNK by 20 μM cadmium, which was significantly suppressed by $[Ca^{2+}]_i$ chelation (Matsuoka *et al.*, 1998). In our study, 10 μM TPEN itself inhibited mitochondrial activity and cell proliferation, and increased necrotic and apoptotic cell death. However, when 10 μM TPEN was treated

together with 20 μM cadmium, almost similar effect was observed with 1 μM TPEN and 20 μM cadmium. Because of cytotoxicity of TPEN itself, higher than 10 μM TPEN was not tried. Chelating intracellular or extracellular Cd^{2+} showed a similar effect on cadmium-altered MAPKs activity. Free Cd^{2+} mediated cadmium-induced ERK activation but not JNK and p38 MAPK activity in our study. Because chelating intracellular Cd^{2+} was still able to generate H_2O_2 and to elevate $[Ca^{2+}]_i$, the interference with $[Ca^{2+}]_i$ homeostasis and generation of ROS is thought to be important than the direct effect of free Cd^{2+} in cadmium-altered JNK and p38 MAPK activity.

Most (90%) of the cadmium taken up by cells resides in cytosol and is known to be complexed with MT (Korotkov *et al.*, 1999). Exposure of cells as well as animals to cadmium results in the induction of several stress response gene expression, such as those encoding for MT and GSH synthesis (Waisberg *et al.*, 2003). Metallothionein (a low molecular weight protein containing about 30% cysteine) and GSH sequester cadmium with high affinity resulting in decreased availability of free Cd^{2+} capable of interacting with cellular targets to elicit toxicity; Cd-MT and Cd-GSH is regarded as the major underlying causes of tissue susceptibility to cadmium toxicity and carcinogenicity (Waisberg *et al.*, 2003). However, pronounced toxic effect of Cd-MT or cadmium complexes than free Cd^{2+} has been shown in some studies. Toxic effect of Cd-MT complexes on mitochondria was stronger than that of inorganic cadmium salts in vivo experiments (Nordberg, 1978). The organic cadmium complexes affected respiration and perturbed ion permeability significantly stronger than free Cd^{2+}, probably due to Cd^{2+} incorporated into the complexes can easily penetrate to adjacent –SH groups of mitochondrial respiratory enzymes and other molecules of the inner mitochondrial membrane that regulate the mitochondrial ion permeability (Korotkov *et al.*, 1999). These studies are evidence that

cadmium complexes may exert substantial cytotoxic effect on mitochondrial activity compared with free Cd^{2+}.

The mechanisms responsible for cadmium-induced deregulation of gene expression include effects on secondary messengers such as $[Ca^{2+}]_i$, ROS and signal transduction cascades involving kinases (Waisberg *et al.*, 2003). Besides the direct interaction of cadmium with intracellular molecules, altered redox system or $[Ca^{2+}]_i$ homeostasis is considered as a target of toxic action by cadmium. Cadmium has inhibitory effect on Ca^{2+}-ATPase pump for sequestration of $[Ca^{2+}]_i$, and it is also known to disturb Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular stores (Benters *et al.*, 1996; Benters *et al.*, 1997; Hechtenberg *et al.*, 1994). ROS may involve direct interaction with specific receptors and signaling pathways such as protein kinases, protein phosphatases and transcription factors from the plasma membrane to the cell nucleus during cell growth and differentiation (Sauer *et al.*, 2001).

In summary, we have demonstrated here that free Cd^{2+} was responsible for cadmium-induced ERK activation and necrosis. Initial response of $[Ca^{2+}]_i$ elevation and H_2O_2 production by cadmium was not inhibited through chelating Cd^{2+}, indicating that cadmium complexes rather than free Cd^{2+} may contribute to early signaling of Ca^{2+} and oxidative stress. Inhibition of proliferation and mitochondrial activity also still observed with treatment of TPEN and cadmium. Free Cd^{2+} was not a requirement in cadmium-induced apoptotic responses, including caspase-3 activation and DNA fragmentation. ERK activation by cadmium was responsible for necrosis and $[Ca^{2+}]_i$-mediated JNK activation for apoptosis in J774A.1 cells as reported earlier (Kim *et al.*, 2004). The selective inhibitory effect of TPEN on cadmium-activated ERK and necrosis demonstrated that free Cd^{2+} plays a role in ERK-induced signaling leading to necrosis rather than

$[Ca^{2+}]_i$-JNK-csapase-3 pathway. Altered $[Ca^{2+}]_i$ level or redox system by other cadmium complexes is considered as an initiation of toxic action leading to mitochondrial impairment, growth arrest and apoptosis.

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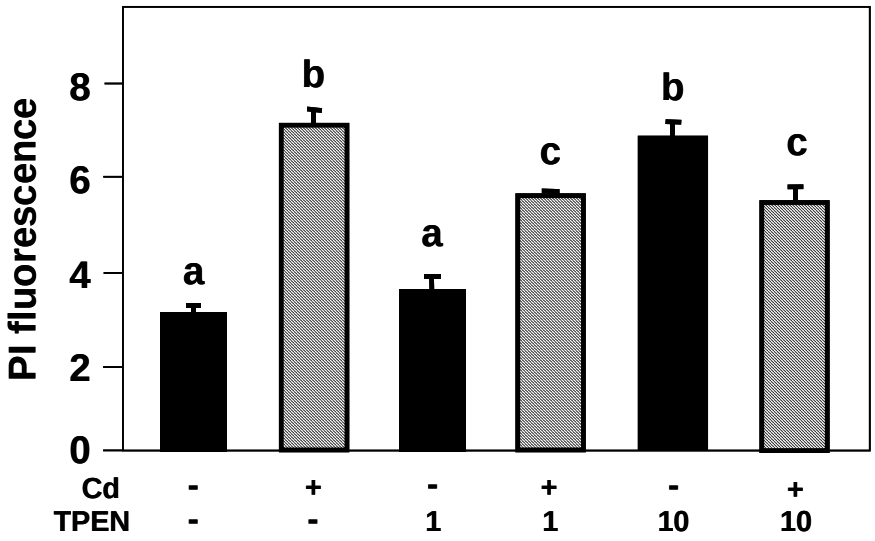

Fig. 6.1. Effect of TPEN on $CdCl_2$-induced necrosis. J774A.1 cells were pretreated with 1 or 10 μM TPEN for 30 min and then exposed to $CdCl_2$ (20 μM) for 24 h. The intensity of PI fluorescence after $CdCl_2$ exposure was read by microplate spectrofluorometer. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$.

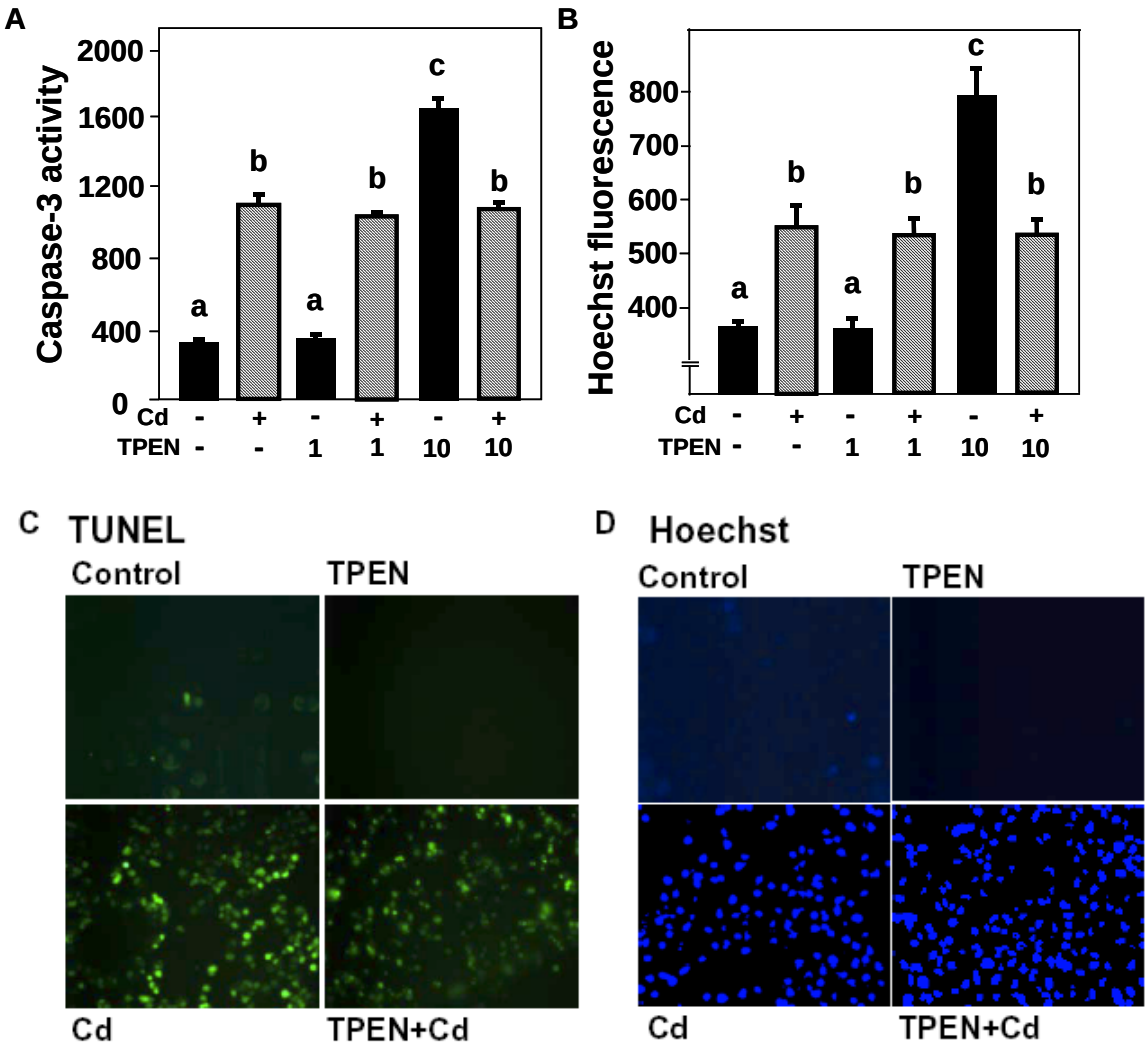

Fig. 6.2. Effect of TPEN on $CdCl_2$-induced caspase-3 activation and DNA fragmentation. J774A.1 cells were pretreated with 1 or 10 μM TPEN for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 h. (A) The intensity of fluorescence on caspase-3 enzyme activity was measured. (B) The intensity of fluorescence on apoptotic nuclei stained by Hoechst 33258 read by spectrofluorometer. (C) Fluorescence on TUNEL positive nuclei was visualized under fluorescence microscope. (D) Cell nuclei visualized under fluorescence microscope with Hoechst 33258 staining. For A and B, mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$.

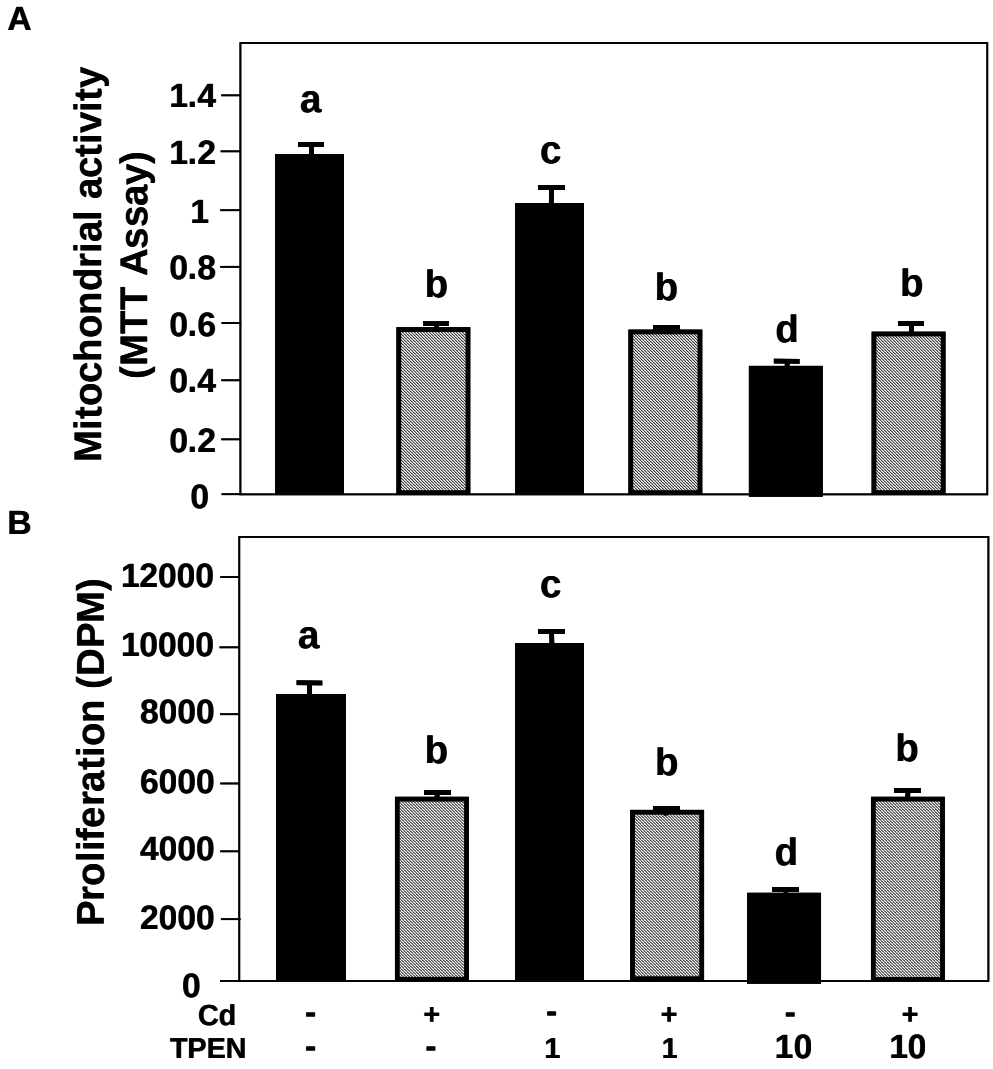

Fig. 6.3. Effect of TPEN on $CdCl_2$–induced mitochondrial activity impairment and growth arrest. J774A.1 cells were pretreated with TPEN (1 or 10 μM) for 30 min and then exposed to $CdCl_2$ (20 μM). (A) Mitochondrial activity measured by MTT assay after 24 h $CdCl_2$ exposure. (B) Proliferation measured by [^{3}H]thymidine incorporation after 16 h $CdCl_2$ exposure. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$.

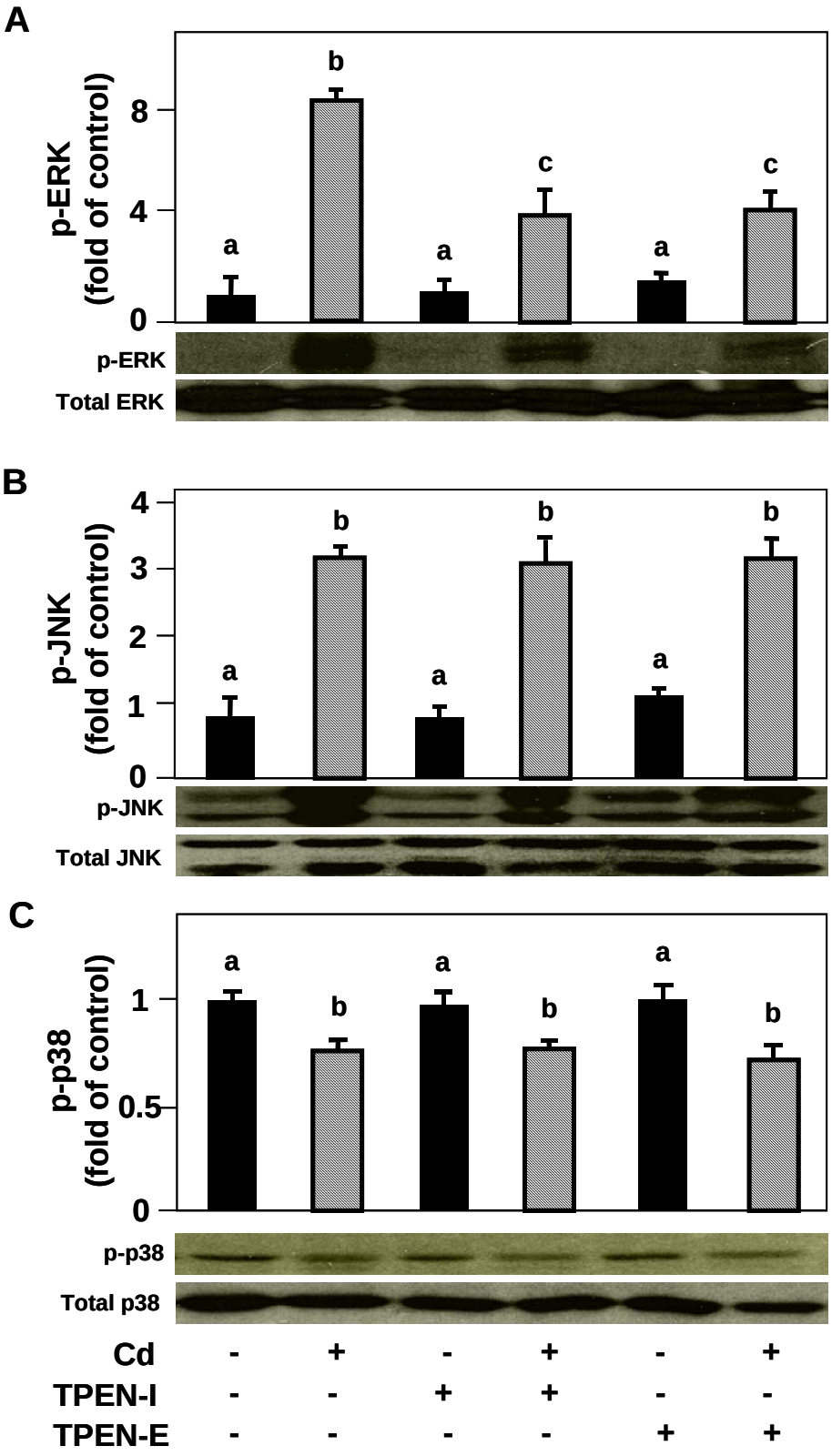
A
p-ERK
(fold of control)
0
4
8
a
b
a
c
a
c
p-ERK
Total ERK
B
p-JNK
(fold of control)
0
1
2
3
4
a
b
a
b
a
b
p-JNK
Total JNK
C
p-p38
(fold of control)
0
0.5
1
a
b
a
b
a
b
p-p38
Total p38
Cd - + - + - +
TPEN-I - - + + - -
TPEN-E - - - - + +

Fig. 6.4. **Effect of TPEN on $CdCl_2$-induced activation of ERK and JNK and down-regulation of p38.** J774A.1 cells were pretreated with TPEN (1 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 16 h (TPEN-I), or 20 μM cadmium incubated with 1 μM TPEN for 30 min in the absence of cells and then cells exposed to the complex (TPEN-E). (A) p-ERK, (B) p-JNK, and (C) p-p38 were measured by western blot after 16 h $CdCl_2$ exposure, bands for total MAPKs are illustrated to indicate protein loading. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$.

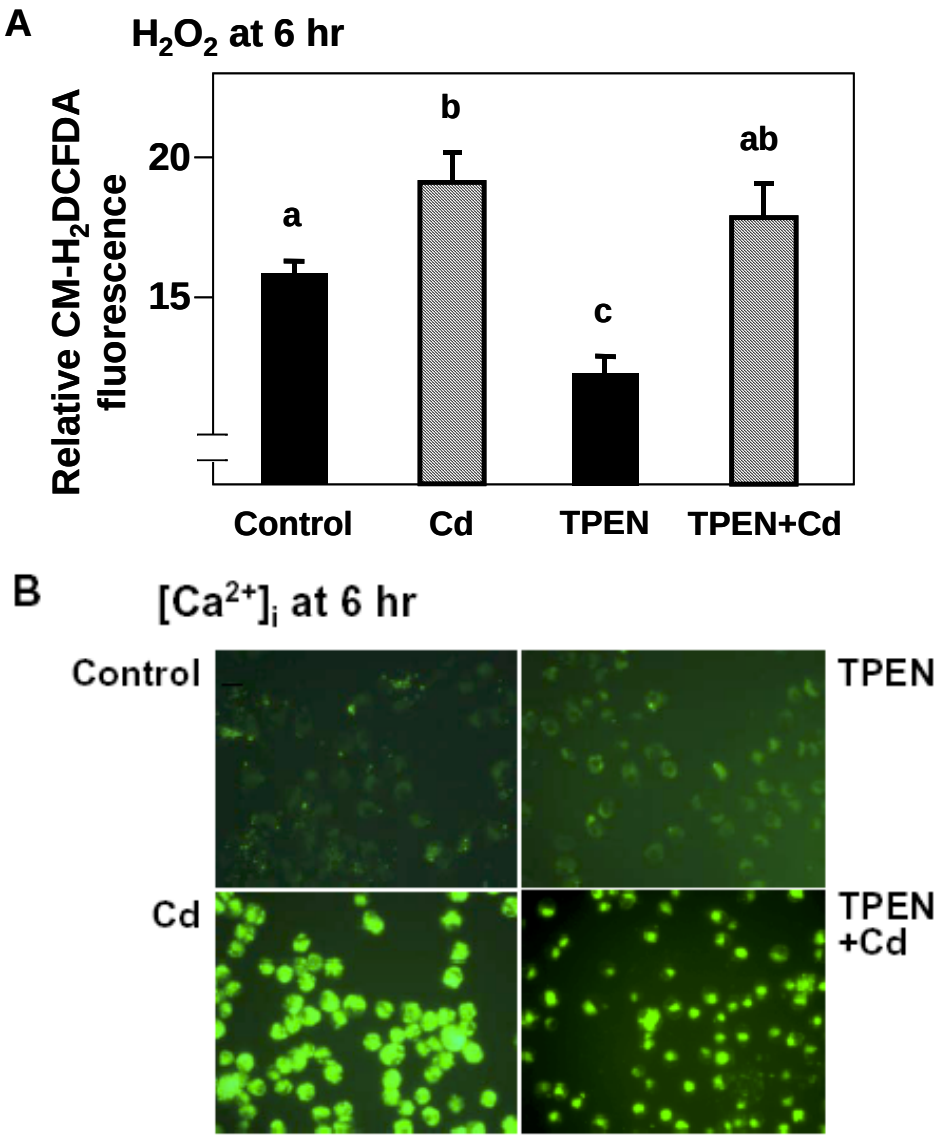

Fig. 6.5. Effect of TPEN on $CdCl_2$-induced generation of H_2O_2 and $[Ca^{2+}]_i$ elevation. J774A.1 cells were pretreated with TPEN (1 μM) for 30 min and then exposed to $CdCl_2$ (20 μM) for 6 h. (A) Fluorescence stained with H_2DCFDA read by microplate spectrofluorometer. (B) Morphological fluorescence intensity of altered $[Ca^{2+}]_i$ level visualized under fluorescence microscope using Fluo-3/AM after 6 h $CdCl_2$ exposure. Representative pictures are shown. Mean + SE (n=3). Different letters on top of bars indicate a significant difference at $p < 0.05$.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Cadmium is a nonessential and toxic heavy metal commonly found in cigarette smoke and industrial effluent. Inhalation of cadmium will directly effect on macrophages in respiratory system without first-pass elimination and be a factor of immunodepression of smokers. The objectives of the present study were to (1) investigate the effect of cadmium on cell cycle progression and cell death via ERK, (2) evaluate cadmium-mediated calcium signaling in activation of JNK and caspase-3, (3) determine the time frame of ROS generation and apoptosis induced by cadmium and (4) examine the role of free versus chelated cadmium in cadmium-induced cytotoxicity in J774A.1 murin macrophages.

In the first study, we indicated that cadmium inhibited proliferation in murine macrophages via G2/M arrest and a gap between viability and proliferation was explained by inhibited cell cycle progression. Cell growth kinetics in a time-dependent fashion showed increased thymidine incorporation at 8 hr and then thymidine uptake was dramatically decreased at 24 hr. This result suggested that cadmium is accelerating DNA content doubling by going through G0/G1 to S at earlier time point and then later arresting cells at G2/M phase. We observed elevated phosphorylation of ERK by cadmium at 16 and 24 hr. Activated ERK by cadmium was a major mediator for inhibition of proliferation and G2/M arrest. Inhibition of ERK inhibited $p21^{WAF1/CIP1}$ expression suggesting ERK-$p21^{WAF1/CIP1}$–G2/M arrest signaling. Regulation of cell death by ERK was more related with necrosis than caspase-3 activation or apoptosis in J774A.1 cells. Our results also suggested that expression of ROS by cadmium play an important role in ERK- $p21^{WAF1/CIP1}$ signaling.

The second study presented that cadmium activates JNK, which plays a critical role in the apoptotic suicide of cells. Cadmium strongly stimulated JNK activity after 8 h exposure of J774A.1 murine macrophage cells and this stimulation persisted until 16 h. The sustained JNK activation was Ca^{2+}-dependent and served as a death signal in cadmium-induced apoptosis.

Chelation of $[Ca^{2+}]_i$ by BAPTA-AM and EGTA prevented the cadmium-induced H_2O_2 generation, hampered mitochondrial activity, JNK, caspase-3 activation and apoptosis, confirming the early mediating role of Ca^{2+} during cadmium-induced apoptosis. We also presented evidence that cadmium down-regulates activation of p38 MAPK. Cadmium-mediated modulation of JNK and p38 MAPK activity was tightly correlated with elevated $[Ca^{2+}]_i$. Chelating $[Ca^{2+}]_i$ reduced H_2O_2 production indicating that ROS act concert with $[Ca^{2+}]_i$ signaling.

In the third study, we observed that cadmium-generated ROS can influence the growth as well as death of murine macrophages *in vitro*, through the activation of multiple signaling pathways including MAPKs and caspases. Cadmium generated ROS in a dose-dependent manner and elevated oxidative stress played a critical role in the apoptotic suicide of cells. Cadmium strongly stimulated H_2O_2 after 6 h exposure to J774A.1 murine macrophage cells and this stimulation persisted until 10 h. The level of H_2O_2 produced by cadmium started to decrease after 10 h and became similar to control at 16 and 24 h, suggesting that the production of H_2O_2 is an initial step of cadmium-mediated cellular signaling. Antioxidation by NAC, a precursor of glutathione or silymarin, a flavonoid type of free radical scavenger, prevented the cadmium-induced H_2O_2 generation, hampered mitochondrial activity, caspase-3 activation and apoptosis, confirming that oxidative stress is an early signal to cadmium-induced cellular damage. The activation of JNK and ERK was ROS dependent and may serve as a death signal in cadmium-induced apoptosis. *N*-acetylcysteine dose-dependently inhibited JNK and ERK. The amount of H_2O_2 produced by cadmium was affected by the presence of caspase-3 inhibitor, but not by inhibitors of MAPKs, indicating that the activation of caspase-3 is an important source for cadmium-elevated ROS generation and MAPKs are activated as downstream of oxidative stress.

In the final study, we found that free Cd^{2+} was responsible for cadmium-induced ERK

activation and necrosis. Chelated intracellular Cd^{2+} was still able to generate H_2O_2 and to elevate $[Ca^{2+}]_i$, indicating that cadmium complexes rather than free Cd^{2+} may contribute to early signaling of Ca^{2+} and oxidative stress. Inhibition of proliferation and mitochondrial activity also still observed with treatment of TPEN and cadmium. Free Cd^{2+} was not a requirement in cadmium-induced apoptotic responses, including caspase-3 activation and DNA fragmentation. ERK activation by cadmium was responsible for necrosis and $[Ca^{2+}]_i$-mediated JNK activation for apoptosis in J774A.1 cells as reported in the second study. The selective inhibitory effect of TPEN on cadmium-activated ERK and necrosis demonstrated that free Cd^{2+} plays a role in ERK-induced signaling leading to necrosis rather than $[Ca^{2+}]_i$-JNK-csapase-3 pathway.

All together, the data presented in this dissertation indicate that cadmium induces elevation of $[Ca^{2+}]_i$ and generation of ROS as an initial signals to give cytotoxic effect on J774A.1 cells. Both level of $[Ca^{2+}]_i$ and oxidative status were balancing each other and showed most important effect on cadmium toxicity including cell death (both apoptosis and necrosis), growth arrest, mitochondrial impairment and MAPKs activation. Cadmium activated ERK and JNK in response to elevated $[Ca^{2+}]_i$ and oxidative stress, however deactivated p38 MAPK. Activated JNK was responsible for cadmium-induced caspase-3 activation and apoptosis, on the other hand, activated ERK by cadmium was related with G2/M arrest and necrosis rather than apoptotic response. Chelated intracellular Cd^{2+} was still able to generate H_2O_2 and to elevate $[Ca^{2+}]_i$, indicating that cadmium complexes may contribute to early signaling of Ca^{2+} and oxidative stress. Free Cd^{2+} was responsible for cadmium-induced ERK activation and necrosis but not JNK-caspase-3-apoptosis signaling. Altered $[Ca^{2+}]_i$ level or redox system by other cadmium complexes is considered as an initiation of toxic action leading to mitochondrial impairment, growth arrest and apoptosis.

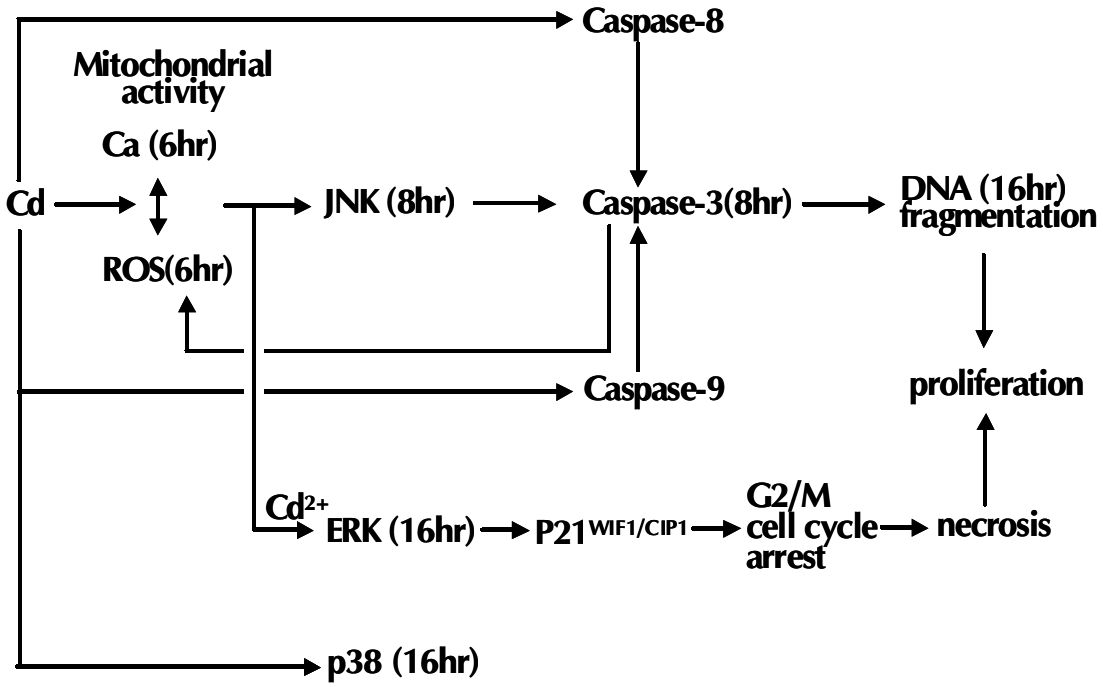

Fig. 7.1. Possible signal transduction mechanism of cadmium toxicity leading to apoptosis and growth arrest in J774A.1 murine macrophages.