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Cadmium Induces Apoptosis Through Oxidative Stress in Human Breast Cancer MCF-7 Cells

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인간 유방암 세포 MCF-7에서 산화적 스트레스에 의한 카드뮴의 아폽토시스

오지영, 이수정, 신재호, 김태성, 문현주, 강일현 강태석, 김안근[1], 한순영[※]

식품의약품안전청 국립독성연구원 내분비장애물질과, [1]숙명여자대학교 약학대학

요 약

본 연구는 건전지나 플라스틱 등 산업물질, 식품, 흡연 그리고 공기, 물 등을 통해 인간과 생태계에 노출되고 있는 중금속 카드뮴을 인간 유방암 세포 MCF-7에 처리하였을 때 일어나는 현상을 살펴보고 나아가 카드뮴의 독성기전을 규명하기 위해 시행되었다. 카드뮴으로 인한 아폽토시스는 DNA분절 현상과 핵의 쪼개짐, 세포주기에 있어서 sub-G1의 출현 그리고 아폽토시스시에 발현되는 단백질 caspase의 발현, 특히 산화적 스트레스상태에서 마이토콘드리아가 손상을 입었을 때 발현되는 caspase-9의 발현을 통해 확인하였다. 카드뮴으로 인한 산화적 스트레스는 활성 산소종이 대조군에 비해 증가하고 이를 방어하기 위한 항산화효소 superoxide dismutase, catalase, glutathion reductase가 감소함을 통하여 확인하였다. 이상의 결과들을 통해 카드뮴은 인간 유방암 세포 MCF-7에서 산화적 스트레스를 유발시켜 아폽토시스를 일으키는 것으로 추정할 수 있다.

Key words: Cadmium, Apoptosis, Reactive oxygen species (ROS), Anti-oxidative enzyme.

INTRODUCTION

Apoptosis is a positive programmed form of cell death, which eliminates genetically damaged and infected cells from the body, which is important for the control of development and homeostasis in multi-cellular organisms (Li *et al.*, 2000; Owuor and Kong, 2002). The morphological and biochemical features of cells undergoing apoptosis are characterized by cell shrinkage, membrane blebbing, nuclei breaks, DNA fragmentation, and by protein expression as like Bcl-2 family proteins and caspases (Thornberry and Lazebnik, 1998). Oxidative stress is defined as an imbalance between pro-oxidants or free radicals and anti-oxidizing enzymes such as superoxide

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dismutase (SOD), glutathion peroxidase (GPx), catalase (CAT), glutathion (GSH) and glutathion reductase (GR) (Mates *et al.*, 1999). Reactive oxygen species (ROS), which include the free radical superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical (OH·), and non-free radical hydrogen peroxide (H_2O_2) are the byproducts of the normal cellular metabolism or are formed by extracellular environment challenges e.g., UV radiation, ionization and cigarette smoking (Kumar and Jugdutt, 2003). ROS has sufficient reactivity to cause protein oxidation and degradation, lipid peroxidation and DNA damage. Severe oxidative stress progressively leads to cell toxicity and ultimately to cell death (Bonnefoy *et al.*, 2002). Furthermore, ROS could be a signal for the initiation and execution of apoptosis (Carmody and Cotter, 2001).

Cadmium is a highly toxic metal to humans because it affects various metabolic processes, e.g., membrane transport system and protein synthesis. In addition, cadmium may act directly or indirectly on DNA and interfere with gene regulation and repair processes (Beyersmann and Hechtenberg, 1997; Hartwig, 1994; Hartwig and Beyersmann, 1989). Environmentally, human are exposed to cadmium as a food contaminant, in industrial products such as paints, plastics, batteries, and by inhaling cigarette smoke. Also, nature surrounding us as like air, soil, water could be an exposure route of cadmium.

Moreover, several reports have demonstrated that cadmium can induce apoptosis in many organs and cells, e.g., in the liver (Habeebu *et al.*, 1998), kidney (Hamada *et al.*, 1996), lung epithelial cells (Hart *et al.*, 1999), and immune cells (El Azzouzi *et al.*, 1994; Tsangaris and Tzortzatou-Stathopoulou, 1998). Also, Farris (1991) suggested that cadmium toxicity may be related to ROS production and could have a negative influence on the intracellular enzymatic system (Hussein *et al.*, 1987). Despite several researches, there is no report about cadmium-induced apoptosis in human breast cancer MCF-7 cells. So, we investigated the mechanism of cadmium-induced apoptosis particularly focused on ROS generation and enzymatic system.

MATERIALS AND METHODS

1. Chemicals

Cadmium chloride ($CdCl_2$) was purchased from Sigma (St. Louis, MO, USA). This reagent was dissolved in dimethylsulphoxide (DMSO), also from Sigma.

2. Cell culture conditions

MCF-7 cells were obtained from the ATCC (American Type Culture Collection) and grown in RPMI 1640 media (Gibco BRL, Grand Island, N.Y., USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL), 10,000 units/ml penicillin and 10 mg/ml streptomycin. Cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C and detached with 0.25% trypsin containing 1mM EDTA when 70% confluent.

3. MTT reduction assay

The MTT reduction assay was based on the ability of viable cells to reduce MTT reagent (Sigma) from a yellow water-soluble dye to a dark blue-insoluble formazan product (Julio, 1994). MCF-7 cells (1×10^4/well) were seeded in 96-well plates in medium containing $CdCl_2$ at various concentrations (0-200 μM) and incubated for 12 h. MTT (100 μl) was added to each well for 4 h and then dissolved in DMSO, and absorbance was measured at 570 nm using an ultra microplate reader (Bio-Tech Instruments, Inc., USA). Mean value of cell viability at each concentration was used to calculate the IC_{50} (the concentration required to reduce cell viability by 50%).

4. Tryphan blue assay

Cadmium treated cell suspension (1 ml) was mixed with 200 μl tryphan blue solution (0.4% w/v, Sigma) for 10 min and live cells were counted. Live cells were confirmed by dye-exclusion using a hemocytometer (Bright-Line, USA) under an optical micro-

scope (Olympus, Japan) (David, *et al.*, 1997).

5. Quantitative DNA fragmentation assay

We measured DNA fragmentation assay as one of the hallmarks of apoptosis (David, *et al.*, 1997). MCF-7 cells were plated in 100 cm^2 at a density of 10^5 cells/well and was treated with cadmium for 12 h. Suspensed cells was dissolved in lysis buffer [10mM Tris/HCl, 1 M NaCl, 10 mM EDTA, 0.6% (w/v) SDS, pH 8.0] and incubated overnight at 4°C. DNA was extracted twice using phenol: chloroform: isoamyl alcohol (25 : 24 : 1) (Sigma) and isopropyl alcohol. RNA was removed by incubating the above extracted pellet with RNase (10 μg/ml). Extracted DNA was washed with 70% ethanol and visualized by electrophoresis in a 1.5% agarose gel containing ethidium bromide in TAE buffer. The DNA in the gel was detected by using UV light (312 nm, Vilber lourmat, France) and was photographed the DNA mobility.

6. Morphological evaluation of nuclei

Cells were washed twice with cold PBS and fixed with formalin solution for 3 h. Following a second washing with PBS, cells were incubated with 4', 6-diamidino-2-phenylindole (DAPI, Sigma) solution (1 μg/ml) for 2h and apoptotic bodies were observed through fluorescence microscopy (Olympus, Japan) (David *et al.*, 1997).

7. Cell cycle analysis

To estimate that the proportion of MCF-7 cells in different phases of cell cycle was affected by cadmium, we conducted FACS scanning for DNA content of each cell cycle phase. Cells were harvested after chemical treatment and washed with PBS. Fixed cells (10^6/ml) with 70% ethanol were washed PBS and incubated with RNase (1 mg/ml) and PI (400 μg/ml, Sigma). Percentage of degraded DNA was examined by flow cytometry (Beckman Coulter, USA) using EPICS system II software (Ver. 3.0) (Julio, 1994). And then cell cycle was determined.

8. Detection of caspase-9 expression

After supernatant was separated by centrifugation, cells were resuspended in lysis buffer (0.5% triton X-100, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4) and cytoplasmic extracts were separated by centrifugation at 15,000 rpm for 15 min. Protein concentration was determined using the Bradford method (Bio-Rad protein assay kit, CA, USA). Same protein (20 μg/ml) was denaturated for 5 min at 92°C and transferred onto 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membrane (Bio-Rad) and blocked overnight with 5% skimmed milk in PBST [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20]. Membrane was then incubated overnight with primary anti-rabbit caspase-9 (1 : 500) (Santacruz, CA, USA) at 4°C. After incubation with goat anti-rabbit IgG-horseradish peroxidase secondary antibody (1 : 1,000) (Santacruz) for 2 h, membrane was developed by enhanced chemiluminescence (ECL, Amersham Biosciences, UK, England) (Julio, 1994).

9. Determination of peroxides production

The level of peroxides was determined by measuring Fe^{2+} oxidation at an acidic pH (Jiang *et al.*, 1991). A standard curve for tert-butyl hydroperoxide was prepared, 90% methanol reagent containing 4 mM butylated hydroxytoluene (BHT) and 125 μM xylenol orange was added to cytoplasmic fractions. After approximately 30 min at room temperature, absorbance was read at 560 nm and converted to peroxide concentration. Peroxides level was determined using a Peroxi Detect kit (Sigma), according to the manufacturer's instructions.

10. Measurement of anti-oxidative enzyme activities

1) Superoxide dismutase assay

SODs were measured using SOD kit (OxisResearch, Inc. USA). SODs were determined by measuring increased rate of autoxidation of 5, 6, 6a, 11b-

tetrahydro-3, 9, 10-trihydroxybenxofluorene at pH 8.8 (Nebot *et al.*, 1993). A mixture of 1, 4, 6-trimethyl-2-vinylpyridinium trifluoromethanesulfonate in HCl and the individual homogenized cytoplasmic enzyme samples were incubated for 1 min at 37°C and then 30 µl of 5, 6, 6a, 11b-tetrahydro- 3, 9, 10-trihydroxybenxofluorene in HCl containing diethylenetriaminepentaacetic acid (DTPA) and ethanol was added and absorbance read at 525 nm as 6sec intervals, 10 times. One unit of SODs activity was calculated using the following formula.

Units / ml = A / B

A: $0.93\{(V_S/V_C)-1\}$

B: $1.073 - 0.073 \times (V_S/V_C)$

(V_S: slope of the linear portion of the sample absorbance curve,

V_C: slope of the linear portion of the blank absorbance curve)

2) Catalase assay

CAT (EC 1.11.1.6) activities were determined using the CAT kit (OxisResearch). Catalase was messured by a quenched H_2O_2 amount by 4-aminophenazone 4-aminoantipyrene (AAP), 3, 5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) and horseradish peroxidase (HRP) (Wakimoto *et al.*, 1998; Aebi, 1984). Briefly, 500 µl sodium azide was added to cytosolic fraction containing 10 mM H_2O_2. Two ml of HRP/Chromogen reagent was then added and incubated for 10 min. Catalase activity was defined as the catalase enzyme's standard curve at 520 nm.

3) Glutathion reductase assay

This assay was performed using a GR kit (Sigma). GR (EC 1.6.4.2) was determined by following the reduced absorbance caused by the oxidation of NADPH (Garcia-Alfonso *et al.*, 1993). Briefly, the enzyme reaction was conducted in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. Absorbance of the mixed solution containing 2 mM EDTA, oxidized glutathion 500 µl, homogenated protein 100 µl and 2 mM NADPH 50 µl was read at 340 nm as 10 sec intervals, 10 times. The oxidation of NADPH was calculated using a mM extinction coefficient of 6.22 for NADPH.

11. Statistical analysis

Values are expressed as means ± S.D. from three independent experiments and statistical analysis was performed by student t-test (*Sigma stat*, ver. 2.03, Jandel Scientific Co., Erkrath, Germany).

RESULTS AND DISCUSSION

Cadmium induced-apoptosis has been observed *in vitro* and *in vivo* (Habeebu *et al.*, 1998; Yu and Chen, 2004). A number of theories about cadmium-induced apoptosis have been suggested. In genotoxicity respects, cadmium ions bind to nucleic acids or chromatin, and then cause conformational DNA changes, chromosomal aberrations, and DNA strand breaks (Hamada *et al.*, 1996; Habeebu *et al.*, 1998). When cells are exposed to cadmium, several genes with an important role in apoptosis are induced (Matsuoka and Call, 1995; Zheng *et al.*, 1996). In addition, cadmium causes lipid peroxidation (Stacey *et al.*, 1980), and may substitute for zinc in zinc-finger proteins (Hamada *et al.*, 1996), and ultimately lead to apoptosis. From the view of oxidative stress, the main point of our study, cadmium can affect on mitochondria (Koizumi *et al.*, 1994).

Initially, to evaluate the cytotoxic concentration of cadmium in human breast cancer MCF-7 cells, cells were plated in a medium and then treated with cadmium (0~200 µl) for 12 h. Cell viability was decreased in a dose-dependent manner. MCF-7 cells showed 50% cell viability at approximately 100 µM cadmium (Fig. 1A). In tryphan blue exclusion assay, dye exclusion was reduced dosedependently. In particular, cells excluding tryphan blue reagent were significantly reduced at 100 µM cadmium (Fig. 1B). To observe whether the cell death by cadmium is due to an apoptotic mechanism and to determine appro-

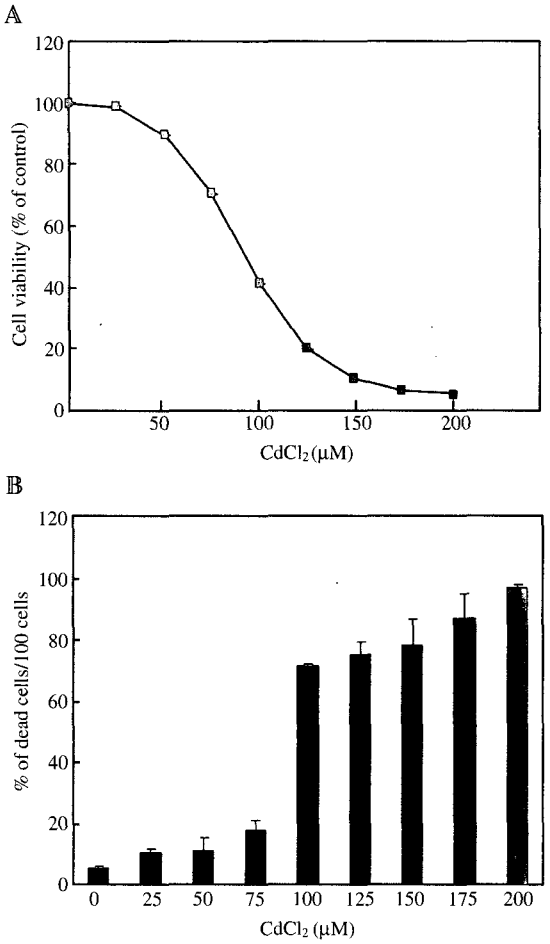

Fig. 1. Dose dependent course of MCF-7 cell viability after exposure $CdCl_2$. The cells were exposed to the various concentrations of $CdCl_2$ (0~200 μM) for 12h. Cell viability was determined by MTT assay (panel A). And dead cells were counted by tryhpan blue exclusion assay (panel B). Values on panel A show the mean (n = 6), and the % of dead cells among total 100 cells in panel B were obtained by triplicate experiments.

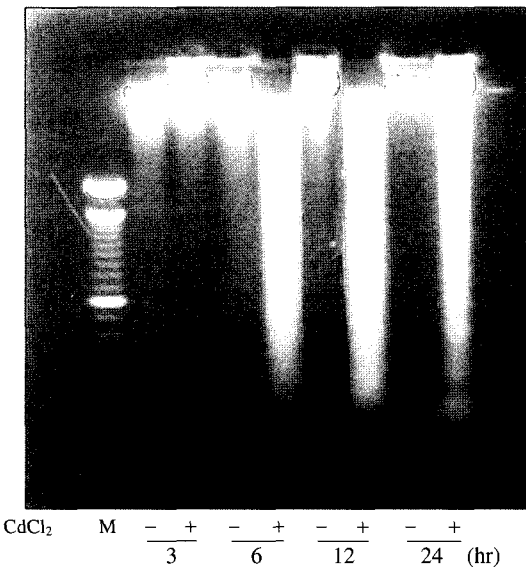

Fig. 2. Time dependent course of $CdCl_2$ induced DNA fragmentation. MCF-7 cells were treated with 100 μM $CdCl_2$ for various times (3, 6, 12 and 24 h). DNA migration was detected by eletrophoresis. Results are representative of three independent experiments (M: DNA 100 bp size marker).

Table 1. Occurrence of apoptotic bodies induced by cadmium

Concentration (μM)	% of apoptotic bodies
0	0.67±0.29
25	3.00±0.87
50	2.67±0.58
75	6.33±0.77
100	15.00±0.50
125	17.67±0.50
150	17.00±1.04
175	22.33±0.77
200	28.33±0.77

MCF-7 cells were exposed to various concentrations of $CdCl_2$ (0~200 μM) for 12h, Nuclei were stained with DAPI (1 μg/ml) and apoptotic cells were counted among 100 cells under a fluorescence microscope. % of apoptotic bodies is the mean value± S.D. of three independent experiments.

priate dose levels and times, DNA fragmentation, nuclei breaks, sub-G1 cell populations and caspase-9 expression were evaluated as apoptotic hallmarks. Fig. 2 illustrates that DNA fragmentation by cadmium gradually increased time-dependently and peaked 12 h after treating 100 μM cadmium. In addition, after fixing an experiment time of 12 h, we counted apoptotic bodies to study dose-dependent responses to cadmium. The number of nuclei breaks, cell shrinkage and nuclei condensation increased at higher cadmium concentrations (Table 1). As shown

in Fig. 3, the apoptotic bodies were dose-dependently increased when 100 μM cadmium was treated in MCF-7 cells. We used 100 μM cadmium for 12 h as an exposure in this study because we observed representative apoptotic features under this condition, which equated to cell viability of ca. 50%. In addition, we analyzed DNA content to confirm the

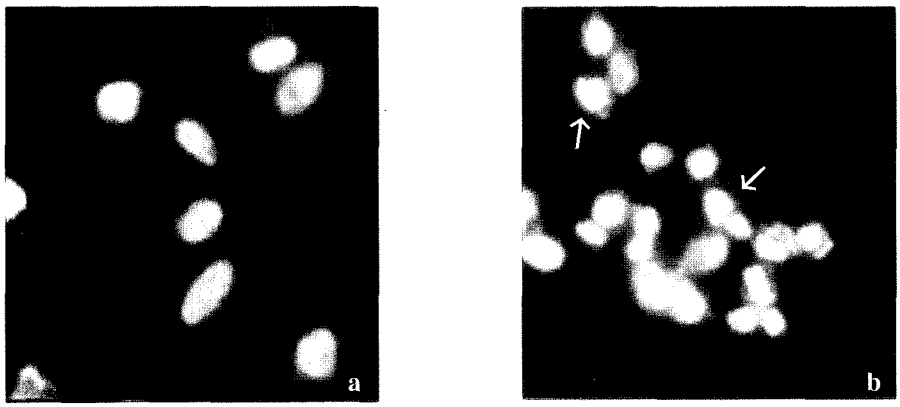

Fig. 3. Morphological change in nuclei. Cells were exposed to 100 μM $CdCl_2$ and stained with DAPI (1 μg/ml). Apoptotic bodies were observed under a fluorescence microscope (×400). Pictures are the representative of three independent tests and arrows point at the apoptotic bodies. (a: control, b: $CdCl_2$ 100 μM).

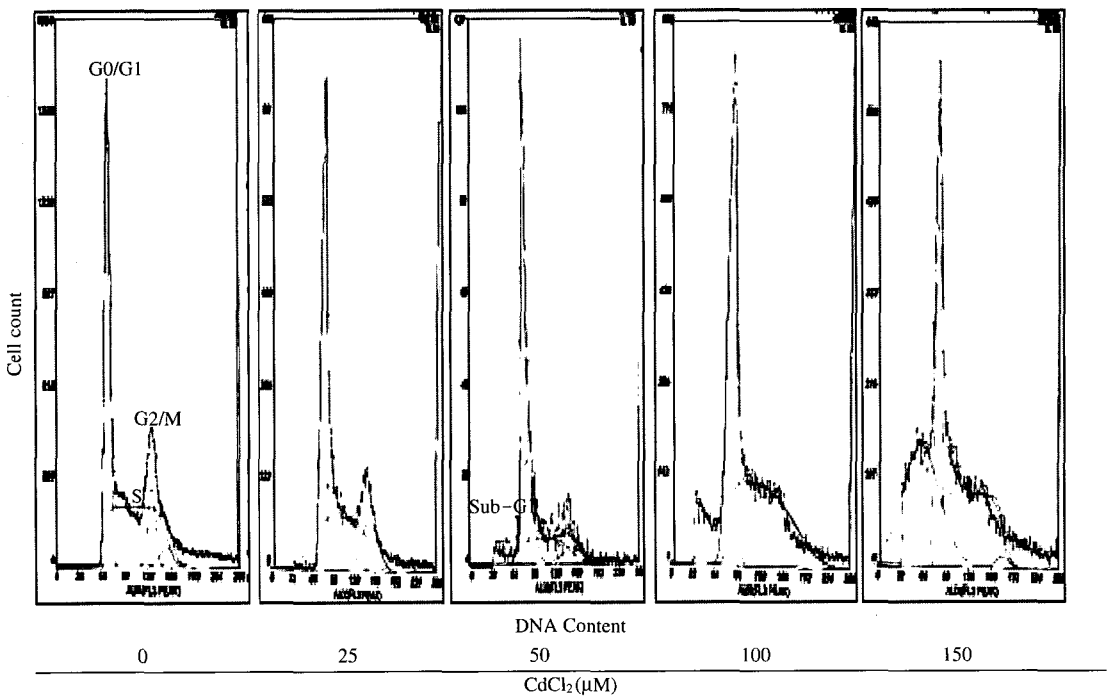

Fig. 4. Change of DNA content in the cell cycle. After exposing $CdCl_2$ (25, 50, 100 and 150 μM) for 12 h, degraded DNA was measured by flow cytometry using EPICS system II software (Ver. 3.0). The above data are representative of three independent tests.

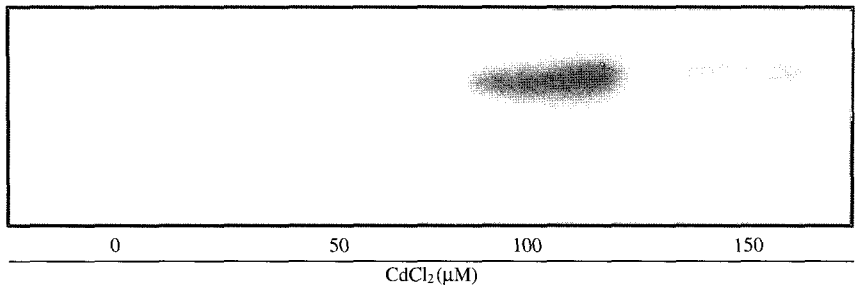

Fig. 5. Dose response course for caspase-9 expression. MCF-7 cells were exposed to $CdCl_2$ (50, 100 and 150 μM) for 12 h, Caspase-9 was detected by Western immuno-blotting. Results are representative of two independent experiments.

sub-G1 peak occurrence. The DNA content of sub-G1 was elevated on increasing cadmium concentration (Fig. 4).

We supposed that cadmium-induced apoptosis is partially induced through a mitochondrial dependent pathway. If cells are going to be under apoptosis, apoptosis-related protein such as caspase, cytochrome *c* is released from mitochondria. The caspases, a family of cysteine proteases, are major participants in apoptotic signal transduction (ElinosBaez *et al.*, 2003). These proteins exist in the cytosol as inactive proenzymes that become activated during apoptosis. Caspase-9 is apoptosis initiator protein and caspase-3 is executor protein that dose not exist in MCF-7 cells. Therefore, we detected mitochondria-dependent caspase-9 expression. When we exposed 50, 100 and 150 μM of cadmium for 12 h, we found caspase-9 expression at all doses. In particular, the expression of caspase-9 was strongly increased at 100 μM cadmium. However, the weak expression of caspase-9 at 150 μM cadmium was believed to be due to a change in the cell death pattern to necrosis from apoptosis (Fig. 5). We thought that ROS in mitochondria could be related with cell death. When MCF-7 cells were treated with cadmium (12 h, 100 μM), the peroxide level was elevated. When the cells were exposed to 100 μM $CdCl_2$ for 12 h, peroxides level showed the increasing tendency than control (Fig. 6). ROS induced by cadmium increases lipid peroxidation, thiobarbituric acid reactive substance formation, malondialdehyde in the liver, brain,

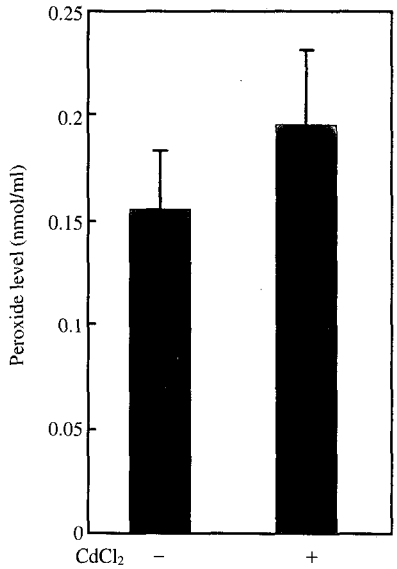

Fig. 6. Peroxides level measurement. MCF-7 cells were treated with $CdCl_2$ (100 μM) for 12 h. Peroxide levels were measured by oxidizing Fe^{2+}. Each column shows the mean value of three experiments ±S.D.

and lung (Manca *et al.*, 1991). Also, ROS directly affects protein and DNA (Mates *et al.*, 1999) and leads to apoptosis (Farris *et al.*, 1991). Cadmium has a negative effect on anti-oxidative enzymes that are the major scavengers of intracellular ROS (Hussein *et al.*, 1987). Several researches have approached the solution of cadmium toxicity as the special protein expression. Many detoxifying and cytoprotective proteins, i.e. metallothioneins, glutation, heat hock

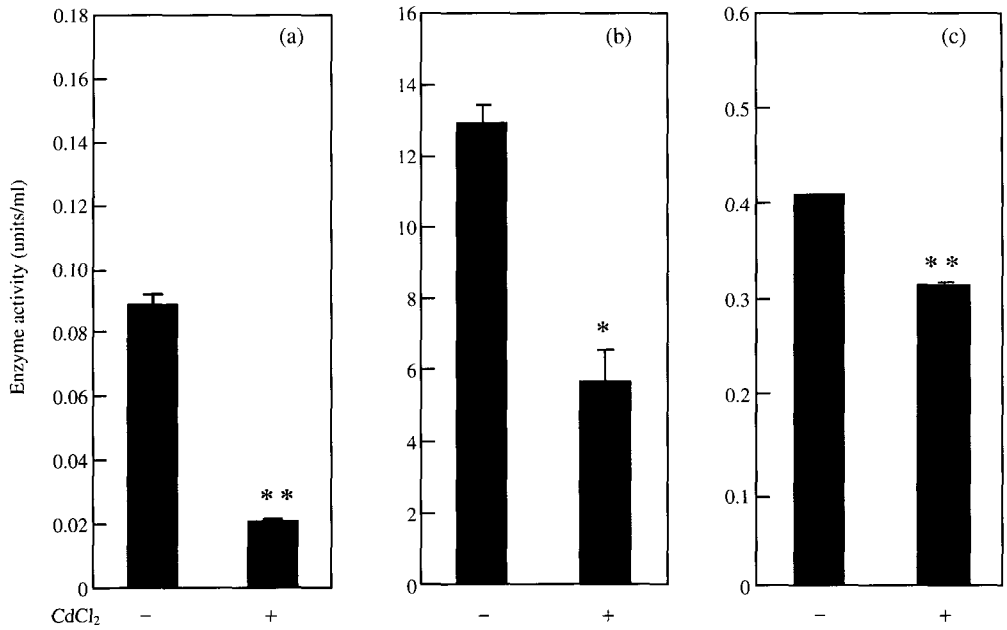

Fig. 7. Cellular anti-oxidative enzyme activities. Chemically treated MCF-7 cells (100 μM $CdCl_2$) were harvested, and their protein was extracted. (a) SODs activity was determined by measuring the increase of autoxidation of 5, 6, 6a, 11b-tetrahydro-3, 9, 10-trihydroxybenxofluorene at 525 nm. (b) The CAT activity was measured by determining the quenched H_2O_2 amount. (c) GR activity was detected by the oxidation of NADPH. Result are expressed as average of triplicate sample with S.D. **$P < 0.001$. *$P = 0.02$ compared with control.

proteins (Hsp), heme oxygenase, and zinc transporter proteins respond to cadmium exposure (Detmar, 2002). Anti-oxidative enzyme level is known to be a modulator of the apoptotic process (Stohs and Bagchi, 1995). If anti-oxidative enzyme level is low, harmful hydroxyl radicals or superoxide anions remain in the body (Pryor, 1986; Fleury *et al.*, 2002). However, cells are exposed to high dose of cadmium (100 μM), anti-oxidative enzyme system that could prevent from oxidative stress is demolished. After exposing cadmium (100 μM, 12 h), we found SOD, CAT, GR activities were reduced significantly than control (Fig. 7).

Through our study, we confirmed that cadmium could induce apoptosis and the mode of action may be associated with the mitochondria dependent apoptosis pathway, as evidenced by increased peroxides level and caspase-9 expression. In addition, our results suggest that increased ROS level and depleted anti-oxidative enzyme activities could be trigger factors for cadmium induced apoptosis in MCF-7 cells.

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