

Reactive oxygen species-dependent HSP90 protein cleavage participates in arsenical As^{+3} - and MMA^{+3} -induced apoptosis through inhibition of telomerase activity via JNK activation

Shing-Chuan Shen^a, Liang-Yo Yang^{b,c}, Hui-Yi Lin^a, Chin-Yen Wu^a,
Tsung-Hsien Su^d, Yen-Chou Chen^{a,e,*}

^a Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan

^b Department of Physiology and Graduate Institute of Neuroscience, Taipei Medical University, Taipei, Taiwan

^c Neuroscience Research Center, Taipei Medical University Hospital, Taipei, Taiwan

^d Mackay Memorial Hospital and Mackay Medicine, Nursing and Management College, Taipei, Taiwan

^e Cancer Research Center and Orthopedics Research Center, Taipei Medical University Hospital, Taipei, Taiwan

Received 22 October 2007; revised 19 December 2007; accepted 17 January 2008

Available online 31 January 2008

Abstract

The effects of six arsenic compounds including As^{+3} , MMA^{+3} , DMA^{+3} , As^{+5} , MMA^{+5} , and DMA^{+5} on the viability of NIH3T3 cells were examined. As^{+3} and MMA^{+3} , but not the others, exhibited significant cytotoxic effects in NIH3T3 cells through apoptosis induction. The apoptotic events such as DNA fragmentation and chromosome condensation induced by As^{+3} and MMA^{+3} were prevented by the addition of NAC and CAT, and induction of HO-1 gene expression in accordance with cleavage of the HSP90 protein, and suppression of telomerase activity were observed in NIH3T3 cells under As^{+3} and MMA^{+3} treatments. An increase in the intracellular peroxide level was examined in As^{+3} - and MMA^{+3} -treated NIH3T3 cells, and As^{+3} - and MMA^{+3} -induced apoptotic events were blocked by NAC, CAT, and DPI addition. HSP90 inhibitors, GA and RD, significantly attenuated the telomerase activity in NIH3T3 cells with an enhancement of As^{+3} - and MMA^{+3} -induced cytotoxicity. Suppression of JNKs significantly inhibited As^{+3} - and MMA^{+3} -induced apoptosis by blocking HSP90 protein cleavage and telomerase reduction in NIH3T3 cells. Furthermore, Hb, SnPP, and dexferosamine showed no effect against As^{+3} - and MMA^{+3} -induced apoptosis, and overexpression of HO-1 protein or inhibition of HO-1 protein expression did not affect the apoptosis induced by As^{+3} or MMA^{+3} . These data provide the first evidence to indicate that apoptosis induced by As^{+3} and MMA^{+3} is mediated by an ROS-dependent degradation of HSP90 protein and reduction of telomerase via JNK activation, and HO-1 induction might not be involved.

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Keywords: Arsenics; Apoptosis; ROS; Telomerase; Heme oxygenase 1; Heat shock protein 90

Introduction

Arsenic is a ubiquitous contaminant, and millions of people are at risk from drinking arsenic-contaminating water. Data of epidemiology studies have indicated that chronic exposure of humans to inorganic arsenic is positively correlated with hepatic damage, peripheral neuropathies, and cancers, particularly of the skin, liver, and bladder (Waalkes et al., 2004; Yoshida et al., 2004). The cytotoxic effects of arsenics have also been reported, and previous studies indicated that arsenic exposure causes ovarian dysfunction, aberrant embryo development, and postnatal growth retardation (Waalkes et al., 2003). In addition to previous

Abbreviations: NAC, *N*-acetyl-L-cysteine; HO-1, heme oxygenase-1; HSP90, heat shock protein 90; JNKs, c-Jun NH_2 -terminal kinases; ERKs, extracellular signal-regulated protein kinases; ROS, reactive oxygen species; DCHF-DA, dichlorodihydrofluorescein-diacetate; As^{+3} , arsenite; MMA^{+3} , monomethylarsonous acid; DMA^{+3} , dimethylarsinous acid; As^{+5} , arsenate; MMA^{+5} , monomethylarsonic acid; DMA^{+5} , dimethylarsinic acid; GA, geldanamycin; RD, radicicol, SnPP, tin protoporphyrin; DFX, desferoxamine; CAT, catalase; DPI, diphenyleneiodonium; LDH, lactate dehydrogenase; BSO, buthionine sulfoximine.

* Corresponding author. Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan. Fax: +886 2 23787139.

E-mail address: yc3270@tmu.edu.tw (Y.-C. Chen).

biological roles, arsenicals also have been used for many years as therapeutic agents, and arsenic trioxide (As_2O_3) has attracted great attention due to its efficiency in both newly diagnosed and relapsed patients with acute promyelocytic leukemia (Davison et al., 2004). Several previous studies demonstrated that arsenicals reduce the viability of cultured cells via apoptosis, and the formation of reactive oxygen species (ROS) has been shown in apoptosis induced by arsenicals (Liu et al., 2003). Our previous study demonstrated that As_2O_3 treatment induced apoptosis in NIH3T3 cells through production of ROS and activation of caspase cascades and the NADPH oxidase enzyme (Chen et al., 1998). Arsenic exists in inorganic and organic forms in different oxidation states, and both arsenate and arsenite are the more commonly known inorganic arsenic compounds. Monomethylarsonic acid (MMA^{+5}) and dimethylarsinic acid (DMA^{+5}) are stable methylated metabolites of inorganic arsenicals and are rapidly excreted in the urine. In addition, monomethylarsonous acid (MMA^{+3}) and dimethylarsinous acid (DMA^{+3}) have been detected in the urine of humans with chronic exposure to inorganic arsenic (Mandal et al., 2004). DMA^{+5} induces cellular toxicity and carcinogenesis through formation of ROS-dependent DNA damage in humans. MMA^{+3} and DMA^{+3} have high affinity for certain cellular proteins, and MMA^{+3} inhibits glutathione reductase, NADPH-dependent oxidoreductase, and thioredoxin reductase (Chou et al., 2004; Sakurai et al., 2002). Aposhian et al. (2000) indicated that MMA^{+3} exists in the urine of some humans exposed to inorganic arsenic in drinking water. However, little is known about the toxicity of MMA^{+3} and DMA^{+3} .

Telomeres are the nucleoprotein structures at the termini of chromosomes which are composed of simple repetitive G/C-rich DNA. Telomeres play an important role in maintaining chromosome stability through inhibiting the degradation of chromosome ends, and chromosomes shorten during active cell division and triggering of apoptosis was observed when the telomere length became too short. Telomerase, a ribonucleoprotein reverse transcriptase, has been shown to maintain telomere length. Activation of telomerase is necessary for the continued growth of several human tumors such as lung cancer, colorectal carcinoma, and hepatocarcinoma, and plays a critical role in tumorigenesis (Deng and Chang, 2007; Goldkorn and Blackburn, 2006; Smith and Higgs, 1999). Zhang et al. (2003a) showed that arsenic (As_2O_3) inhibits the proliferation of human keratinocytes by reducing telomerase enzyme activity. Chou et al. (2001) indicated that arsenic treatment suppresses telomerase gene expression at the messenger (m)RNA level, which leads to chromosomal lesions in vitro. However, upregulation of telomere-binding factors, TRF1 and TRF2, by As_2O_3 was also observed in human gastric cancer MGC-803 cells (Zhang et al., 2005). The relationships of the effects of arsenicals and their methylated metabolites on the activity of telomerase enzyme are still undefined.

Heme oxygenase (HO) and heat shock protein 32 (HSP32) are rate-limiting enzymes in the metabolism of heme, during which process, biliverdin, carbon monoxide (CO), and free iron are generated. HO-1 is an inducible form of HO and is induced by various oxidative agents such as heme, hyperoxia, and arsenicals. The functions of HO-1 induction have been shown by examining the ability of HO-1-overexpressing cells to resist

various stress insults, and this suggests that induction of HO-1 elicits an important cytoprotective role against oxidative damages (Kikuchi et al., 2005; Liu et al., 2005). Our previous studies showed that induction of HO-1 protected glial cells and macrophages from H_2O_2 - or chemical anoxia-induced apoptosis, and inhibited lipopolysaccharide- (LPS) or lipoteichoic acid (LTA)-induced nitric oxide (NO) production in macrophages (Chen et al., 2006; Lin et al., 2007). Heat shock protein 90 (HSP90) is one of the most abundant cellular proteins under non-stressful conditions, and contributes to several biological processes such as signal transduction, protein folding, and protein degradation. HSP90 might confer a survival advantage to tumor cells through association with several regulators of apoptosis (Zhang et al., 2007). HSP90 plays antiapoptotic roles through regulating the Akt-mediated survival cascade (Pespeni et al., 2007). Lewis et al. (2000) demonstrated that overexpression of HSP90 protein in NIH3T3 cells suppresses tumor necrosis factor α ($\text{TNF}\alpha$)-induced apoptosis. Interfering with the caspase apoptotic pathway and preventing the cleavage of Bid proteins are possible antiapoptotic actions of HSP90. The role of HSP90 in arsenic-induced apoptosis is still undefined.

We show that oxidative stress induced by As^{+3} and MMA^{+3} , but not As^{+5} , MMA^{+5} , DMA^{+5} , or DMA^{+3} , reduced the viability of NIH3T3 fibroblasts via apoptosis induction, inhibition of telomerase activity, and induction of HSP90 protein cleavage and HO-1 protein expression. The antioxidants, *N*-acetylcysteine (NAC), catalase (CAT), and diphenyleneiodonium (DPI), effectively prevented As^{+3} - and MMA^{+3} -induced ROS production, telomerase inhibition, HSP90 protein cleavage, and HO-1 protein induction. The roles of telomerase, HSP90, and HO-1 in As^{+3} - and MMA^{+3} -induced apoptosis via an ROS-dependent manner are first demonstrated herein.

Materials and methods

Cell culture. NIH3T3 cells from American Type Culture Collection (Manassas, VA) were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin), and 10% heat-inactivated fetal bovine serum (FBS), and maintained at 37 °C in a humidified incubator containing 5% CO_2 .

Chemicals. Arsenite (As^{+3}) and arsenate (As^{+5}) (sodium salts, at least 99% pure) were purchased from Sigma-Aldrich (St. Louis, MO). Monomethylarsonous acid (MMA^{+3}), dimethylarsinous acid (DMA^{+3}), monomethylarsonic acid (MMA^{+5}), and dimethylarsinic acid (DMA^{+5}) were obtained from Chem Service (West Chester, PA). (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), and 2',7'-dichlorodihydrofluorescein-diacetate (DCHF-DA), NAC, and CAT were obtained from Sigma Chemical (St. Louis, MO). The antibodies of HO-1, pERK, tERK, tJNK, Cyt *c* were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies for detecting HSP90 and HSP110 proteins were obtained from BD Biosciences (Franklin Lakes, NJ). The pJNK antibody was obtained from Cell Signaling (Beverly, MA). PD98059, SB203580, SP600125, and LY294002 were obtained from Calbiochem (La Jolla, CA).

Cell viability. MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well in 24-well plates for 12 h, followed by treatment with indicated compounds for a further 12 h. Cells were washed with phosphate-buffered saline (PBS) three times, and MTT (50 mg/ml) was added to the medium for 4 h. Then, the supernatant was removed, and the formazone crystals were

dissolved using 0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an enzyme-linked immunosorbent assay (ELISA) analyzer (Dynatech MR-7000; Dynatech Laboratories, Alexandria, VA).

LDH release assay. The percentage of LDH release was expressed as the proportion of LDH released into the medium compared to the total amount of LDH present in cells treated with 2% Triton-X-100. The activity was monitored as the oxidation of the reduced form of nicotinamide-adenine dinucleotide (NADH) at 530 nm by an LDH assay kit (Roche, Indianapolis, IN, USA). Cytotoxicity was determined by the equation: $[(\text{OD}_{530} \text{ of the treated group} - \text{OD}_{530} \text{ of the control group}) / (\text{OD}_{530} \text{ of the Triton X-100-treated group} - \text{OD}_{530} \text{ of the control group})] \times 100\%$.

DNA gel electrophoresis. Cells under different treatments were collected, washed with PBS twice, and lysed in 80 μl of lysis buffer (50 mM Tris (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium sarkosinate, and 1 mg/ml proteinase K) for 3 h at 56 °C and then treated with 0.5 mg/ml RNase A for another hour at 56 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting-point agarose, and 0.025% (w/w) bromophenol blue) and loaded onto a pre-solidified 2% agarose gels containing 0.1 mg/ml ethidium bromide. The agarose gels were run in TBE buffer and observed and photographed under UV light (Chen et al., 2006). Data of DNA analysis have been repeated at least three times using the DNA samples derived from three-independent experiments.

Western blotting. Total cellular extracts were prepared as described in our previous paper (Chen et al., 2006), and an equal amount of proteins from each group was separated on 8%–12% sodium dodecylsulfate (SDS)-polyacrylamide minigels, followed by transfer to Immobilon polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, Mass., USA). Membranes were incubated with 1% bovine serum albumin (BSA) and then incubated with specific antibodies including anti-HO-1, anti-tJNK, anti-pJNK, anti-pERK, anti-tERK, anti-HSP90, anti-caspase 9, and anti-HSP110 overnight at 4 °C. Expression of indicated proteins including HO-1, tJNK, pJNK, tERK, pJNK, HSP90, caspase 9, and HSP110 was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma). Data of Western blotting have been repeated at least three times using the proteins derived from three-independent experiments.

Telomerase activity assay. Cells were treated with the indicated compounds for 12 h. Total cell extracts were prepared as described (Kim and Wu, 1997). Briefly, ice-cold lysis buffer (10 mM Tris-HCl (pH 7.5), 1 mM MgCl_2 , 1 mM ethyleneglycoltetraacetic acid (EGTA), 10% glycerol, 0.5% 3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulfonate (CHAPS), 0.1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride (PMSF), and 5 mM β -mercaptoethanol) were added to cells and stored at –20 °C. Protein (0.5 μg) was taken from the cell lysate, and 0.1 μg TS primer (AATCCGTCGAGCAGAGTT), 5 μl of 10 $\times$ reaction buffer (200 mM Tris-HCl (pH 8.0), 15 mM MgCl_2 , 630 mM KCl, 0.05% Tween-20, and 10 mM EGTA), and 46 μM dNTP were added at 30 °C for 30 min and then 85 °C for 5 min to stop the reaction. CX primer (0.1 μg CCCTTACCCTTACCCTTACCCTAA), 0.1 μg TSNT primer (AATCCGTCGAGCAGAGTTAAAGGCCGAGAAGC), and 0.1 μg NT primer (ATCGTCTCTCGGCCTTTT) were added to the mixture, and then the polymerase chain reaction (PCR) was run with 15 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Finally, 15 μl of the reaction mixture was used to run on 12% non-denatured polyacrylamide minigels in 1 $\times$ TBE with visualization by silver staining (GE Healthcare Biosciences AB, Uppsala, Sweden). Data of TRAP assay have been repeated at least three times using the proteins derived from three-independent experiments.

Reverse-transcriptase (RT) PCR. Cells were treated with the indicated compounds for 6 h and then washed with ice-cold PBS. Total RNA was isolated using a total RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the total RNA concentration was detected using a spectrophotometer. Total RNA (2 μg) was converted to complementary (c)DNA with oligo d(T). The PCR was performed on cDNA using the following respective sense and antisense primers for HO-1: CTGTGTAACCTCTGCTGTCC and CCACACTACCTGAGTCTACC amplifying a 667-bp product, hTERT: CGGAAGAGTGCTGTG-

GAGCAA and GGATGAAGCGGAGTCGGA (146 bp), and GAPDH: TGAAGGTCGGTGTGAACGGATTGGC and CATGTAGGCCATGAGGTCCACCAC (983 bp). The PCR of the cDNA was performed in a final volume of 50 μl containing the PCR primers, oligo (dT), total RNA, and DEPC H_2O using RT-PCR beads (Amersham Pharmacia). The amplification sequence protocol was 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s. The PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining (Lin et al., 2007). Data of RT-PCR have been repeated at least three times using the RNA samples derived from three-independent experiments.

Determination of ROS production (DCHF-DA assay). Production of reactive oxygen species (ROS) was monitored by flow cytometry using DCHF-DA. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped within cells. Hydrogen peroxide or low-molecular-weight hydroperoxides produced by cells oxidize DCHF to the highly fluorescent compound, 2',7'-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. The DCHF-DA (100 μM) green fluorescence was excited using an argon laser and was detected using a 525-nm (FL1-H) band-pass filter by flow cytometric analysis.

Measurement of the mitochondrial membrane potential. Cells under different treatments were incubated with DiOC6(3) (40 nM) at 37 °C for 30 min, and washed with cold PBS. The cell pellet was collected by centrifugation at 500 $\times g$ for 10 min, and resuspended in 500 μl of PBS. Fluorescence intensities of DiOC6(3) in cells were analyzed by flow cytometric analysis (FACScan, Becton Dickinson, San Jose, CA, USA) with excitation and emission settings of 484 and 500 nm, respectively (Chow et al., 2006).

Cytochrome c release from mitochondria of NIH3T3. Both mitochondrial and cytosolic fractions were prepared for detection of cytochrome c (Cyt c) protein expression by Western blotting. Cells under different treatments were washed once with ice-cold PBS, and resuspended in 5 volumes of hypotonic buffer (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose). Cells were homogenized and centrifuged at 750 $\times g$ for 10 min at 4 °C. The supernatants were centrifuged at 10,000 $\times g$ for 15 min at 4 °C, and the supernatant (cytosolic fraction) and the pellets (mitochondrial fraction) were collected. The pellet was dissolved in 0.1 ml of lysis buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.15 M NaCl, 5 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 mM PMSF, 2 mM sodium orthovanadate, and 1% SDS). The expressions of Cyt c in both the mitochondrial and cytosolic fractions were analyzed by Western blotting. Data of Cyt c protein detection have been repeated at least three times using the proteins derived from three-independent experiments.

Establishment of HO-1 transfectants. pCMV-HO-1, a constitutive expression vector, carries full-length human HO-1 cDNA under control of the CMV promoter/enhancer sequence. We transfected pCMV-HO-1 or pCMV into cells using the Transfast™ transfection reagent (Invitrogen, Carlsbad, CA, USA). After 48 h, cells were trypsinized and replated in DMEM with 10% FBS and 400 $\mu\text{g}/\text{ml}$ G418. G418-resistant cells were selected and expanded. The level of HO-1 was analyzed by Western blotting.

Transfection of HO-1 oligonucleotides, dominant-negative JNK plasmids, and HO-1 siRNA. HO-1 sense and antisense oligonucleotides were directed against the flanking translation initiation codon and six base pairs on either side of the mouse HO-1 cDNA, and modified with phosphorothioate. The sequence of the HO-1-specific antisense oligonucleotide was 5'-ACGCTCCATCACCAG-3', and that of the sense oligonucleotide was 5'-CCGGTGATGGAGCGT-3'. Briefly, cells were placed in serum-free medium and then transfected with the phosphorothioated specific sense or antisense oligonucleotides (1 μg), dominant-negative JNK plasmids, or HO-1 siRNA using the Transfast™ transfection reagent (Promega) for 48 h. After 48 h of incubation, cells were treated with the indicated compounds for an additional 12 h, and the expressions of HO-1 and JNK protein were analyzed by Western blotting.

Statistical analysis. Values are expressed as the mean $\pm$ S.E. The significance of the difference from the respective controls for each experimental test

condition was assayed using Student's *t*-test for each paired experiment. A *p* value <0.05 or 0.01 was regarded as indicating a significant difference.

Results

Differential apoptotic effects of arsenic metabolites including As^{+3} , MMA^{+3} , DMA^{+3} , As^{+5} , MMA^{+5} , and DMA^{+5} on NIH3T3 cells

We examined the effect of arsenic metabolites on the viability of NIH3T3 cells by MTT and LDH release assays. In NIH3T3 cells, As^{+3} and MMA^{+3} , but not DMA^{+3} , As^{+5} , MMA^{+5} , or DMA^{+5} , produced effective dose-dependent cytotoxic effects according to the MTT assay (Figs. 1A, B). Data from the LDH release assay showed that As^{+3} and MMA^{+3} at the doses of 5, 10, 20, and 40 μM

induced LDH release into the medium (Fig. 1C). We further examined if the reduction in cell viability by As^{+3} and MMA^{+3} occurs through apoptosis. Results of Fig. 1D show that As^{+3} and MMA^{+3} (but not DMA^{+3} , As^{+5} , MMA^{+5} , or DMA^{+5}), at a dose of 10 μM , induced DNA fragmentation in NIH3T3 cells. DNA ladders induced by As^{+3} and MMA^{+3} were inhibited by the addition of the antioxidants, NAC and CAT (Fig. 1E). The prevention of NIH3T3 cells from As^{+3} - and MMA^{+3} -induced cytotoxic effects by NAC and CAT was detected by the MTT assay (Fig. 1F). Results of microscopic observations showed that an occurrence of condensed chromatin was detected in the nuclei of As^{+3} - and MMA^{+3} -treated NIH3T3 cells via Giemsa staining (Fig. 1G). These data indicate that As^{+3} and MMA^{+3} possess the ability to reduce the viability of NIH3T3 cells via apoptosis induction.

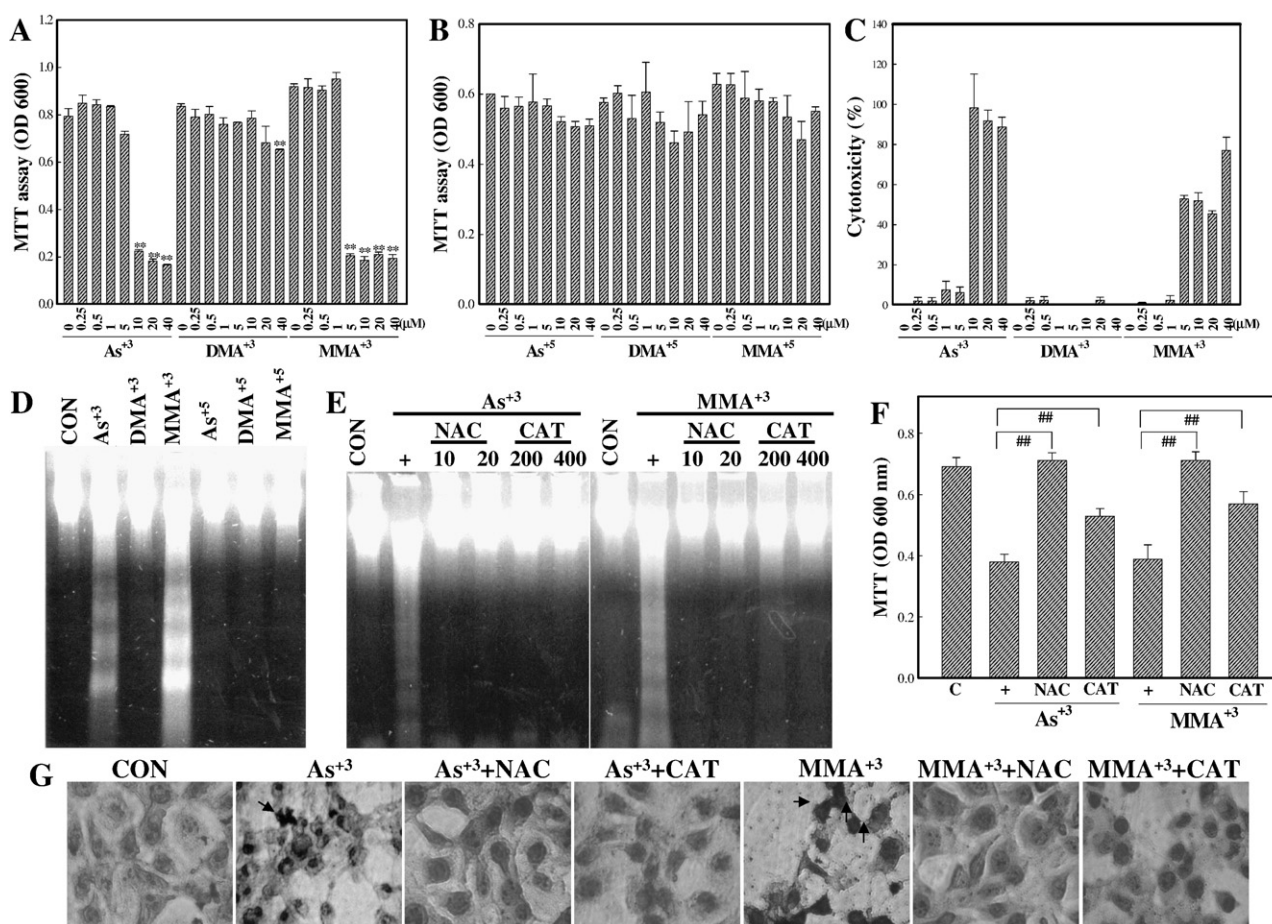


Fig. 1. As^{+3} and MMA^{+3} induction of apoptosis in NIH3T3 cells. (A) As^{+3} and MMA^{+3} , but not DMA^{+3} , induction of cell death in NIH3T3 cells. NIH3T3 cells were treated with different doses of the indicated compounds for 12 h, and the viability of cells was detected by the MTT assay as described in Materials and methods. (B) Neither As^{+5} , MMA^{+5} , nor DMA^{+5} reduced the viability of NIH3T3 cells. As described in (A), the viability of NIH3T3 cells under different treatments was analyzed by the MTT assay. Data were derived from three-independent experiments, and are analyzed and expressed as the mean $\pm$ SE. (C) As^{+3} and MMA^{+3} induced LDH release in NIH3T3 cells. As described in (A), the amount of LDH in the medium was examined, and data are expressed as the cytotoxicity (%). (D) As^{+3} and MMA^{+3} , but not the others, induced DNA fragmentation in NIH3T3 cells. Cells were treated with the indicated compounds (10 μM) for 12 h, and the integrity of DNA was analyzed. (E, F) *N*-acetyl-L-cysteine (NAC) and catalase (CAT) prevented As^{+3} - and MMA^{+3} -induced cytotoxic effects by reducing DNA fragmentation. Cells were treated with different doses of NAC (10 and 20 mM) or CAT (200 and 400 U/ml) for 30 min, and extracellular NAC and CAT was removed by washing three times with fresh medium, followed by As^{+3} or MMA^{+3} (10 μM) treatment for an additional 12 h. The integrity of DNA (E) and the cytotoxicity (F) of cells under different treatments were examined by DNA electrophoresis and MTT assay, respectively. Data were derived from three-independent experiments and are expressed as the mean $\pm$ SE. (G) NAC (10 mM) and CAT (100 U/ml) suppressed chromatin condensation elicited by As^{+3} and MMA^{+3} (10 μM). As described in (E), chromatin-condensed cells were examined by Giemsa staining under microscopic observations. ** *p* < 0.01 indicates a significant difference from control group (0), and ## *p* < 0.01 indicates a significant difference between the indicated groups by Student's *t*-test.

Induction of HO-1 gene expression, HSP90 protein cleavage, and reduction of telomerase activity in As³⁺- and MMA³⁺-treated NIH3T3 cells

We examined the effect of inorganic and organic arsenics on the expressions of HO-1 protein and HSP90 protein, and telomerase activity in NIH3T3 cells. As illustrated in Fig. 2A, 10 μ M As³⁺ and MMA³⁺ significantly induced HO-1 protein expression with the occurrence of cleaved fragments of HSP90 protein in NIH3T3 cells; however, HSP110 protein remained unchanged. In the same part of the experiment, As³⁺ and MMA³⁺ time-dependently induced HO-1 protein expression and cleaved fragments of HSP90 in NIH3T3 cells without affecting HSP110 protein expression (Fig. 2B). We further analyzed the telomerase activity in NIH3T3 cells under different treatments by the TRAP assay. Data of Fig. 2C (left panel) show that As³⁺ and MMA³⁺, but not the others, significantly reduced the telomerase activity of NIH3T3 cells. Inhibition of hTERT mRNA expression by As³⁺ and MMA³⁺ was identified in NIH3T3 cells by RT-PCR analysis (Fig. 2C, right panel). Additionally, an increase in the HO-1 mRNA level was detected in As³⁺- and MMA³⁺-treated NIH3T3 cells by RT-PCR using specific primers, and GAPDH was used as an internal control to verify that the same amount of RNA was in each lane (Fig. 2D). A noncytotoxic dose (2 μ M) of As³⁺ or MMA³⁺ showing no effect on HO-1 protein expression, DNA fragmentation, and HSP90 protein cleavage was detected in NIH3T3 cells (data not shown).

As³⁺ and MMA³⁺ induction of HO-1 protein expression, HSP90 protein cleavage, and inhibition of telomerase activity occur through stimulating ROS production in NIH3T3 cells

Our previous study indicated that an increase in ROS production participated in As³⁺-induced apoptosis (Chen et al., 1998). Buthionine sulfoximine (BSO) is an inhibitor of γ -glutamylcysteine synthetase to block the biosynthesis of glutathione. In the present study, incubation of NIH3T3 cells with BSO, an inhibitor enhances the cytotoxic effect of a low dose (2 μ M) of As³⁺ and MMA³⁺ by MTT assay (Fig. 3A). We further examined the intracellular peroxide level in As³⁺- and MMA³⁺ (10 μ M)-treated NIH3T3 cells by DCHF-DA assay. Data of Fig. 3B show that a significant elevation in the intracellular peroxide level was detected in As³⁺- and MMA³⁺-treated NIH3T3 cells, which was blocked by the addition of NAC or CAT. Incubation of NIH3T3 cells with NAC dose-dependently attenuated As³⁺- and MMA³⁺-induced HO-1 protein expression and HSP90 protein cleavage by Western blotting (Fig. 3C; upper panel). Equal amounts of proteins loaded in each lane were examined by Coomassie blue staining (Fig. 3C; lower panel). Data of the TRAP assay indicated that NAC addition inhibited telomerase reduction elicited by As³⁺ and MMA³⁺ (Fig. 3D). Inhibition of As³⁺- and MMA³⁺-induced HO-1 protein and HSP90 protein cleavage with increasing telomerase activity by CAT was also observed (data not shown). These data support ROS generation being involved in As³⁺- and MMA³⁺-

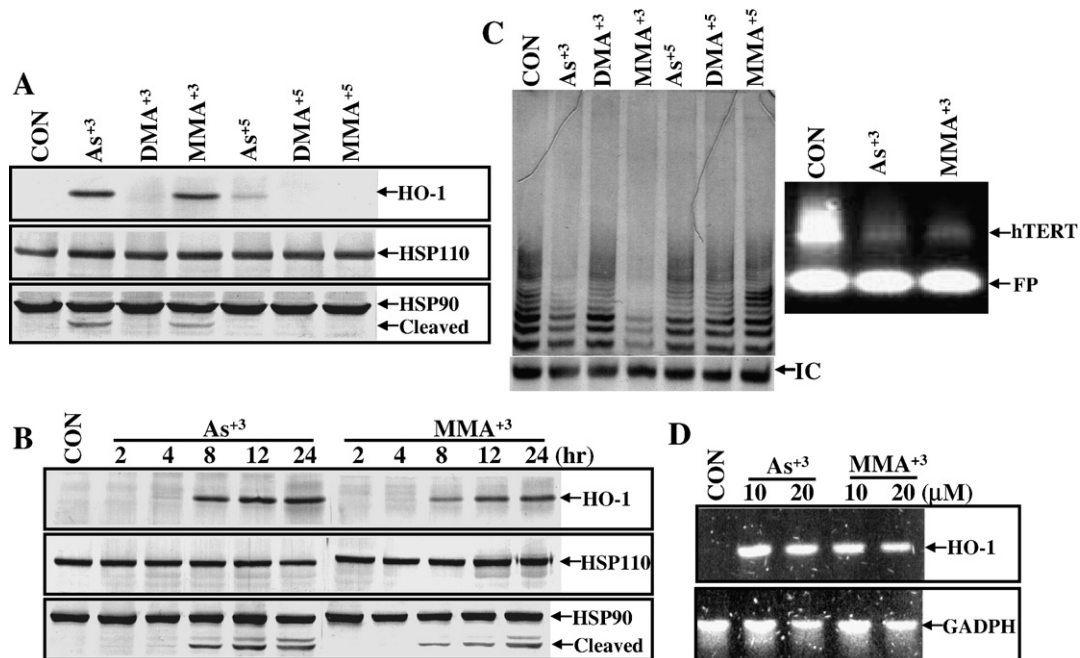


Fig. 2. Induction of heme oxygenase (HO)-1 gene expression at both the protein and mRNA levels, inhibition of telomerase activity with reducing hTERT gene expression, and induction of heat shock protein (HSP)90 cleavage were detected in As³⁺- and MMA³⁺-treated NIH3T3 cells. (A) As³⁺ and MMA³⁺, but not the others, induced HO-1 and HSP90 protein cleavage in NIH3T3 cells. Cells were treated with the indicated compounds (10 μ M) for 12 h, and the expressions of HO-1, HSP110, and HSP90 proteins were examined by Western blotting using specific antibodies. (B) As³⁺ and MMA³⁺ time-dependently induced the expression of HO-1 and cleaved the HSP90 protein. Cells were treated with As³⁺ or MMA³⁺ (10 μ M) for different times, and the expressions of HO-1, HSP110, and HSP90 proteins were analyzed by Western blotting. (C) As³⁺ and MMA³⁺ suppressed telomerase activity and hTERT mRNA expression. (Left panel) As described in A, the telomerase activity of NIH3T3 cells under different treatments was analyzed by the TRAP method as described in Materials and methods. (Right panel) Cells were treated with As³⁺ and MMA³⁺ for 6 h, and the expression of hTERT mRNA was examined by RT-PCR. FP, free probe. (D) As³⁺ and MMA³⁺ induced HO-1 mRNA expression in NIH3T3 cells. Cells were treated with As³⁺ or MMA³⁺ (10 and 20 μ M) for 6 h, and the expression of HO-1 mRNA was detected by RT-PCR.

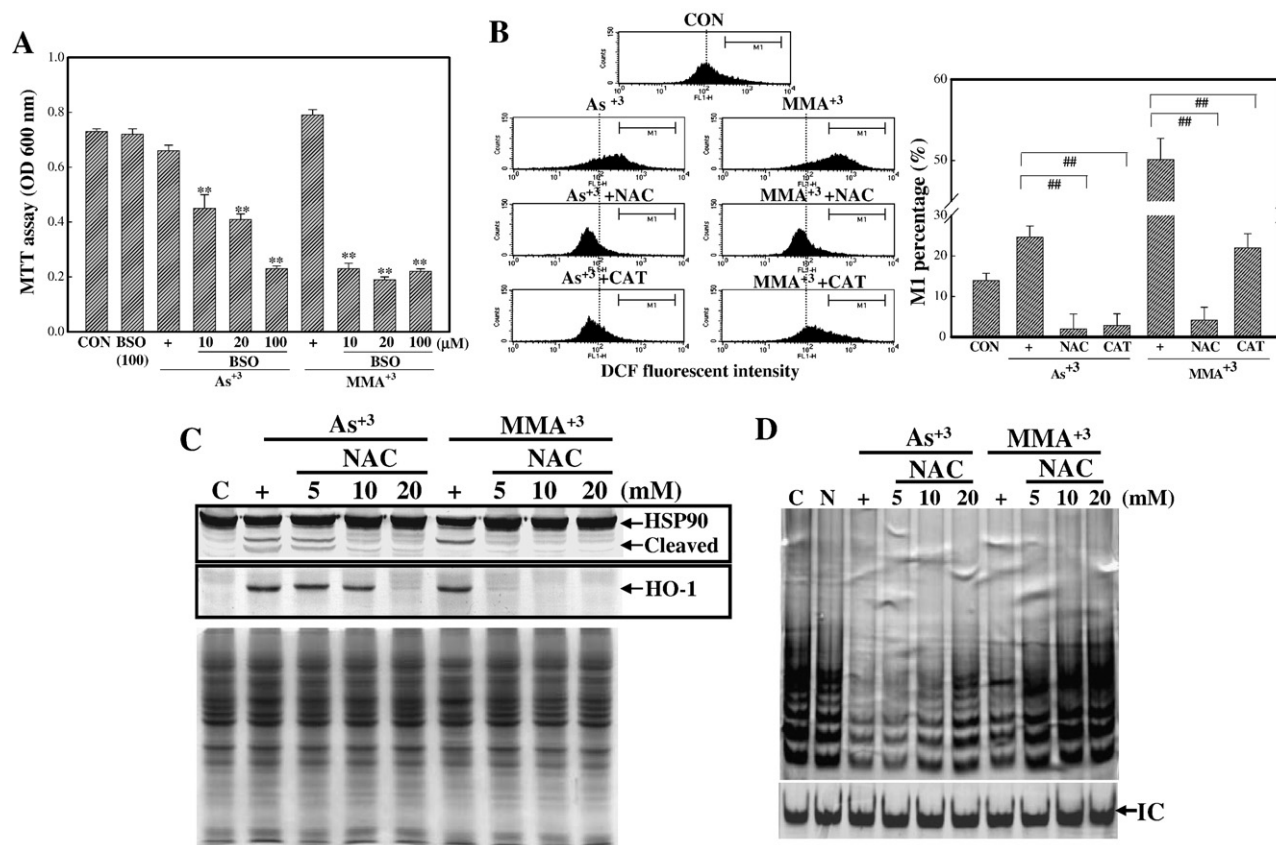


Fig. 3. Reactive oxygen species (ROS) production is involved in As^{+3} - and MMA^{+3} -induced apoptosis of NIH3T3 cells. (A) BSO enhancement of As^{+3} - and MMA^{+3} (2 μ M)-induced cytotoxic effect in NIH3T3 cells. Cells were treated with indicated BSO (10, 20, and 100 μ M) for 30 min followed by As^{+3} and MMA^{+3} treatment for 12 h. The viability of cells under different treatments was analyzed by MTT assay. (B) *N*-acetyl cysteine (NAC) and catalase (CAT) inhibited As^{+3} - and MMA^{+3} -induced peroxide production in NIH3T3 cells. Cells were treated with or without NAC (10 mM) or CAT (100 U/ml) for 30 min, then washed three times with fresh medium to remove NAC or CAT, followed by As^{+3} or MMA^{+3} (10 μ M) treatment for a further 2 h. The peroxide level was detected by a DCHF-DA assay via flow cytometric analysis. (Left panel) A representative of the flow cytometric analysis; (right panel) data derived from three-independent experiments are analyzed and expressed as the mean $\pm$ SE. (C) NAC addition inhibited As^{+3} - and MMA^{+3} -induced HO-1 and HSP90 protein cleavage. As described in (A), expressions of the indicated proteins were examined by Western blotting after As^{+3} or MMA^{+3} treatment for 12 h. (D) NAC inhibition of As^{+3} - and MMA^{+3} -reduced telomerase activity in NIH3T3 cells. As described in (C), the telomerase activity under different treatments was evaluated by the TRAP assay. Data were derived from three-independent experiments and are expressed as the mean $\pm$ SE. ** p < 0.01 indicates a significant difference from control group (CON), ## p < 0.01 indicates a significant difference between the indicated group by Student's *t*-test.

induced HO-1 protein expression, HSP90 protein cleavage, and a decrease in telomerase activity.

A decrease in mitochondria permeability in As^{+3} - and MMA^{+3} -induced apoptosis in NIH3T3 cells via ROS production

A decrease in mitochondria permeability has been shown in As^{+3} -induced apoptosis; however, it is still uncertain if mitochondrial dysfunction is involved in MMA^{+3} -induced apoptosis. DiOC6 was used to examine the mitochondrial membrane potential in the present study via flow cytometric analysis. Data of the flow cytometric analysis indicated that As^{+3} and MMA^{+3} treatments reduced the loss in mitochondrial membrane potential in NIH3T3 cells, which was blocked by the addition of NAC (Fig. 4A). A release of Cyt *c* from mitochondria (M) to the cytosol (C) and a decrease in pro-caspase 9 protein were detected in mitochondrion-dependent apoptosis. Therefore, expression of

the Cyt *c* protein in both the mitochondrial and cytosolic fractions, and pro-caspase 9 protein level in cell lysate under different treatments were examined by Western blotting. Data of Fig. 4B show that As^{+3} and MMA^{+3} treatments induced the release of Cyt *c* from mitochondria to the cytosol, and a decrease in pro-caspase 9 protein, and those events were attenuated by the addition of NAC.

NADPH oxidase inhibitor, DPI, inhibits As^{+3} - and MMA^{+3} -induced apoptosis by blocking HO-1 protein expression, HSP90 protein cleavage, and telomerase activity

DPI was shown to block As^{+3} -induced cell death in our previous study; however, the effect of DPI on As^{+3} - and MMA^{+3} -induced events in NIH3T3 cells is still undefined. As illustrated in Fig. 5A (upper panel), the addition of DPI suppressed As^{+3} - and MMA^{+3} -induced HO-1 protein expression and HSP90 protein cleavage in cells by Western blotting,

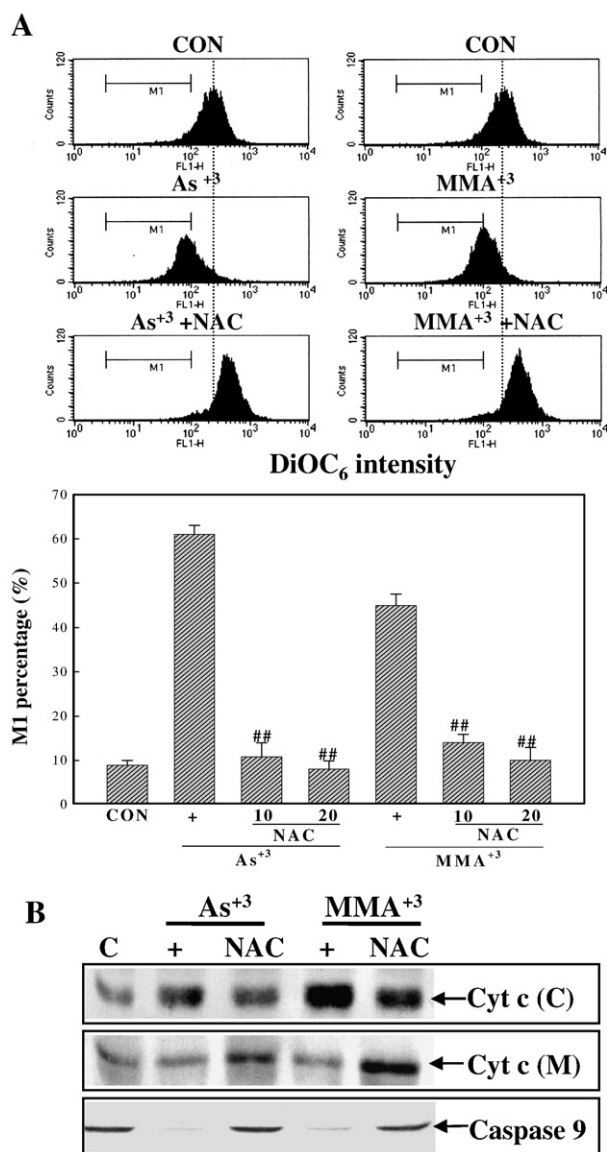


Fig. 4. *N*-acetyl cysteine (NAC) protects NIH3T3 cells from As³⁺- and MMA³⁺-induced loss of the mitochondria membrane potential. (A) NAC inhibited the loss of the mitochondrial membrane potential stimulated by As³⁺ and MMA³⁺. Cells were treated with NAC (10 mM) for 30 min, then washed three times with fresh medium to remove NAC, followed by As³⁺ or MMA³⁺ (10 μ M) for a further 2 h. The mitochondrial membrane potential under different treatments was detected by DiOC₆ staining via flow cytometric analysis (upper panel). (Lower panel) Data derived from three-independent experiments are analyzed and expressed as the mean $\pm$ SE. (B) NAC inhibited As³⁺- and MMA³⁺-induced cytochrome c (Cyt c) release and caspase 9 activation. The cytosolic (C) and mitochondrial (M) fractions were used to detect Cyt c protein, and the total cellular lysate was used to detect caspase 9 protein by Western blotting with specific antibodies. ^{##}*p* < 0.01, indicates a significant difference respectively compared to the As³⁺- or MMA³⁺-treated group by Student's *t*-test.

and an equal amount of loaded proteins in each lane was determined by Coomassie blue staining (lower panel). Data of the DNA fragmentation assay showed that DPI addition inhibited As³⁺- and MMA³⁺-induced DNA ladder formation (Fig. 5B). Similarly, DPI addition protected NIH3T3 cells from As³⁺- and MMA³⁺-induced reduction in cell viability according to the MTT assay (Fig. 5C). Results of the TRAP assay indicated

that the reduction in telomerase activity by As³⁺ and MMA³⁺ was blocked by DPI incubation in NIH3T3 cells (Fig. 5D). This suggests that activation of NADPH oxidase is involved in As³⁺- and MMA³⁺-induced cytotoxic events.

HSP90 inhibitors, geldanamycin and radicicol, potentiate As³⁺- and MMA³⁺-induced apoptosis by reducing telomerase activity

In order to elucidate the role of HSP90 in As³⁺- and MMA³⁺-induced apoptosis in NIH3T3 cells, two chemical HSP90 inhibitors, geldanamycin (GA) and radicicol (RD), were used in the present study. As illustrated in Fig. 6A, GA and RD attenuated the telomerase activity in NIH3T3 cells according to the TRAP assay. In the presence of GA plus As³⁺ or MMA³⁺, GA potentiated telomerase inhibition elicited by As³⁺ and MMA³⁺ (Fig. 6B). Furthermore, data of the MTT assay showed that the enhancement of As³⁺- and MMA³⁺-induced cell death by GA and RD acted against the viability of NIH3T3 cells (Fig. 6C). This suggests that inhibition of HSP90 may suppress telomerase activity and enhance the cytotoxic effects of As³⁺ and MMA³⁺.

Activation of JNKs participates in As³⁺- and MMA³⁺-induced apoptosis

We further investigated if MAPK activation is involved in As³⁺- and MMA³⁺-induced NIH3T3 apoptosis. Four specific chemical inhibitors, including PD98059, SB203580, SP600125, and LY294002, which respectively block ERKs, p38, JNKs, and PI₃K, were used in the present study. Data of Fig. 7A show that adding the JNK inhibitor, SP600125, but not the others, inhibited As³⁺- and MMA³⁺-induced cytotoxic effects in NIH3T3 cells according to the MTT assay. An increase in phosphorylated JNK and ERK proteins was detected in As³⁺- and MMA³⁺-treated NIH3T3 cells (Fig. 7B). The addition of SP600125 inhibited As³⁺- and MMA³⁺-induced HSP90 protein cleavage and telomerase inhibition; however, HO-1 protein expression was not changed (Figs. 7C, D). However, the ERK inhibitor, PD98059, inhibited As³⁺- and MMA³⁺-induced ERK protein phosphorylation, but had no effect on HSP90 protein cleavage (Fig. 7E). Another ERK inhibitor U0126 inhibition of As³⁺- and MMA³⁺-induced ERKs protein phosphorylation, but not cell death, was identified by Western blotting and MTT assay, respectively (Fig. 7F). Transfection of the dominant-negative JNK plasmid (DN-JNKs) blocked As³⁺- and MMA³⁺-induced cytotoxic effects in NIH3T3 cells according to the MTT assay (Fig. 7G). This suggests that JNK activation participates in As³⁺- and MMA³⁺-induced cell death.

Induction of HO-1 gene expression is not involved in the As³⁺- or MMA³⁺-induced cytotoxic effect

Production of ferric and carbon monoxide (CO) has been identified in HO-overexpressing cells. In order to provide direct evidence to investigate the role of HO-1 induction in As³⁺- and MMA³⁺-induced cell death, the ferric chelator, desferoxamine (DFx), the HO inhibitor, tin protoporphyrin (SnPP), and the CO

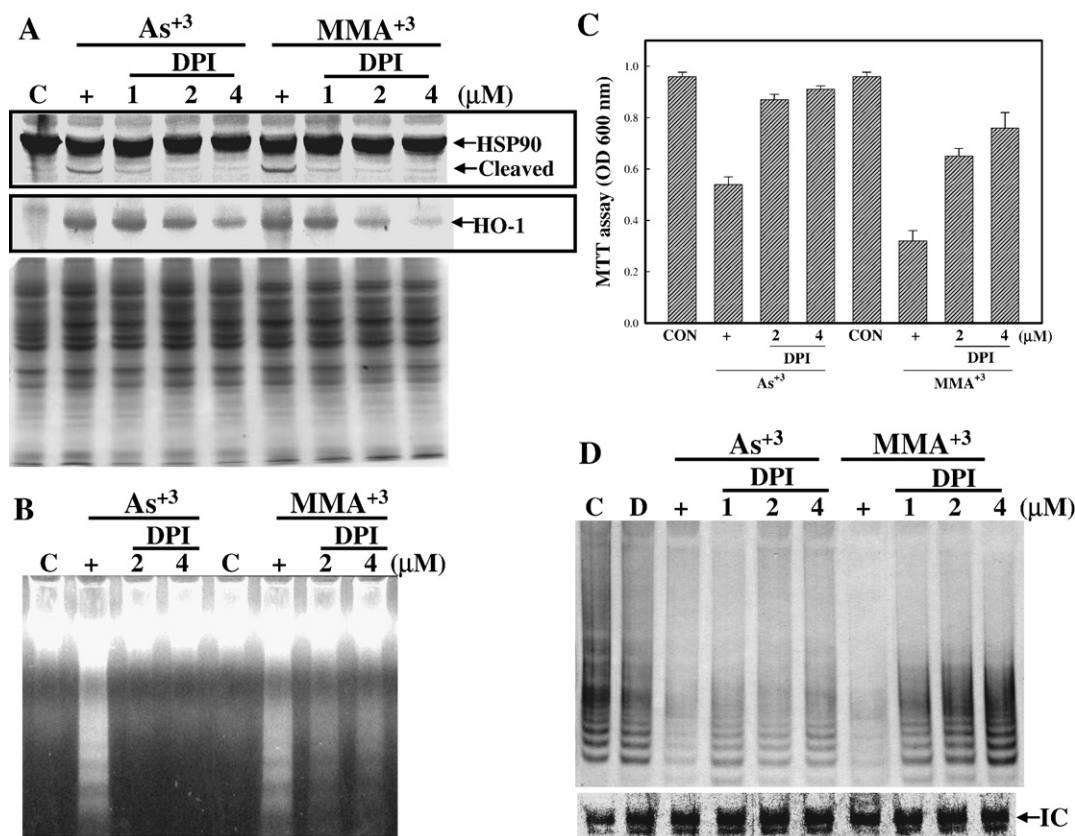


Fig. 5. The NADPH oxidase inhibitor, DPI, reverses the cytotoxic effects of As³⁺ and MMA³⁺. (A) DPI inhibited As³⁺- and MMA³⁺-induced HO-1 and HSP90 protein cleavage in NIH3T3 cells. Cells were treated with different doses of DPI (1, 2, and 4 μ M) for 30 min followed by As³⁺ or MMA³⁺ (10 μ M) treatment for 12 h. (Upper panel) The protein expressions of HSP90 and HO-1 were detected by Western blotting. (Lower panel) Equal amounts of total proteins loaded in each lane were confirmed by Coomassie blue staining. (B, C) DPI addition protected NIH3T3 cells from As³⁺- and MMA³⁺-induced cell death. As described in (A), the integrity of DNA (B) and viability of cells (C) under different treatments were analyzed by electrophoresis and an MTT assay, respectively. (D) As³⁺ and MMA³⁺ reductions of telomerase activity were blocked by DPI. Cells were treated as described in (A), and the telomerase activity was analyzed by a TRAP assay. C, control; D, 0.2% DMSO treatment.

scavenger, hemoglobin (Hb), were used in the present study. Data of the MTT assay showed that DFX, SnPP, and Hb incubation exhibited no effects on As³⁺- and MMA³⁺-induced cell death in NIH3T3 cells (Fig. 8A). HO-1-overexpressing NIH3T3 cells were established, and two clones of HO-1-overexpressing cells, including HO-1/A and HO-1/B, were established. We further analyzed the cytotoxic effect of As³⁺ and MMA³⁺ in HO-1/A, HO-1/B, and pcDNA cells by the MTT assay. As shown in Fig. 8B, no significant difference in the cytotoxicity of As³⁺ or MMA³⁺ was detected in HO-1/A, HO-1/B, pcDNA, or parental (CON) NIH3T3 cells. Blocking HO-1 gene expression by transfection of NIH3T3 cells with HO-1 antisense HO-1 oligonucleotides or siRNA was performed, and a reduction in HO-1 protein expression induced by As³⁺ and MMA³⁺ by the HO-1 antisense oligonucleotide or siRNA was observed (Fig. 8C). However, As³⁺- and MMA³⁺-induced cytotoxic effects did not change when NIH3T3 cells were transfected with HO-1 antisense oligonucleotides or siRNA (Fig. 8D). Additionally, transfection of NIH3T3 cells with HO-1 antisense oligonucleotides or siRNA was unable to inhibit As³⁺- and MMA³⁺-induced HSP90 protein cleavage in NIH3T3 cells (Fig. 8E). These data suggest that HO-1 induction is not involved in As³⁺- or MMA³⁺-induced cytotoxic actions.

Discussion

NIH3T3 cells have been used to study the action of arsenics. Lim et al. (1998) indicated that As³⁺ induced the activation of p38 and JNK1 in NIH3T3 cells. Kim et al. (2002) suggested that growth inhibition induced by As³⁺ was mediated by inducing p21 protein expression in NIH3T3 cells. Our previous study indicated that As³⁺, but not As⁵⁺, induced apoptosis through ROS-dependent caspase 3 activation in NIH3T3 cells (Chen et al., 1998). However, the effects of MMA³⁺, DMA³⁺, MMA⁵⁺, and DMA⁵⁺ on the viability of NIH3T3 cells are still undefined. In this study, we investigated the effects of inorganic arsenics (As³⁺ and As⁵⁺) and their methylated metabolites (MMA³⁺, DMA³⁺, MMA⁵⁺, and DMA⁵⁺) on cell viability in vitro to better understand the mechanism of arsenic carcinogenesis. We found that As³⁺ and MMA³⁺ exerted inhibitory effects on telomerase activity by inducing HSP90 protein cleavage and HO-1 gene expression in an ROS-dependent manner. Furthermore, activation of JNKs, but not ERKs, was involved in As³⁺- and MMA³⁺-induced cell death in accordance with suppressing telomerase activity and HSP90 protein cleavage. All these lines of evidence suggest that oxidative stress resulting from As³⁺ or MMA³⁺ leads to telomerase reduction, JNK activation, and

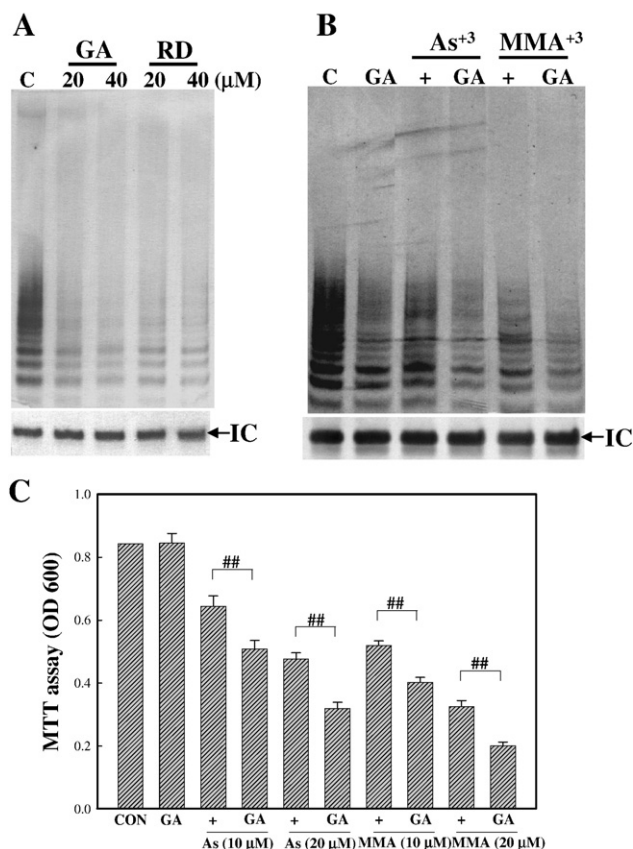


Fig. 6. The heat shock protein (HSP) 90 inhibitors, geldanamycin (GA) and radicicol (RD), inhibited telomerase activity and potentiated the cytotoxicity of As^{+3} and MMA^{+3} in NIH3T3 cells. (A) Inhibition of telomerase activity by GA and RD in NIH3T3 cells. Cells were treated with GA or RD (20 and 40 μM) for 12 h, and the endogenous telomerase activity was analyzed by a TRAP assay. (B) GA potentiated the inhibitory effects of As^{+3} and MMA^{+3} on telomerase activity in NIH3T3 cells. Cells were treated with GA (20 μM) for 30 min followed by As^{+3} or MMA^{+3} (10 μM) for a further 12 h. The telomerase activity under different treatments was analyzed by a TRAP assay. (C) GA potentiated the cytotoxic effects of As^{+3} and MMA^{+3} against the viability of NIH3T3 cells. As described in (B), the viability of cells under different treatments was detected by an MTT assay. Data derived from three-independent experiments are expressed as the mean $\pm$ SE, and $^{##}p < 0.01$ indicates a significant difference between indicated groups by Student's *t*-test.

HSP90 protein cleavage, and disrupts the viability of cells through apoptosis induction.

Arsenics have several biological effects on different cells. Low doses of arsenic induce differentiation and promote proliferation in cells, and higher doses of arsenic produce toxic effects in cells. The cytotoxic effect of arsenic was shown to be attributable to apoptosis induction in several cells (Tapio and Grosche, 2006). Our group reported that As^{+3} , but not As^{+5} , induced apoptosis in NIH3T3 cells through ROS-dependent caspase 3 activation. However, the apoptotic effects of methylated arsenics are still unclear. Data of the present study indicated that As^{+3} and MMA^{+3} , but not the others, showed an apoptosis induction ability against the viability of NIH3T3 cells, and the apoptosis induced by As^{+3} and MMA^{+3} was blocked by the addition of the antioxidants, NAC, DPI, and CAT. ROS-dependent apoptosis was demonstrated in As^{+3} - and MMA^{+3} -induced cell death.

Activation of MAPKs by arsenics has been reported. It is generally accepted that activation of JNKs is associated with apoptosis induction, while activation of ERKs plays a role as survival-promoting kinases. Nevertheless, there are multiple exceptions in relation to MAPK activation and arsenic-induced apoptosis. Fernandez et al. (2004) showed that ERK activation participates in As_2O_3 plus protein kinase C activator-induced apoptosis. Inhibition of JNKs was reported to potentiate As_2O_3 -stimulated apoptosis in several cell models (Ramos et al., 2006). In the present study, activation of ERK and JNK proteins via phosphorylation induction was identified in As^{+3} - and MMA^{+3} -treated NIH3T3 cells, and the addition of the JNK inhibitor, SP600125, or transfection of cells with a dominant-negative JNK plasmid significantly attenuated the cell death induced by As^{+3} or MMA^{+3} . SP600125 addition also inhibited As^{+3} - and MMA^{+3} -induced HSP90 protein cleavage and telomerase reduction, but not HO-1 protein expression. This suggests that activation of JNKs participates in As^{+3} - and MMA^{+3} -stimulated apoptosis.

Previous studies showed that arsenic exerts its cytotoxicity via ROS generation. Mitochondria are the main source of ROS production, and have been implicated in arsenic-induced apoptosis (Wang et al., 2007). Our previous study demonstrated that As^{+3} -induced apoptosis occurs through elevating ROS production and loss of the mitochondrial membrane potential in NIH3T3 cells. This suggests that mitochondrial dysfunction may contribute to the cytotoxicity induced by arsenics. In addition, a significant increase of cytochrome P450 total content and generation of hydroxyl radicals in the liver with an early elevation of 8-OHdG in DMA^{+5} - and MMA^{+5} -treated male F344 rats (Kinoshita et al., 2007; Nishikawa et al., 2002). However, the roles of ROS and mitochondrial dysfunction in methylated arsenics are still unclear. In the present study, an increase in intracellular peroxide was examined in NIH3T3 cells treated with As^{+3} and MMA^{+3} , but not the others, and NAC, CAT, and DPI effectively protected cells from As^{+3} - and MMA^{+3} -induced cell death by reducing ROS production. These data suggest that ROS generation is involved in As^{+3} - and MMA^{+3} -stimulated cell death.

Telomerase is the enzyme which adds telomere repeats to chromosomes at each round of cell division, and a previous study showed that arsenic is a selective inhibitor of telomerase (Zhou et al., 2007). Zhang et al. (2003b) showed that telomerase-active cells are sensitive to arsenic-induced apoptosis, while telomerase-negative cells are more resistant. Liu et al. (2003) indicated that oxidative stress may contribute to arsenic-induced telomere attrition and apoptosis. Chou et al. (2001) indicated that inhibition of telomerase transcription by arsenic leads to genetic instability. Zhang et al. (2003a) showed that low concentrations of arsenic increase telomerase which promotes cell proliferation, and high concentrations of arsenic decrease telomerase activity which promotes apoptosis. Although the effect of As^{+3} on telomerase activity has been reported, whether inorganic arsenic (As^{+3} and As^{+5}) and its methylated metabolites (MMA^{+3} , DMA^{+3} , MMA^{+5} , and DMA^{+5}) affect telomerase activity in relation to apoptosis is still unclear. Data of the present study show that As^{+3} and MMA^{+3} , but not the others, inhibited

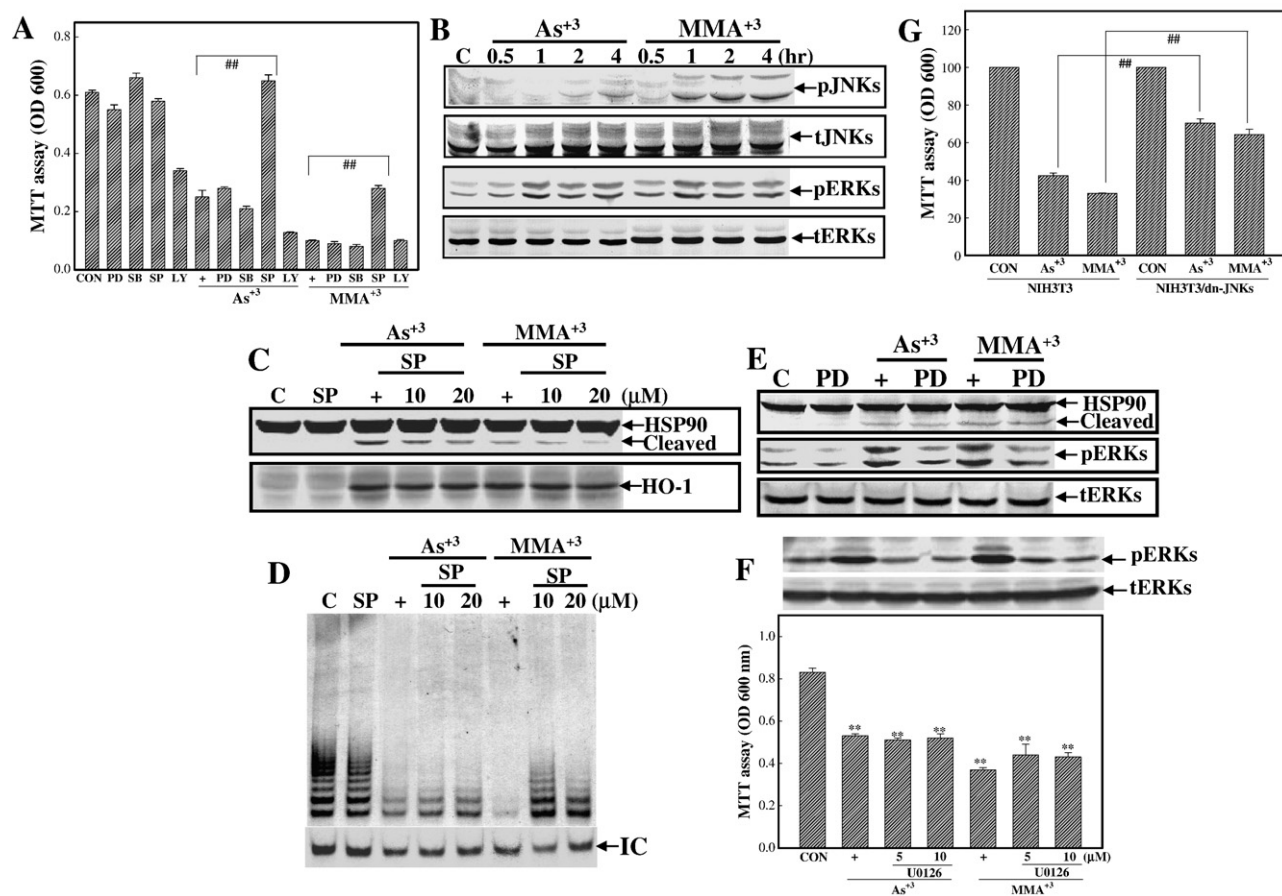


Fig. 7. JNK, but not ERK, activation is involved in As³⁺- and MMA³⁺-induced cytotoxicity. (A) Addition of the JNK inhibitor, SP600125, but not PD98059 (PD), SB203580 (SB), or LY294002 (LY), attenuated the cytotoxic effects of As³⁺ and MMA³⁺. Cells were treated with the indicated inhibitors (20 μM) for 30 min followed by As³⁺ or MMA³⁺ (10 μM) treatment for an additional 12 h. The viability of cells was analyzed by an MTT assay. (B) Induction of ERK and JNK protein phosphorylation by As³⁺ and MMA³⁺ in NIH3T3 cells. Cells were treated with As³⁺ or MMA³⁺ (10 μM) for different times (0.5, 1, 2, and 4 h), and total and phosphorylated ERK and JNK proteins were detected by Western blotting using specific antibodies. (C) SP inhibition of As³⁺- and MMA³⁺-elicited HSP90 protein cleavage, but not HO-1 protein induction. Cells were treated with SP (5, 10, and 20 μM) for 30 min followed by As³⁺ or MMA³⁺ (10 μM) treatment for 12 h. The expressions of HO-1 and the HSP90 protein were examined by Western blotting. (D) SP600125 reversed the inhibitory effect of As³⁺ and MMA³⁺ on telomerase activity in NIH3T3 cells. As described in (C), the endogenous telomerase activity was analyzed by a TRAP assay. (E) PD98059 (PD) showed no effect on As³⁺- or MMA³⁺-induced HSP90 protein cleavage. Cells were treated with PD (20 μM) for 30 min followed by As³⁺ or MMA³⁺ (10 μM) treatment for 1 h (for ERKs) or 12 h (for HSP90), and the expressions of HSP90, phosphorylated ERK (pERK), and total ERK (tERK) proteins were examined by Western blotting. (F) U0126 inhibition of As³⁺- and MMA³⁺-induced ERKs protein phosphorylation, but not cell death. Cells were treated with U0126 (5 and 10 μM) for 30 min followed by As³⁺ or MMA³⁺ (10 μM) treatment for 1 h, and the expressions of phosphorylated ERK (pERK), and total ERK (tERK) proteins were examined by Western blotting (Upper panel). As the same part of experiment, the viability of cells after 12 h treatments was analyzed by MTT assay (Lower panel). (G) Transfection of dominant-negative JNK plasmids reversed the inhibitory effect of As³⁺ and MMA³⁺ against the viability of NIH3T3 cells. Cells were transiently transfected with a control pcDNA plasmid (NIH3T3) or dominant-negative JNK plasmids (NIH3T3/dn-JNKs) followed by As³⁺ and MMA³⁺ (10 μM) treatment for 12 h, and the viability of cells was detected by an MTT assay. All data derived from three-independent experiments are expressed as the mean ± SE, and ***p* < 0.01 indicates a significant difference from CON group by Student's *t*-test. ##*p* < 0.01 indicates a significant difference between indicated groups by Student's *t*-test.

telomerase activity and mRNA expression in NIH3T3 cells, and the inhibitory effect of As³⁺ and MMA³⁺ on telomerase activity was blocked by the ROS scavengers, NAC, CAT, and DPI. This suggests that ROS production participates in the inhibition of telomerase by As³⁺ and MMA³⁺, leading to apoptosis.

Exposure to arsenicals has been shown to cause the induction of heat shock proteins (HSPs) in breast cancer cells T47D, melanoma cells M14, human keratinocytes, and livers of mouse (Del Razo et al., 2001; Liu et al., 2001). HO-1, an HSP of 32 kDa, is responsible for the breakdown of heme into equimolar amounts of biliverdin, carbon monoxide, and Fe, and As³⁺, but neither MMA³⁺ nor DMA³⁺ has been shown to be an effective

HO-1 inducer (Brown et al., 1997). Cebrián et al. (1988) reported that acute, but not subchronic, administration of As³⁺ to rats increased hepatic HO-1 activity. HSP90 is a cytosolic protein which coordinates the trafficking and regulates a variety of diverse signaling proteins, and HSP90 synthesis is also induced by As³⁺. The association of HSP90 with several key oncogenic client proteins, including ERBB2, CDK4, AKT/PKB, mutant p53, and telomerase hTERT, has been shown in several previous studies (Powers and Workman, 2006). Keppler et al. (2006) demonstrated a telomerase complex containing HSP90, and its telomerase activity was sensitive to the HSP90 inhibitor, geldanamycin. The relationship of HO-1 and HSP90 to

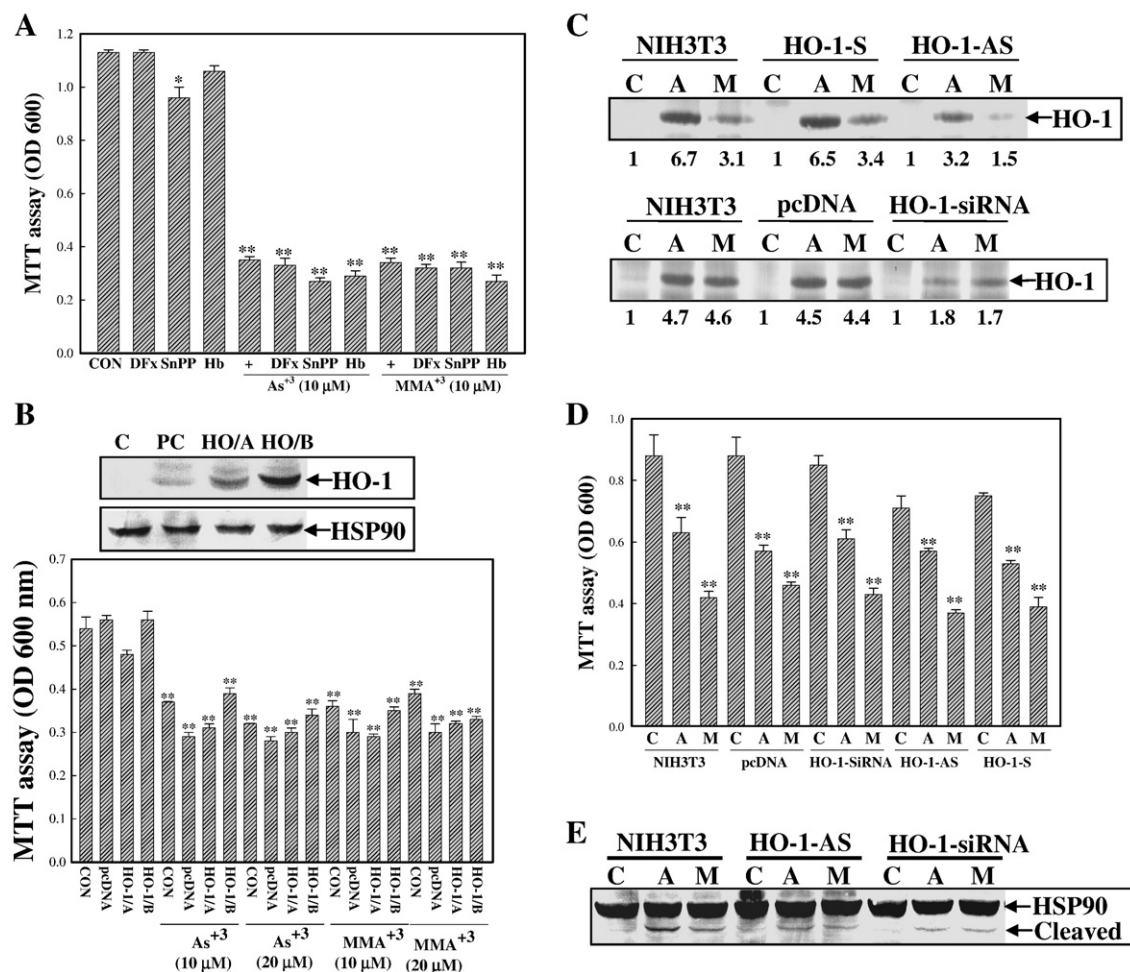


Fig. 8. Heme oxygenase (HO)-1 induction might not be involved in As³⁺- and MMA³⁺-stimulated cytotoxic effects in NIH3T3 cells. (A) An iron chelator, desferoxamine (DFx), an HO inhibitor, tin protoporphyrin (SnPP), and a CO scavenger, hemoglobin (Hb), showed no effects on As³⁺- or MMA³⁺-induced cell death. Cells were treated with the indicated compounds for 30 min followed by As³⁺ or MMA³⁺ (10 μM) treatment for 12 h. The viability of cells under different treatments was analyzed by an MTT assay. (B) Overexpression of HO-1 protein did not alter the cytotoxic effects of As³⁺ and MMA³⁺ in NIH3T3 cells. Two stable HO-1-overexpressing cells (HO-1/A and HO-1/B) were established in the study, and the expression of HO-1 protein in cells was detected by Western blotting (upper panel). The cytotoxic effects of As³⁺ and MMA³⁺ in four cells including parental (C), pcDNA plasmid-transfected cells (PC), HO-1/A, and HO-1/B were analyzed by an MTT assay (lower panel). (C) HO-1 antisense oligonucleotides or siRNA transfection attenuated As³⁺- or MMA³⁺-induced HO-1 protein expression in NIH3T3 cells. Cells were transfected with HO-1 sense or antisense oligonucleotides or siRNA followed by As³⁺ and MMA³⁺ (10 μM) treatment for 12 h, and the expression of HO-1 protein was detected by Western blotting. (D, E) Attenuation of HO-1 protein expression was unable to inhibit the As³⁺- or MMA³⁺-induced cytotoxic effects (D) or HSP90 protein cleavage (E) in NIH3T3 cells. As described in (C), the viability of cells and the expression of HSP90 protein were examined by an MTT assay and Western blotting, respectively. Data derived from three-independent experiments are expressed as the mean ± SE, and * *p* < 0.05, ** *p* < 0.01 indicates a significant difference from control (CON or C) group. CON or C, control; A, As³⁺; M, MMA³⁺.

arsenical-induced apoptosis is still unclear. In the present study, As³⁺ and MMA³⁺, but not the others, induced HO-1 gene expression and HSP90 protein cleavage in NIH3T3 cells. Blocking HO-1 gene expression using an antisense HO-1 oligonucleotide or HO-1 siRNA showed no effect on As³⁺- and MMA³⁺-induced apoptosis; however, the HSP90 inhibitor, geldanamycin, and radicicol potentiate the cytotoxic effects of As³⁺ and MMA³⁺ in accordance with inhibiting telomerase activity. This suggests that telomerase inhibition via HSP90 protein cleavage may play an important role in arsenical-induced cell death.

Inorganic arsenic compounds have been shown to be more toxic than organic arsenic compounds, and approximately 60%–75% of ingested inorganic arsenic was excreted into

urine which contained 10%–15% inorganic arsenic, 10%–15% monomethylarsonic acid (MMA⁵⁺), and 60%–80% dimethylarsinic acid (DMA⁵⁺) (Vahter and Concha, 2001). Cullen et al. (1984) postulated that MMA⁵⁺ and DMA⁵⁺ are then reduced to MMA³⁺ and DMA³⁺. This is the first study to investigate the differential cytotoxic actions of inorganic arsenics (As³⁺ and As⁵⁺) and organic arsenics (MMA³⁺, DMA³⁺, MMA⁵⁺, and DMA⁵⁺) in a model system. The molecular events including telomerase inhibition, ROS generation, HSP90 protein cleavage, HO-1 induction, and JNK activation in As³⁺- and MMA³⁺-induced apoptosis are demonstrated. In summary, the present results demonstrate that telomerase inhibition and HSP90 protein cleavage are located downstream of JNK activation, and ROS generation may explain at least a portion of the apoptosis capacity

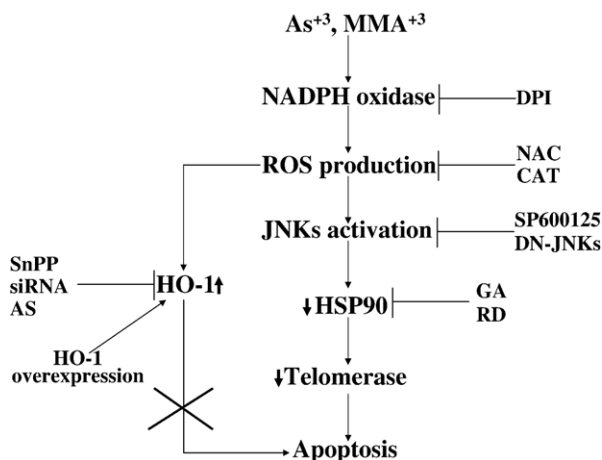


Fig. 9. A tentative apoptosis mechanism elicited by As^{+3} and MMA^{+3} is proposed. As^{+3} , arsenite; MMA^{+3} , monomethylarsonous acid; NAC, *N*-acetyl-L-cysteine; HO-1, heme oxygenase-1; HSP90, heat shock protein 90; JNKs, c-Jun NH_2 -terminal kinases; ROS, reactive oxygen species; GA, geldanamycin; RD, radicicol; SnPP, tin protoporphyrin; CAT, catalase; DPI, diphenyleneiodonium; DN-JNKs, dominant-negative-JNKs; AS, antisense.

of As^{+3} and MMA^{+3} . Hence, our results suggest that HSP90 inhibitors might be exploited as a tool to improve the apoptosis efficacy of arsenics. A tentative apoptotic mechanism via ROS-dependent JNKs activation, HSP90 protein cleavage, and telomerase inhibition elicited by As^{+3} and MMA^{+3} is proposed (Fig. 9).

Acknowledgments

This study was supported by the National Science Council of Taiwan (NSC95-2320-B-038-029-MY2, NSC96-2320-B-038-031-MY3, NSC96-2320-B-038-022), 96TMU-WFH-04, and 93MMH-TMU-05.

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