



# JWA is required for arsenic trioxide induced apoptosis in HeLa and MCF-7 cells via reactive oxygen species and mitochondria linked signal pathway

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## ARTICLE INFO

### Article history:

Received 13 September 2007

Revised 8 January 2008

Accepted 30 January 2008

Available online 20 February 2008

### Keywords:

Arsenic trioxide

JWA

Reactive oxygen species

MAPK

Bad

Caspase

Apoptosis

## ABSTRACT

Arsenic trioxide, emerging as a standard therapy for refractory acute promyelocytic leukemia, induces apoptosis in a variety of malignant cell lines. JWA, a novel retinoic acid-inducible gene, is known to be involved in apoptosis induced by various agents, for example, 12-O-tetradecanoylphorbol 13-acetate, *N*-4-hydroxy-phenyl-retinamide and arsenic trioxide. However, the molecular mechanisms underlying how JWA gene is functionally involved in apoptosis remain largely unknown. Herein, our studies demonstrated that treatment of arsenic trioxide produced apoptosis in HeLa and MCF-7 cells in a dose-dependent manner and paralleled with increased JWA expression. JWA expression was dependent upon generation of intracellular reactive oxygen species induced by arsenic trioxide. Knockdown of JWA attenuated arsenic trioxide induced apoptosis, and was accompanied by significantly reduced activity of caspase-9, enhanced Bad phosphorylation and inhibited MEK1/2, ERK1/2 and JNK phosphorylations. Arsenic trioxide induced loss of mitochondrial transmembrane potential was JWA-dependent. These findings suggest that JWA may serve as a pro-apoptotic molecule to mediate arsenic trioxide triggered apoptosis via a reactive oxygen species and mitochondria-associated signal pathway.

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## Introduction

Epidemiological evidence implicated exposure to arsenic in drinking water with induction of human cancers such as the skin, bladder, liver, kidney and lung cancers (Liu et al., 2001; Eblin et al., 2006). Paradoxically, however, arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) has been used therapeutically for over 2000 years in the world (Florea et al., 2007) and received more attention since the discovery due its clinical efficacy in acute promyelocytic leukemia (APL), syphilis and psoriasis in some traditional Chinese remedies (Liu and Huang, 2006; Yu et al., 2007). The effectiveness of As<sub>2</sub>O<sub>3</sub> in treating APL led to its use in various clinical studies for the treatment of solid tumors, such as prostate cancer, cervical cancer and ovarian cancer (Cheung et al., 2007). As<sub>2</sub>O<sub>3</sub> apparently affects numerous intracellular signal transduction pathways and produces alterations in cellular functions. These actions of As<sub>2</sub>O<sub>3</sub> may result in the induction of apoptosis depending on cell type, exposure concentrations, and time (Miller et al., 2002). As<sub>2</sub>O<sub>3</sub> was also shown to (1) increase the activities of caspases-3, -8 and -9, suppress Bcl-2 family expression, (2) generate reactive oxygen species (ROS), (3) disrupt mitochondrial membrane potential ( $\Delta\psi/m$ ), and (4) activate MAPK and release cytochrome *c*, resulting in apoptosis (Nimmanapalli and Bhalla, 2002; Tapio and Grosche, 2006). A key component underlying the As<sub>2</sub>O<sub>3</sub> generated-effects is that ROS plays an important role (Shi et al., 2004).

The JWA gene, also known as ARL6IP5, jmx, hp22, PRAF3, DERP11, HSPC127, addicisin and GTRAP3-18 in GenBank (Butchbach et al., 2002;

Schweneker et al., 2005), was initially cloned from human tracheal bronchial epithelial cells by treatment with all-trans retinoic acid (ATRA) (Zhou et al., 1999). Our previous studies showed that JWA encoding a novel microtubule-associated protein (Li et al., 2003), was associated with malignant cell differentiation and apoptosis induced by various chemicals such as 12-O-tetradecanoylphorbol 13-acetate (TPA), ATRA, *N*-4-hydroxy-phenyl-retinamide(4HPR), As<sub>2</sub>O<sub>3</sub> (Cao et al., 2002; Mao et al., 2006) and HEK-293T cell apoptosis induced by cadmium (Cao et al., 2007). In addition, JWA is responsive to stress stimulations, such as heat shock, H<sub>2</sub>O<sub>2</sub>, and benzo[a]pyrene (B[a]P)-induced oxidative stress (Zhu et al., 2005; Chen et al., 2007b). However, the underlying mechanisms involved in the regulation of JWA and its function in apoptosis remains to be elucidated. The present study was designed to investigate how JWA functions in As<sub>2</sub>O<sub>3</sub> triggered cell apoptosis and in an attempt to clarify the underlying molecular mechanisms.

## Materials and methods

**Cell lines and reagents.** HeLa and MCF-7 cells were purchased from the Institute of Biochemistry and Cell Research, China Life Science Academy (Shanghai, China), and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies/Gibco, Gaithersburg, MD). For experiments, cells were grown at 37 °C in a humidified incubator with 5% CO<sub>2</sub> (HERA Cell, Heraeus, Germany). A stock solution of As<sub>2</sub>O<sub>3</sub> was prepared by dissolving As<sub>2</sub>O<sub>3</sub> powder (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS, pH 7.4) to a concentration of 10 mM. MIT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide], 2,7-dichlorofluorescein diacetate (DCFH-DA) dyes and catalase were purchased from Sigma (St. Louis, MO). Antibodies specific for rabbit anti-MEK1/2, phospho-MEK1/2 (Ser217/221), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), JNK, phospho-JNK (Thr183/Tyr185), Bad, phospho-Bad (ser112), caspase-9 and monoclonal mouse anti-caspase-8 were purchased from Cell Signaling Technology (Beverly, MA, USA); Rabbit anti-Bcl-2 was purchased from

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Biosynthesis Biotechnology Company (Beijing, China). JWA protein c-terminal 20 amino acid peptide and the anti-JWA polypeptide antisera (dilution at 1:200) were produced by Research Genetics, Inc. (S.W. Huntsville, AL).

**Stable transfection.** Construction of JWA expression vectors and stable transfection: the pEGFP-C1 expression vector was purchased from Clontech (Palo Alto, CA). The antisense initiation JWA coding region sequence (from 409 to 71 bp) (339 bp in length) was inserted into pEGFP-C1 vector using both BamH I and Hind III restriction endonucleases. Transfection of pEGFP-C1-as JWA DNA and pEGFP-C1 vector DNA into the HeLa and MCF-7 cells was carried out with polyfect reagent following the manufacturer's (Qiagen, Valencia, CA) protocol. G418 was added to each dish at a final concentration at 1000 µg/ml until the positive cells were screened out. The resulting HeLa and MCF-7 cells were verified and found to possess stable knockdown of JWA expression (asJWA-HeLa, asJWA-MCF-7), and vector controls (C1-HeLa, C1-MCF-7), respectively.

**Cell viability assay.** The effect of As<sub>2</sub>O<sub>3</sub> on cell viability was assayed by the MTT colorimetric method, which measures viable cells by assessing metabolic integrity (Mosmann, 1983). The two groups of cells (asJWA-HeLa, C1-HeLa and asJWA-MCF-7, C1-MCF-7 cells) were harvested by trypsinization; subsequently, cells were thoroughly washed, and seeded in 96-well micro titer plates at a density of 2 × 10<sup>4</sup> cells/well. After 24 h culture, the cells were treated with indicated concentrations of As<sub>2</sub>O<sub>3</sub> (0, 1, 2 or 5 µM for asJWA-HeLa, C1-HeLa; and 0, 2, 5 or 10 µM for asJWA-MCF-7, C1-MCF-7 cells) respectively for 48 h. Briefly, 20 µl MTT solution (1 mg/ml) was added to each well, after 4 h, 150 µl DMSO was added into each well and incubated for 10 min. Then optical density at 490 nm was read by an enzyme-linked immunosorbent assay (ELISA) reader (Bio-TEK Instruments, Winooski, VT). Samples of at least three independent experiments were analyzed in duplicate.

**Apoptosis assay.** In this study, several assays were used to determine As<sub>2</sub>O<sub>3</sub>-induced cellular apoptosis. Apoptotic morphological changes in the nuclear chromatin of cells were detected by staining with the DNA-binding fluorochrome Hoechst 33258 (bis-benzimidazole). Treated cells were washed in PBS (pH 7.4) and then fixed with 4% paraformaldehyde for 10 min at room temperature. Following fixation, cells were rinsed 3 times in PBS (pH 7.4) and then stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, Jiangsu, China). Stained nuclei were observed under a fluorescence microscope (IX70, Olympus, Japan). Five fields were randomly sampled from each coverslip for fluorescence microscopy observation. All the cells stained with Hoechst dye in each field were counted as either pyknotic or viable. The number of pyknotic cells was counted in the 5 sampled regions and expressed as % apoptotic cells per coverslip = (total number of pyknotic cells)/(total number of pyknotic cells + total number of viable cells) × 100%.

To confirm the results of morphologic analysis, apoptosis was also determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL), as well as Annexin V-PE assay. For TUNEL assay, APO-DIRECT™ Kit (BD PharMingen, San Diego, CA) was used. Cells were fixed in freshly diluted 1% paraformaldehyde in PBS (pH 7.4) for 30 min on ice and stored in 70% ethanol at -20 °C for at least 18 h, then followed by two washes with PBS containing 0.2% BSA. To label DNA strand breaks, 1 ~ 2 × 10<sup>6</sup> cells were incubated with 50 µl of TUNEL reaction buffer containing terminal deoxynucleotidyl transferase and fluorescein-BrdUTP, and it was then incubated for 1 h at 37 °C in a humidified 5% CO<sub>2</sub>-in-air atmosphere. Cells were washed twice with PBS, suspended in PBS containing 50 µg/ml of PI and 50 µg/ml of DNase-free RNase A for 30 min in room temperature in the dark room and then analyzed by a FACScan flow cytometry (Becton Dickinson Inc.). For Annexin V-PE assay, the Annexin V-PE Apoptosis Detection Kit (BD PharMingen, San Diego, CA) was used. Apoptotic cells were detected by annexin V binding to phospholipid phosphatidylserine (PS), which is translocated from the inner to the outer leaflet of the plasma membrane of apoptotic cells. Approximately 1 × 10<sup>6</sup> cells were resuspended in 1 × binding buffer and incubated with phycoerythrin (PE)-conjugated annexin V and the fluorescent DNA-binding dye 7-AAD for 15 min at room temperature in the dark room. The cells were analyzed by flow cytometry using the PE signal detector (FL1) and the 7-AAD signal detector (FL2) in a FACScan flow cytometry (Becton Dickinson Inc.). Cells negative for both annexin V and 7-AAD staining are definite as live cells; annexin V-positive and 7-AAD-negative stained cells are as early apoptotic cells; both 7-AAD and annexin V-positive stained cells are necrotic and/or late apoptotic cells; and 7-AAD-positive and annexin V-negative stained cells are necrotic cells.

**Determination of mitochondrial transmembrane potentials (Δψ<sub>m</sub>).** Changes of mitochondrial transmembrane potentials (Δψ<sub>m</sub>) are considered to be an indicator of mitochondrial damage. The Δψ<sub>m</sub> was quantified by means of a cationic fluorescent probe, rhodamine 123 (Rh-123) (Molecular Probes, Eugene, OR, USA), which accumulates in functional mitochondria and shows high Δψ<sub>m</sub>. Active mitochondria in viable cells was stained as bright green. Loss of gradient within nonviable cells results in loss of fluorescence. In this study, both asJWA-HeLa and C1-HeLa cells were harvested following treatment with As<sub>2</sub>O<sub>3</sub> (0, 5 µM) for 24 h; after washing twice with PBS (pH 7.4), about 1 × 10<sup>6</sup> cells were incubated (37 °C, 30 min) with 10 mg/ml Rh-123, a cationic lipophilic fluorochrome taken up by mitochondria in proportion to the Δψ<sub>m</sub>. Fluorescent intensities were determined by flow cytometry (Becton Dickinson Inc.).

**Western blots.** Western blots were performed based on the standard procedures. Briefly, harvested cells were washed with PBS (pH 7.4) twice and lysed by scraping into

100 ~ 200 µl keratin extraction buffer (1% Triton-X 100, 0.02 mM Tris, 0.6 M KCl, and 1 mM PMSF, pH 7). The samples were centrifuged at 12,000 ×g for 20 min at 4 °C and the pellets were discarded. After detecting the protein concentration of samples using bicinchoninic acid (BCA) protein assay, aliquots of protein were then resolved in 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoreses (SDS-PAGE). The proteins were further transferred onto polyvinylidene membrane for blotting. After blocking with 5% non-fat dry milk for 1 h, membranes were incubated with the specific primary antibodies overnight at 4 °C. After washing with PBST (5 min × 5), the membranes were incubated with horseradish peroxidase (HRP)-linked second antibody for 1 h at 37 °C. After washing (5 min × 10), the immuno-complexes were detected with an enhanced chemiluminescent kit (Amersham Biosciences). Results shown are representative of three independent experiments. The immunoreactive bands were scanned for semiquantitation and normalized by β-actin bands of the same membrane.

**Detection of cellular reactive oxygen species (ROS).** 2', 7'-dichlorofluorescein diacetate (DCFH-DA), dyes specific binding to intracellular H<sub>2</sub>O<sub>2</sub> (Zamzami et al., 1995), was used to detect the intracellular levels of the H<sub>2</sub>O<sub>2</sub>. It was dissolved in DMSO at 2 mM and kept at -20 °C. DCFH-DA was added into the cell culture medium in each well 15 min before the treatments were completed, and the staining was carried out at 37 °C. The final DMSO concentration in the cell culture media was 0.1%. In the case of cellular imaging, the cells (2 × 10<sup>4</sup>/well) were placed onto glass coverslips in 24-well plates. After being stained, the cells were washed in PBS (pH 7.4) and fixed with 10% buffered paraformaldehyde. The coverslips were mounted on glass slides and observed using a fluorescence microscope (IX70, Olympus, Japan) fitted with an argon-ion laser.

**Statistical analysis.** Data were presented as the means ± SD. Two-way analysis of variance (ANOVA) procedures were used to assess significant difference between treatment and control groups. A probability level of 0.05 was used to establish significance and *p* values < 0.05 were considered statistically significant.

## Results

### As<sub>2</sub>O<sub>3</sub> induces apoptosis and JWA expressions in HeLa and MCF-7 cells

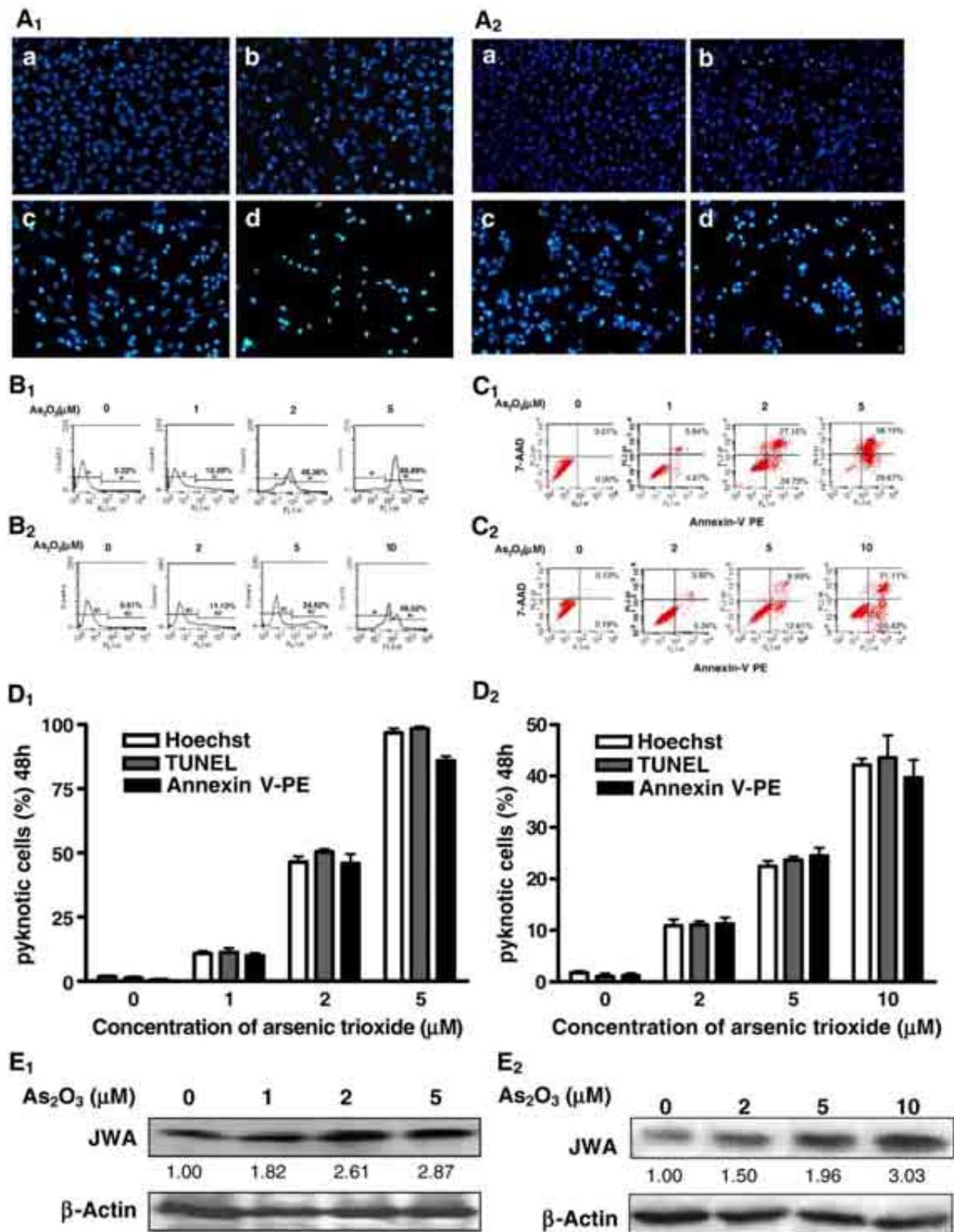
The Hoechst 33258 staining assay showed that As<sub>2</sub>O<sub>3</sub> induces morphological apoptosis in both HeLa and MCF-7 tumor cells in a dose-dependent manner (Fig. 1A<sub>1</sub>–A<sub>2</sub>). However, the two cell lines indicated differential sensitivity to As<sub>2</sub>O<sub>3</sub> exposure. For HeLa cells, treatment with 5 µM As<sub>2</sub>O<sub>3</sub> for 48 h induced almost 100% apoptosis; however, induced only 25% apoptosis in MCF-7 cells (Fig. 1D<sub>1</sub>–D<sub>2</sub>). Similarly, both TUNEL and Annexin V-PE assays confirmed that the same treatments induced but with differential potentiation of apoptosis in HeLa and MCF-7 cells (Fig. 1B<sub>1</sub>–B<sub>2</sub>; C<sub>1</sub>–C<sub>2</sub>). JWA expressions in both HeLa and MCF-7 cells also showed a dose-dependent increasing manner following treatment of cells with As<sub>2</sub>O<sub>3</sub>. The induction of As<sub>2</sub>O<sub>3</sub> on JWA expression, however, also indicated differential sensitivities between the cells (Fig. 1E<sub>1</sub>–E<sub>2</sub>).

### JWA is required for As<sub>2</sub>O<sub>3</sub>-inducing apoptosis

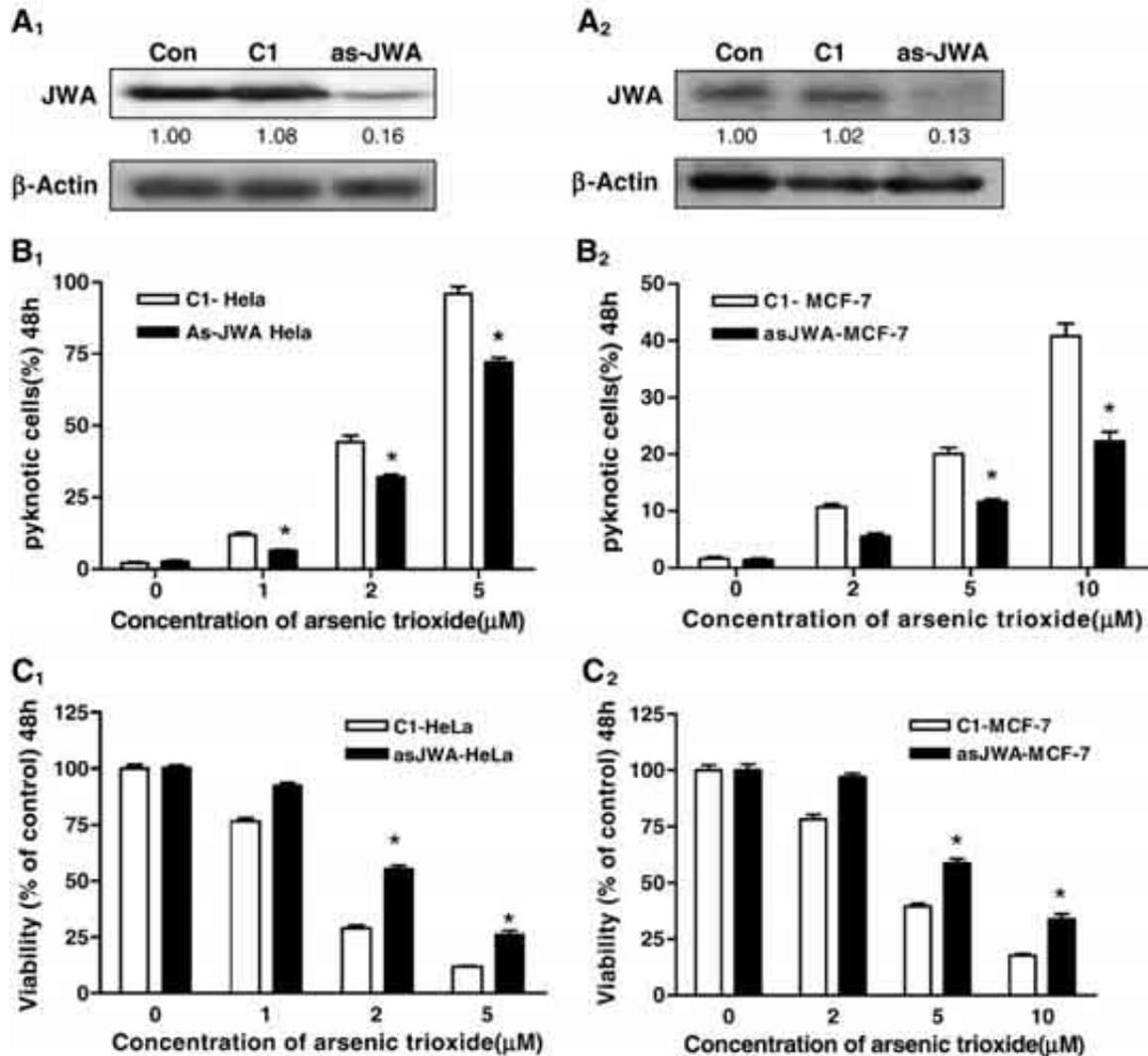
As<sub>2</sub>O<sub>3</sub> is a well-established anti-proliferative or pro-apoptotic chemical (Park et al., 2005a). To evaluate whether increased JWA expression is required for As<sub>2</sub>O<sub>3</sub> inducing apoptosis in both HeLa and MCF-7 cells, we established stable JWA deficient HeLa and MCF-7 cells and to repeat the cell culture models. As predicted, both cells showed a significant down regulation of JWA protein (Fig. 2A<sub>1</sub>–A<sub>2</sub>). Interestingly, these JWA deficient cells showed a significant resistance to As<sub>2</sub>O<sub>3</sub> treatment when compared with their vector controlled cells. As a result, knock-down JWA resulted in only about 75% apoptosis in HeLa cells (compared to about 100% in vector control) and 12% in MCF-7 cells (compared to about 22% in vector control) after treatment with 5 µM As<sub>2</sub>O<sub>3</sub> for 48 h (Fig. 2B<sub>1</sub>–B<sub>2</sub>). In contrast of this, the MTT assay demonstrated significantly increased viabilities in both asJWA-HeLa and asJWA-MCF-7 cells compared with their vector controls after As<sub>2</sub>O<sub>3</sub> treatment (Fig. 2C<sub>1</sub>–C<sub>2</sub>).

### JWA enhances As<sub>2</sub>O<sub>3</sub>-induced apoptosis through mitochondrial pathway

Genetic and biochemical evidences indicate that apoptosis proceeds in two major cell death pathways: an intrinsic pathway that involves mitochondrial membrane permeabilization and release of



**Fig. 1.** As<sub>2</sub>O<sub>3</sub> induces apoptosis in HeLa and MCF-7 cells is paralleled with increased JWA expression: (A<sub>1</sub>, A<sub>2</sub>) As<sub>2</sub>O<sub>3</sub> induces apoptosis in HeLa and MCF-7 cells: (A<sub>1</sub>) HeLa cells were incubated with As<sub>2</sub>O<sub>3</sub> at 0, 1, 2, or 5 μM for 48 h, Hoechst 33258 staining assay was examined (a) control, (b) 1 μM As<sub>2</sub>O<sub>3</sub>, (c) 2 μM As<sub>2</sub>O<sub>3</sub>, (d) 5 μM As<sub>2</sub>O<sub>3</sub>. (A<sub>2</sub>) MCF-7 cells were incubated with As<sub>2</sub>O<sub>3</sub> at 0, 2, 5, or 10 μM for 48 h, Hoechst 33258 staining assay was examined (a) control, (b) 2 μM As<sub>2</sub>O<sub>3</sub>, (c) 5 μM As<sub>2</sub>O<sub>3</sub>, (d) 10 μM As<sub>2</sub>O<sub>3</sub>. (B<sub>1</sub>, B<sub>2</sub>) TUNEL assay for detecting As<sub>2</sub>O<sub>3</sub>-induced apoptosis in HeLa and MCF-7 cells: (B<sub>1</sub>) for HeLa cells, (B<sub>2</sub>) for MCF-7 cells. The concentrations of As<sub>2</sub>O<sub>3</sub> and the percentages of apoptosis were indicated in the figure. (C<sub>1</sub>, C<sub>2</sub>) Annexin V-PE assay for detecting As<sub>2</sub>O<sub>3</sub>-induced apoptosis in HeLa and MCF-7 cells: (C<sub>1</sub>) for HeLa cells, (C<sub>2</sub>) for MCF-7 cells. The concentrations of As<sub>2</sub>O<sub>3</sub> and the percentages of apoptosis were indicated in the figure. (D<sub>1</sub>–D<sub>2</sub>) The quantitative data for three assays in detecting As<sub>2</sub>O<sub>3</sub>-induced apoptosis in HeLa and MCF-7 cells. (D<sub>1</sub>) for HeLa cells, (D<sub>2</sub>) for MCF-7 cells. (E<sub>1</sub>–E<sub>2</sub>) The expression of JWA was determined by Western blot analysis after exposure to indicated concentrations of As<sub>2</sub>O<sub>3</sub> for 48 h in HeLa (E<sub>1</sub>) and MCF-7 (E<sub>2</sub>) cells. The immunoreactive bands of JWA in Western blot were normalized to β-actin bands on the same membrane. The quantitative data below the bands show the band intensities compared with the control group.



**Fig. 2.** JWA expression is required for  $As_2O_3$ -induced anti-proliferation and apoptosis: (A<sub>1</sub>–A<sub>2</sub>) JWA knock-down produced its significant reduced expression in HeLa (A<sub>1</sub>) and MCF-7 (A<sub>2</sub>) cells: Western blot was employed to detect the knock-down efficiency of JWA protein. JWA expression in blank control, vector control and JWA knock-down cells was determined. The immunoreactive bands were normalized to  $\beta$ -actin bands on the same membrane. The quantitative data below the bands show the band intensities compared with the control group. (B<sub>1</sub>–B<sub>2</sub>): Effects of  $As_2O_3$  on groups of C1-HeLa, AsJWA-HeLa (B<sub>1</sub>) and C1-MCF-7, AsJWA-MCF-7 (B<sub>2</sub>) cells apoptosis: cells incubated with indicated concentrations of  $As_2O_3$  for 48 h were fixed and stained with Hoechst 33258, the frequency of apoptotic cells was calculated. Data are showed as mean  $\pm$  SD of five independent experiments, asterisks indicate significant at  $p < 0.05$  when compared with vector control. (C<sub>1</sub>–C<sub>2</sub>): Effects of  $As_2O_3$  on groups of C1-HeLa, AsJWA-HeLa (C<sub>1</sub>) and C1-MCF-7, AsJWA-MCF-7 (C<sub>2</sub>) cell growth: cells were treated with indicated concentrations of  $As_2O_3$  for 48 h. Suppression of cell proliferation was estimated with the following equation:  $(1 - a/b) \times 100\%$  where  $a$  is the optical density value of the treatment groups and  $b$  is optical density value of the control groups. Data are presented as means  $\pm$  SD from 6 separate experiments. Asterisks indicate significant differences from control values ( $p < 0.05$ ).

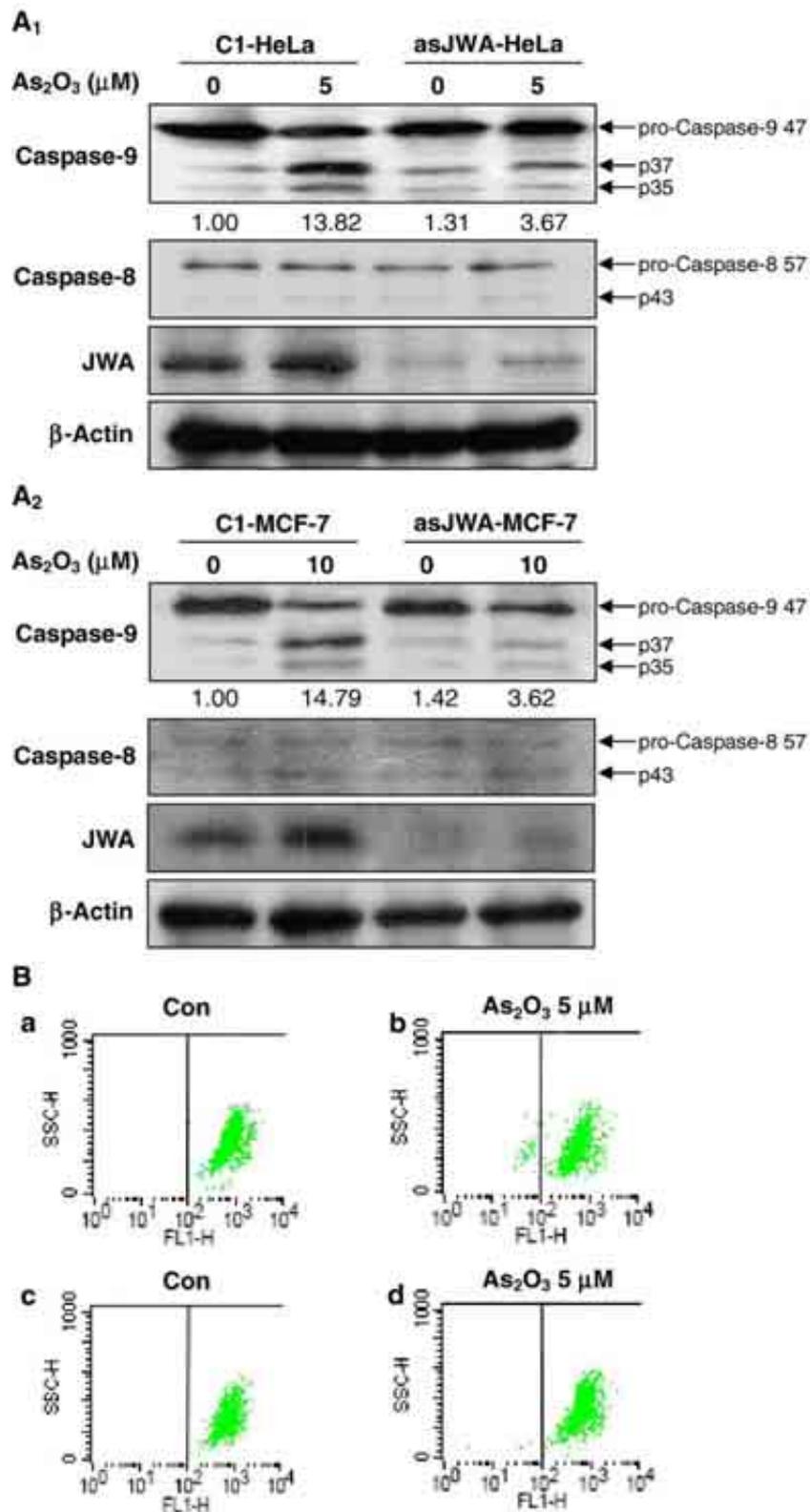
several apoptogenic factors, followed by caspase-9 activation; and an extrinsic apoptotic signaling pathway that occurs via caspase-8 activation (Chauhan et al., 2005; Ishioka et al., 2007). To determine whether caspases signal pathways were involved in  $As_2O_3$  triggered and JWA-dependent cell apoptosis, studies examined expressions of caspase-8 and caspase-9 and their potential cleavages. Data showed that  $As_2O_3$  treatment (5  $\mu$ M for 24 h) significantly triggered caspase-9 but not caspase-8 cleavage, this phenomenon was obviously attenuated in asJWA-HeLa cells (Fig. 3A<sub>1</sub>). Similar phenomena occurred in  $As_2O_3$  treated MCF-7 cells (Fig. 3A<sub>2</sub>).

Since caspase-9 activation is predominantly triggered by the changes of mitochondrial membrane potential ( $\Delta\psi/m$ ), we further investigated that  $As_2O_3$  treatment induced caspase-9 activation was due to the changes of  $\Delta\psi/m$  and JWA was also functionally involved in these consequence. Results of FACS analysis showed that cell treatment with  $As_2O_3$  (5  $\mu$ M for 24 h) led to lost  $\Delta\psi/m$  as indicated by a decrease in Rh-123 fluorescence (Fig. 3Bb). In contrast, asJWA-HeLa cells still maintained their  $\Delta\psi/m$  and only displayed minimal

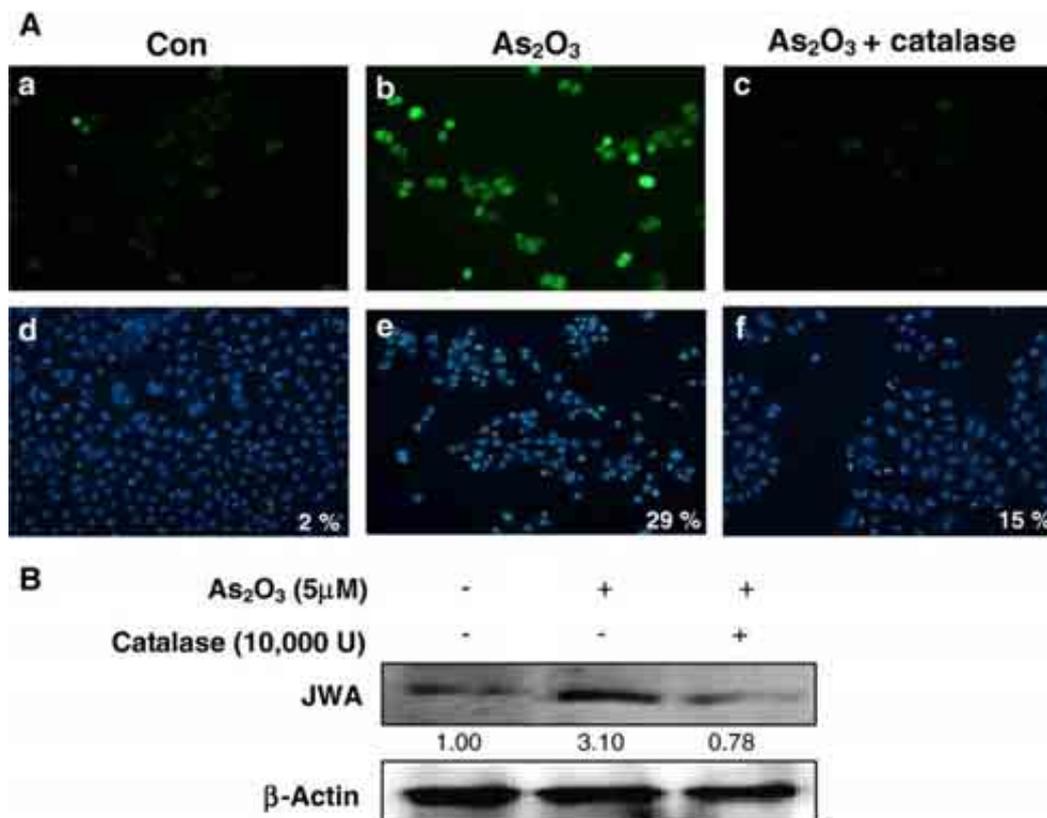
changes in Rh-123 fluorescence (Fig. 3Bd), indicating that loss of mitochondria membrane potential in the presence of JWA expression may be linked to caspase-9 activation.

#### *As<sub>2</sub>O<sub>3</sub> generated ROS induces JWA expression*

ROS are known to affect mitochondria membrane potential, therefore trigger a series of mitochondria-associated events including apoptosis (Tan et al., 1998; Park et al., 2005b).  $As_2O_3$  was found to induce the formation of ROS in a wide variety of cells (Qian et al., 2003). As shown in Fig. 4, when HeLa cells exposed to 5  $\mu$ M  $As_2O_3$  for 1 h, the generation of intracellular ROS ( $H_2O_2$ ) was significantly increased (Fig. 4Ab). This phenomenon was completely suppressed by the addition of the antioxidant catalase (10,000 U/ml) (Fig. 4Ac). As a linked consequence,  $As_2O_3$  (5  $\mu$ M  $As_2O_3$  for 24 h) induced percentage of apoptotic cells from 29% reduced to 15% by addition of catalase (Fig. 4Ae–f). As Fig. 4B indicated,  $As_2O_3$  exposure (5  $\mu$ M for 24 h) resulted in significant up-regulation of JWA in HeLa cells, the



**Fig. 3.** Knock-down JWA against As<sub>2</sub>O<sub>3</sub>-induced apoptosis: knock-down JWA significantly attenuates As<sub>2</sub>O<sub>3</sub>-induced apoptosis in HeLa and MCF-7 cells in a caspase-9-dependent manner: groups of C1-HeLa, asJWA-HeLa (A<sub>1</sub>) and C1-MCF-7, asJWA-MCF-7 (A<sub>2</sub>) cells were adjusted to a density of 1 × 10<sup>6</sup> cells/ml and treated with indicated concentrations of As<sub>2</sub>O<sub>3</sub> for 24 h. Western blot assay was employed to detect the expression of JWA, caspase-9, and caspase-8. The immunoreactive bands were normalized to β-actin bands on the same membrane. The quantitative data below the bands show the band intensities compared with the control group. (B) Knock-down JWA preserves mitochondrial integrity after As<sub>2</sub>O<sub>3</sub> treatment: C1-HeLa and asJWA-HeLa cells were treated with As<sub>2</sub>O<sub>3</sub> 5 μM for 24 h, cells were then recovered and labeled with the RH 123 dye, and the Δψ<sub>m</sub> was detected by FACS in fluorescence channel 1 (FL-1) for green fluorescence. Cells with normal Δψ<sub>m</sub> exhibit strong rhodamine fluorescence. Cells with low fluorescence reflect loss of the Δψ<sub>m</sub>. (a) C1-HeLa cells untreated for control, (b) C1-HeLa cells treated with 5 μM As<sub>2</sub>O<sub>3</sub>, (c) asJWA-HeLa cells untreated for control, and (d) asJWA-HeLa cells treated with 5 μM As<sub>2</sub>O<sub>3</sub>.



**Fig. 4.** As<sub>2</sub>O<sub>3</sub> generated ROS induces JWA expression in HeLa cells. (Aa–c) Detection of ROS generated by 5 μM As<sub>2</sub>O<sub>3</sub> exposure in HeLa cells or after catalase (10,000 U/ml) treatment for 1 h; the intracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels of the cells were detected by fluorescence microscope (IX70, Olympus, Japan) after staining with 2, 7-dichlorofluorescein diacetate (DCFH), as described in Materials and methods. (Ad–f) Detection of cell apoptosis induced by 5 μM As<sub>2</sub>O<sub>3</sub> exposure in HeLa cells or after catalase (10,000 U/ml) treatment for 24 h, cells fixed and stained with Hoechst 33258, the according data in the right corner of the pictures show the percentage of apoptotic cells. (B) Corresponding JWA expression in HeLa cells treatment with 5 μM As<sub>2</sub>O<sub>3</sub> or further with catalase (10,000 U/ml). The JWA immunoreactive bands were normalized to β-actin bands on the same membrane. The quantitative data below the bands show the band intensities compared with the control group.

expression of JWA was also down to levels even below the control after removing H<sub>2</sub>O<sub>2</sub> by addition of catalase.

#### *Molecular mechanisms underlying the effect of JWA on As<sub>2</sub>O<sub>3</sub>-induced apoptosis*

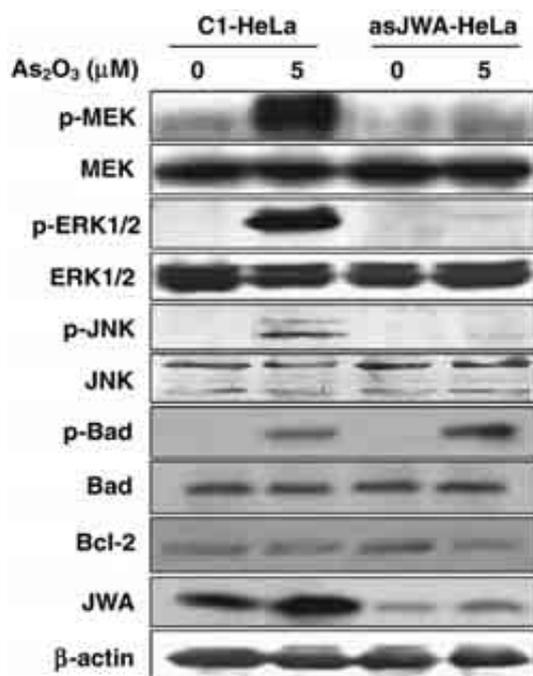
MAPK signal pathways seem to be responsible for mitochondria mediated cell apoptosis (Liu et al., 2006). The Bcl-2 family proteins are also known to be important for apoptosis-regulating molecules that modulate mitochondrial dependent signal pathways (Xia et al., 2004). To understand whether the role of JWA was associated with MAPK or Bcl-2 family proteins, cell culture models were designed to observe the effects of JWA on As<sub>2</sub>O<sub>3</sub> (0, 5 μM for 24 h) induced expression or phosphorylation of MAPK and Bcl-2/Bad molecules. Interestingly, the results showed that the As<sub>2</sub>O<sub>3</sub> activated phosphorylations of MEK, ERK1/2 and JNK were almost completely blocked by knock-down JWA expression; although the total expressions (unphosphorylation level) of these signal molecules were unaffected. The increased Bad phosphorylation was obviously occurred after As<sub>2</sub>O<sub>3</sub> treatment in HeLa cells (Fig. 5). The Bad phosphorylation was further enhanced in As<sub>2</sub>O<sub>3</sub> treated asJWA-HeLa cells.

#### **Discussion**

As<sub>2</sub>O<sub>3</sub>-induced apoptosis was shown to be associated with MAPK, caspase and ROS. However, the mechanisms whereby these molecules cross-talk to each other, and the full spectrum of genetic targets of As<sub>2</sub>O<sub>3</sub>-induced ROS are yet to be established (Maeda et al., 2001;

Chou et al., 2005). Here we provide evidences that JWA may be one of the important molecular targets in mediating As<sub>2</sub>O<sub>3</sub>-induced apoptosis in both HeLa and MCF-7 cancer cells. Firstly, increased JWA expression was observed and paralleled with As<sub>2</sub>O<sub>3</sub>-induced apoptosis, and removing of intracellular H<sub>2</sub>O<sub>2</sub> by addition of catalase resulted in reduced apoptosis and JWA expression. These evidences indicated that H<sub>2</sub>O<sub>2</sub> is a metabolite of As<sub>2</sub>O<sub>3</sub> via activating JWA and then induces apoptosis. Intracellular H<sub>2</sub>O<sub>2</sub> could effectively activate JWA via NFI transcription factor and binds to a CCAAT response element of JWA promoter (Zhu et al., 2006; Chen et al., 2007b). In these linked context events, H<sub>2</sub>O<sub>2</sub> triggered mitochondrial membrane damage and the activation of caspase-9 was necessary for apoptosis. Interestingly, these were also JWA-dependent, suggesting JWA via certain mechanisms to mediate mitochondrial membrane damage.

The changes in Δψ<sub>m</sub>, generation of ROS, and caspase-9 activation are known to be major elements of the mitochondrial pathway of apoptosis (Gupta et al., 2003; Fan et al., 2005). Caspases activation may act as the central executioner in As<sub>2</sub>O<sub>3</sub>-induced apoptosis. Caspase-9 was activated in As<sub>2</sub>O<sub>3</sub>-induced apoptosis in chronic myelogenous leukemia (CML) cells (Slack et al., 2002; Potin et al., 2007). In general, caspase-9 is activated through a mitochondria dependent signal pathway which may be activated by chemotherapeutic agents, UV radiation, oxidative stress, and loss of mitochondrial membrane potential. In contrast, caspase-8 was activated through death receptor pathway by TNF/FAS-family cytokine receptors (Byrd et al., 2002; Evens et al., 2004). It is feasible to differentiate the two pathways by evaluating the activities of caspase-8 and caspase-9 (Liu et al., 2004).



**Fig. 5.** JWA activated MEK–ERK1/2 and JNK phosphorylation. MAPK and Bcl-2/Bad activations in the process of As<sub>2</sub>O<sub>3</sub>-induced apoptosis: C1-HeLa and asJWA-HeLa cells were adjusted to a density of  $1 \times 10^6$  cells/ml and treated with 5 μM As<sub>2</sub>O<sub>3</sub> for 24 h, cell lysates were subjected to Western blot with phospho-MEK, MEK, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-Bad ser112, Bad, and Bcl-2 antibodies. The immunoreactive bands were normalized to β-actin bands on the same membrane.

Data in the present study indicated that JWA exerts effects in HeLa and MCF-7 cells to mediate As<sub>2</sub>O<sub>3</sub>-induced apoptosis via a caspase-9 associated signal pathway.

Secondary, JWA has been proposed as a putative MAPK activating protein (Matsuda et al., 2003) and required for activation of MEK–ERK signal pathways (Chen et al., 2007a). In this study, it was further confirmed that JWA was required for As<sub>2</sub>O<sub>3</sub>-induced MEK–ERK activation; in addition, the activation of JNK was also happened and in a JWA-dependent manner. Studies indicate that As<sub>2</sub>O<sub>3</sub> is an activator of MAPK signaling pathways including ERKs, JNK, and p38 kinase (Cooper et al., 2004). However, the As<sub>2</sub>O<sub>3</sub> dependent induction of different MAPK families is highly dependent on the concentration, the time of exposure, and cell type; and may mediate opposite effects in terms of the biological pathway chosen (Tapio and Grosche, 2006). Among the three main members of the MAPK family in mammalian cells, ERK1/2 is associated with mitogenesis, and as such is generally considered as antiapoptotic; JNK is generally involved in apoptosis induction (Wada and Penninger, 2004). Activation of JNK was shown to correlate with the induction of apoptosis by As<sub>2</sub>O<sub>3</sub> in a variety of cell lines (Miller, 2002; Potin et al., 2007). However, additional studies indicated that the activation of ERK may be pro-apoptotic in certain cell lines, especially in case of oxidative stress-related injuries (Fernandez et al., 2004; Kim et al., 2006; Zhuang and Schnellmann, 2006). Fernandez et al. (2004) demonstrated that ERK activation mediated apoptosis was found after treatment with As<sub>2</sub>O<sub>3</sub> plus protein kinase C activator TPA in promonocytic leukemia cell and that this effect was mediated by intracellular GSH depletion. In this study it was found that MEK1/2, ERK1/2, and JNK phosphorylation was markedly inhibited in the asJWA-HeLa cells. Therefore, the pro-apoptotic effects of JWA may be through pro-apoptotic ERK1/2 and/or JNK phosphorylations.

Finally, the activation of Bad was also found to be associated with As<sub>2</sub>O<sub>3</sub>-induced apoptosis which may not involved in either MEK–ERK

or JNK and via separate signal pathways although JWA was responsible for this process. Bad, a pro-apoptotic Bcl-2 family member, displaces Bax from binding to Bcl-2 and Bcl-xL, thereby promoting apoptosis. On the other hand, phosphorylated Bad ser112 promotes the interaction of Bad with 14-3-3 proteins. The binding of Bad to 14-3-3 correlates with Bad inactivation, thereby inhibiting induction of apoptosis (Datta et al., 2000; Ishitsuka et al., 2005). In this study, only Bad ser112 phosphorylation was increased after treatment with As<sub>2</sub>O<sub>3</sub>, and this role was further enhanced in the asJWA-HeLa cells while the level of Bcl-2, unphosphorylated Bad was not changed. This may give us insight into the fact that the Bad ser112 phosphorylation may be involved in the pathway of JWA inducing its pro-apoptotic effect in As<sub>2</sub>O<sub>3</sub>-induced apoptosis, however, the manner whereby JWA exerts its effect on Bad ser112 phosphorylation remains to be defined. Recent reports demonstrate that Bad ser112 may be phosphorylated by several kinases including AKT, PKA, PKC, Pim-1 and Pim-2, and RSK2, JNK1, MSK1 (Basu and Sivaprasad, 2007; Hindley and Kolch, 2007). Miller et al. (2002) also reported that As<sub>2</sub>O<sub>3</sub> exposure induces translocation of several PKC isoforms from the cytosol to the plasma membrane, where these enzymes play key roles in mediating signal transduction and thus may contribute to the biological effects. Moreover, JWA protein contains SDR–SLR motifs which are essential for C–Raf activation of its downstream effectors MEK–ERK1/2, but not necessary for C–Raf itself. In fact, JWA phosphorylation is required for MEK–ERK MAPK cascades activation. Knock-down JWA results in inactivation of the MEK–ERK and their downstream effectors pathway. Therefore, it is reasonable to postulate that the association between JWA and Bad ser112 phosphorylation may somehow be mediated by PKC activated MEK–ERK signal pathway.

How the phosphorylation of MEK, ERK, JNK and caspase-9 cleavage links with the observed JWA-induced apoptosis needs to be further investigated. Zhuang and Schnellmann (2006) reported that the pro-apoptotic ERK1/2 may act on mitochondria through Bax and/or p53 to regulate activation of caspases-9 and -3 which are downstream of cytochrome *c* release. And JNK activation may transduce apoptotic signals through the posttranslational modification of some Bcl-2 family members phosphorylated by JNK including antiapoptotic Bcl-2 and Bcl-xL and pro-apoptotic Bax, Bak, and Bid (Davison et al., 2004; Gao et al., 2005). Suzuki et al. (2004) reported that when Bad was dephosphorylated, it releases from 14-3-3 protein, dimerizes with antiapoptotic Bcl-xL, and displaces and releases Bax from Bcl-xL. Thereafter, Bax translocates to mitochondria in which it promotes the release of cytochrome *c* and activation of the caspase cascade. With the observation of enhanced Bad phosphorylation in asJWA-HeLa cells treated with As<sub>2</sub>O<sub>3</sub>, JWA might exert its effect on ERK and JNK through Bax and/or p53 to caspase-9.

In summary, the results presented here indicate that (1) As<sub>2</sub>O<sub>3</sub> generated H<sub>2</sub>O<sub>2</sub> via inducing JWA expression and then triggered a series of signal transduction events to induce apoptosis in HeLa and MCF-7 cells. (2) JWA is required for As<sub>2</sub>O<sub>3</sub>-induced activation of MEK–ERK and JNK which further closely linked to changes of mitochondrial membrane potential and the activation of caspase-9; these cascade events play a central role for As<sub>2</sub>O<sub>3</sub> inducing apoptosis. (3) As<sub>2</sub>O<sub>3</sub>-induced Bad activation also contributed to apoptosis and regulated by JWA, however, probably mediated by non-MEK–ERK/JNK mechanism. Data may also be helpful to develop new strategies in using JWA as a potential target for the therapeutic use of As<sub>2</sub>O<sub>3</sub> in certain types of tumors.

#### Acknowledgments

This work was funded in part by National Natural Science Foundation of China (No. 30771828, 30771829); and the key basic research project of Education Department, Jiangsu Province (06KJA33023).

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