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Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome *c* release induced by arsenic trioxide

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Abstract

The precise molecular mechanism underlying arsenic trioxide (As_2O_3)-induced apoptosis is a subject of extensive study. Here, we show that clinically relevant doses of As_2O_3 can induce typical apoptosis in IM-9, a multiple myeloma cell line, in a Bcl-2 inhibitable manner. We confirmed that As_2O_3 directly induced cytochrome *c* (cyto *c*) release from isolated mouse liver mitochondria via the mitochondrial permeability transition pore, and we further identified the voltage-dependent anion channel (VDAC) as a biological target of As_2O_3 responsible for eliciting cyto *c* release in apoptosis. First, pretreatment of the isolated mitochondria with an anti-VDAC antibody specifically prevented As_2O_3-induced cyto *c* release. Second, in proteoliposome experiments, VDAC by itself was sufficient to mediate As_2O_3-induced cyto *c* release, which could be specifically inhibited by Bcl-X_L. Third, As_2O_3 induced mitochondria membrane potential ($\Delta\Psi m$) reduction and cyto *c* release only in the VDAC-expressing, but not in the VDAC-deficient yeast strain. Finally, we found that As_2O_3 induced the increased expression and homodimerization of VDAC in IM-9 cells, but not in Bcl-2 overexpressing cells, suggesting that VDAC homodimerization could potentially determine its gating capacity to cyto *c*, and Bcl-2 blockage of VDAC homodimerization represents a novel mechanism for its inhibition of apoptosis.

Keywords

apoptosis; arsenic trioxide; Bcl-2 proteins; cytochrome *c*; VDAC

Introduction

Apoptosis or programmed cell death is a genetically regulated process that plays an important role in tissue homeostasis and development in multicellular metazoans (Vander Heiden and Thompson, 1999; Green and Evan, 2002). Defects in apoptosis are often

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associated with diseases, such as neuronal degenerative diseases, tumorigenesis, autoimmune disorders, and viral infections (Hickman, 2002; Johnstone *et al*., 2002). Not only are mitochondria the cellular powerhouse for ATP generation but also they play an essential role in regulating apoptosis (Kroemer, 1999; Vander Heiden and Thompson, 1999; Gottlieb, 2000). Mitochondria-dependent apoptosis involves the permeabilization of mitochondrial membranes, which appears to mediate the release from the intermembrane space into the cytosol of apoptogenic factors such as cytochrome *c* (cyto *c*), AIF, Smac/DIABLO, and Endonuclease G (Wang, 2001). Cyto *c*, a soluble protein, normally resides between the inner and outer mitochondrial membrane and participates in oxidative phosphorylation required for energy production (Skulachev, 1998). Once released, it acts as a cofactor to induce the aggregation of Apaf-1 and apoptosome to activate caspases and subsequently the execution of programmed cell death (Wang, 2001).

The mechanisms of how cyto *c* and other apoptogenic factors are released from mitochondria remain elusive. It is generally acknowledged that opening of the permeability transition pore (PTP) located at the contact site of the inner and outer membrane of a mitochondrion could be involved (De Pinto and Palmieri, 1992; Marzo *et al*., 1998a; Desagher and Martinou, 2000). PTP is comprised of the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT) in association with other proteins (Colombini, 1989; De Pinto and Palmieri, 1992; Marzo *et al*., 1998c). ANT is an inner membrane channel that plays a role in ADP/ATP exchanges between the mitochondrial matrix and the intermembranous space (Graham *et al*., 1997; Fiore *et al*., 1998). On the other hand, VDAC is a Ca^{2+}-sensitive channel, which functions as the major pathway for metabolite diffusion across the mitochondrial outer membrane (Colombini, 1989; De Pinto and Palmieri, 1992). VDAC was suggested to form a dimer (Krause *et al*., 1986; Szabo *et al*., 1993; Szabo and Zoratti, 1993), which could represent the PTP-forming component. It is believed that VDAC undergoes extensive conformational changes in response to a variety of stimuli (Szabo *et al*., 1992; Mannella, 1998; Song *et al*., 1998), and facilitates cyto *c* release following some apoptotic insults (Crompton, 1999; Martinou and Green, 2001). Interestingly, Bcl-2 and its family proteins, key regulators of apoptosis and cyto *c* release, are thought to interact physically with VDAC and/or ANT to regulate the opening of PTP during apoptosis (Kroemer, 1997; Shimizu *et al*., 1999). Bcl-2 could modulate the configuration of VDAC and thus the transport of metabolites and permeability transition, although the molecular details of how these proteins are involved in mediating cyto *c* release are a subject of intense debate. Our recent results indicate that, at least *in vitro*, VDAC interacts with both Bax and Bcl-X_L to form a tertiary complex and that the function of VDAC in mediating cyto *c* release could depend on the ratio between Bax and Bcl-X_L (Shi *et al*., 2003a).

Arsenic trioxide (As_2O_3) is a traditional drug that has been widely used for over 2000 years in China (Chen *et al*., 1996; Aposhian, 1997; Miller Jr *et al*., 2002). Recently, clinical data have shown that As_2O_3 induces complete remission of acute promyelocytic leukemia (APL) without any significant side effects (Shen *et al*., 1997; Soignet *et al*., 1998; Zhang *et al*., 2001). There are numerous reports on the proapoptotic effects of As_2O_3 in malignant cell lines through complex signaling pathways, with several clinic trials being conducted on hematopoietic malignancies and solid tumors (Shen *et al*., 1997; Wang *et al*., 1998; Bazarbachi *et al*., 1999; Jing *et al*., 1999; Rousselot *et al*., 1999; Perkins *et al*., 2000; Anderson *et al*., 2002; Miller Jr *et al*., 2002). Remarkably, it has been suggested that arsenite might directly target mitochondrial PTP to induce apoptosis in cancerous cells (Petronilli *et al*., 1994; Costantini *et al*., 1996; Larochette *et al*., 1999), although the precise molecular mechanism is still elusive. Here, we provide for the first time the genetic and biochemical evidence that VDAC is one of the biological targets responsible for induction of cyto *c*

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release and apoptosis by As_2O_3. A full understanding of the molecular mechanism of this ancient remedy may be useful to develop better therapeutic drugs for fighting cancer.

Results

As_2O_3 induces apoptosis, reduction of ΔΨm, and release of cyto c from mitochondria in a Bcl-2 inhibitable manner

We used a clinically relevant concentration of As_2O_3 to treat a multiple myeloma cell line IM-9 for 24 h and assayed for the exposure of phosphotydyl-serine on the plasma membrane, indicative of apoptosis. FACS analysis showed that As_2O_3 induced a significant increase of the population of Annexin V^{high}/PI^{low} IM-9 cells (Figure 1a). This result clearly demonstrated that As_2O_3 could effectively induce apoptosis in multiple myeloma cells. Overexpression of Bcl-2 in IM-9/Bcl-2 cells, which we have shown previously to suppress ionizing radiation-induced apoptosis (Chen *et al*., 2000, 2003), also inhibited As_2O_3-induced apoptosis (Figure 1a). Moreover, the detection of cytoplasmic cyto *c* from IM-9 cells treated with 2 μM As_2O_3 showed the appearance of cyto *c* starting 12 h following treatment (Figure 1c), concomitant with the reduction of ΔΨm (Figure 1b), the onset of caspase 3 activity (data not shown), and Annexin V positivity. On the other hand, As_2O_3-induced release of cyto *c* in the IM-9/Bcl-2 cell line was significantly reduced and the reduction of ΔΨm was prevented (Figure 1b, c), although higher doses of As_2O_3 abrogated the ability of Bcl-2 to suppress apoptosis (data not shown). These results suggest that As_2O_3 evokes a specific apoptotic pathway related to mitochondrial functions. To test this directly, we incubated isolated mouse liver mitochondria with clinically relevant concentrations of As_2O_3 and measured the release of cyto *c* from mitochondria. Indeed, As_2O_3 induced the release of cyto *c* in a dose-(Figure 2a) and time-(Figure 2b) dependent manner. The release of cyto *c* from isolated mitochondria upon treatment with 5 μM As_2O_3 started at 15 min, with mitochondrial cyto *c* being exhausted at 60 min (Figure 2b). These results indicate that As_2O_3 could directly target mitochondria to induce cyto *c* release and apoptosis.

As_2O_3-induced PTP opening and cyto c release is inhibited by PTP-specific inhibitors

To understand the mechanism of cyto *c* release and to determine if PTP is targeted by As_2O_3, we exposed isolated mouse liver mitochondria to various concentrations of As_2O_3 and found that mitochondria swelling, indicative of PTP opening, was evoked in a dose-dependent manner (Figure 3a). A clinically relevant dose of As_2O_3 (5 μM) was sufficient to induce the opening of PTP, starting at about 12 min, while higher doses of As_2O_3 shortened the time required to cause PTP opening. Our results reveal that cyto *c* release occurs concomitantly with the PTP opening and precedes the disruption of ΔΨm (data not shown). Therefore, it is reasonable to propose that As_2O_3 might target the outer mitochondrial membrane (OMM) to induce cyto *c* release, while mitochondrial depolarization and the disruption of oxidative respiratory chain could be secondary to PTP opening and the loss of cyto *c*.

Cyclosporin A (CsA) and bongkrekic acid (BA) are very potent inhibitors of PTP (Marzo *et al*., 1998b). The mitochondrial swelling (Figure 3b) and the efflux of cyto *c* (Figure 3c) induced by As_2O_3 were completely inhibited by pretreatment of isolated mitochondria with either CsA or BA. Bcl-2 and Bcl-X_L have been reported to prevent cyto *c* release and PTP opening induced by various stimuli (Kroemer, 1997; Marzo *et al*., 1998b; Narita *et al*., 1998). As shown in Figure 3d, the addition of Bcl-X_L to isolated mitochondria prevented As_2O_3-induced cyto *c* release in a dose-dependent manner, with higher doses completely preventing cyto *c* release. These results strongly suggest that As_2O_3 might function through PTP to regulate mitochondrial swelling and the associated cyto *c* release.

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VDAC is necessary and sufficient to mediate cyto c release induced by As_2O_3

VDAC is an abundant outer membrane protein and a critical component of the PTP complex (Colombini *et al*., 1996). To determine whether the function of VDAC is required for As_2O_3-induced cyto *c* release, isolated mitochondria were preincubated with an anti-VDAC polyclonal antibody (Ab#25) reported to be a specific steric inhibitor of VDAC-mediated cyto *c* release induced by Bax and Bak (Shimizu *et al*., 2001). As shown in Figure 4a, the antibody could completely prevent the cyto *c* release induced by As_2O_3. In contrast, under identical conditions, the polyclonal antibody raised against the N-terminal peptide of VDAC had no such effect. These data demonstrate unambiguously that VDAC is involved in As_2O_3-induced cyto *c* release.

To investigate whether VDAC is sufficient to mediate As_2O_3-induced cyto *c* release, proteoliposomes encapsulated with FITC-cyto *c* were reconstituted in the presence or absence of VDAC. The reconstituted liposomes were then exposed to 2 μM As_2O_3 for 1 h. The liposomes were not leaky to cyto *c* since there was no detectable FITC fluorescence or cyto *c* protein in the plain liposome preparation. The release of cyto *c*-FITC to the supernatant was found to be strictly dependent on the presence of VDAC (Figure 4b) and such dependence could be abrogated by the presence of Bcl-X_L in the VDAC liposomes, further supporting the notion that a direct interaction between Bcl-X_L and VDAC provides a general yet robust antiapoptotic mechanism in response to As_2O_3. This and the above-mentioned antibody blocking experiment clearly demonstrate that VDAC is necessary and sufficient to mediate the proapoptotic function of As_2O_3, while Bcl-X_L functions to block this effect, possibly by direct interaction with VDAC.

VDAC is required for As_2O_3-induced ΔΨm reduction and cyto c release in yeast

Saccharomyces cerevisiae has been used as a simple model for apoptosis studies (Matsuyama *et al*., 1999; Gross *et al*., 2000). To analyse directly whether VDAC is a target for As_2O_3-triggered apoptosis in intact cells, wild-type (M-3), a VDAC1-deficient (M22-2), or the M22-2 yeast strain supplemented with a human *VDAC1* gene (M22-2/pBDL-VDAC) was treated with 2 μM As_2O_3 for 12 h and ΔΨm was then examined by flow cytometry. As shown in Figure 5a, As_2O_3 significantly decreased ΔΨm in M-3 cells ($P < 0.01$), but had no effect on M22-2 cells. The basal level of ΔΨm in VDAC-deficient yeast cells seemed to be higher than that of wild-type M-3 cells. Interestingly, reintroduction of human VDAC1 into M22-2 cells could partially restore its sensitivity of ΔΨm reduction to As_2O_3 and its basal level of ΔΨm to that of the wild-type yeast M-3 strain. To prove directly that VDAC is responsible for As_2O_3 induced cyto *c* release, we treated isolated mitochondria from the above yeast cells directly with As_2O_3. We found that mitochondria from M-3 and M22-2 (hVDAC1) were permeated by As_2O_3 to release cyto *c* in a dose-dependent manner. Remarkably, the release of cyto *c* was completely blocked in the mitochondria from the VDAC1-deficient yeast M22-2 strain (Figure 5b). These results further support the observation that VDAC is a target of As_2O_3 responsible for eliciting mitochondrial apoptotic changes in intact cells.

As_2O_3 induces VDAC upregulation and dimerization

To further understand the functional aspects of VDAC in mediating As_2O_3-induced cyto *c* release, we examined the expression levels of VDAC following As_2O_3 treatment in IM-9 cells. To our surprise, we found that As_2O_3 upregulated the expression of VDAC in IM-9 cells (Figure 6a). It was suggested, although evidence remains to be provided, that VDAC could potentially form a higher-order complex that participates in gating the efflux of cyto *c* through OMM (Szabo and Zoratti, 1991; Szabo and Zoratti, 1992). To test directly whether As_2O_3 could induce the higher-order complexing of VDAC molecules, we used a cross-linking assay, a commonly used approach to detect the interactions between molecules, to

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examine the VDAC profile in isolated mouse liver mitochondria and intact multiple myeloma cells. First, incubation of isolated mitochondria with the cross-linking reagent DSS after As_2O_3 treatment indeed induced VDAC homodimerization (Figure 6b). We next examined homodimerization of VDAC following As_2O_3 treatment in the multiple myeloma cells. As expected, we found that As_2O_3 induced the homodimerization of VDAC in IM-9 cells (Figure 6c). In contrast, there was no detectable VDAC homodimer present in untreated or in DMSO-treated cells. Also, we did not detect a higher order of oligomerization.

Interestingly, ectopic overexpression of Bcl-2 blocked As_2O_3-induced VDAC upregulation and homodimerization. These results further suggest that VDAC could be a biological stress sensor to As_2O_3, and its homodimerization induced by As_2O_3 could potentially determine its gating capacity to efflux cyto *c*, as Bcl-2 effectively inhibited such a pathway.

Discussion

The present study provides genetic and biochemical evidence that VDAC might play an essential role in As_2O_3-induced apoptotic changes of the mitochondria. First, the anti-VDAC antibody (Ab#25) specifically and effectively prevented As_2O_3-induced cyto *c* release from isolated mitochondria. The same antibody was used as a specific VDAC channel blocker both *in vitro* and *in vivo* (Shimizu *et al*., 2001). Second, liposome and yeast experiments showed that VDAC was necessary and sufficient to mediate cyto *c* efflux caused by As_2O_3. Our results are in good agreement with earlier reports that arsenite might induce mitochondrial PTP opening (Petronilli *et al*., 1994; Costantini *et al*., 1996; Larochette *et al*., 1999). These results are in sharp contrast with a previous report that ANT, rather than VDAC, mediates the mitochondrial membrane permeabilization induced by arsenite (Belzacq *et al*., 2001). The participation of ANT in As_2O_3-mediated stress signal cannot be ruled out, since CsA and BA could inhibit As_2O_3-induced PTP opening and cyto *c* release. Third, Bcl-X_L/Bcl-2 could potently inhibit VDAC-mediated cyto *c* release and its dimerization, possibly through a mechanism of protein–protein interactions (Tsujimoto and Shimizu, 2000; Shi *et al*., 2003a).

Considering the finding that the Bcl-2/Bcl-X_L-VDAC interaction alleviated the apoptotic effect of As_2O_3, we favor the model by which VDAC might be directly targeted by As_2O_3 to mediate cyto *c* release. Our data do not rule out the participation of Bax and/or Bak, commonly acknowledged mediators for cyto *c* release, in As_2O_3-induced cyto *c* release and apoptosis. Further studies are required to understand the exact mechanisms of VDAC in regulating cyto *c* release and how its interactions with Bcl-2 family proteins may determine the outcome of cell fate.

As_2O_3 has been widely used to treat APL and other types of malignant leukemia. Caspase activation (Chen *et al*., 1998b; Soignet *et al*., 1998) and enhanced generation of reactive oxygen species (ROS)(Chen *et al*., 1998b) were suggested to be responsible for the specific cell death in cancer lesions. Our findings could offer an explanation for As_2O_3 apoptotic events, since cyto *c* release from mitochondria into cytosol is causally linked to caspase activation and disruption of mitochondrial respiratory chain, and subsequently the enhanced generation of ROS from the mitochondria. What is puzzling is how clinically relevant doses of As_2O_3 induce apoptosis, leading to tumor cell-specific killing in the clinic. It remains to be investigated whether there exists any tumor-specific target that determines the differential metabolism or distinct responses to As_2O_3-mediated stress between neoplastic and normal cells. Indeed, As_2O_3 induces degradation of the PML/RAR fusion protein in APL patients (Shen *et al*., 1997; Dai *et al*., 1999) or selectively downregulates the Bcl-2 protein via caspase-3 cleavage (Chen *et al*., 1996).

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There are conflicting reports with regard to the Bcl-2 suppression of As_2O_3-induced apoptosis. It was reported that As_2O_3-induced apoptosis of multidrug-resistant acute myelocytic leukemia cells, regardless of whether Bcl-2 and Bcl-X_L were overexpressed (Perkins *et al*., 2000), while other reports suggest that both Bcl-2 and its homologue Bcl-X_L could confer resistance against apoptosis by inhibiting the reduction of $\Delta\Psi m$, cyto *c* release, and caspase activation (Green and Reed, 1998; Cory and Adams, 2002). Our data indicate that overexpression of Bcl-2 could attenuate or delay apoptosis, cyto *c* release, and $\Delta\Psi m$ reduction induced by As_2O_3 in IM-9 cells, with Bcl-X_L potently inhibiting As_2O_3-induced cyto *c* release from isolated mitochondria.

A rather surprising finding was that As_2O_3 upregulated the expression levels of VDAC. This suggests that VDAC could potentially serve as a biological stress sensor to As_2O_3, either directly or indirectly. It is known that radiation could also induce the upregulation of VDAC in LYas cells (Voehringer *et al*., 2000) and increased expression of VDAC is correlated with uterine epithelial apoptosis after estrogen deprivation (Takagi-Morishita *et al*., 2003).

Questions still remain as to how VDAC mediates cyto *c* release induced by As_2O_3. As_2O_3 is a selective dithiol cross-linker (Petronilli *et al*., 1994) that can modulate the levels of the redox modulators of the PT pore, such as GSH and NADH. As_2O_3 cross-linking of cysteine residues within the transmembrane domain of VDAC, if accessible, could lead to the changes of conformation, thus the channel activities, of VDAC and PTP (Jing *et al*., 1999). Alternatively, as we observed, As_2O_3 could induce homodimerization of VDAC molecules, and therefore the VDAC pore activity to mediate cyto *c* release. Previous biochemical and electrophysiological evidence indicates that VDAC tends to form a dimer or oligomer, at least in yeast and in artificial biolipid membranes (Krause *et al*., 1986; Szabo *et al*., 1993; Szabo and Zoratti, 1993). However, we do not observe either the dimers of VDAC in nonapoptotic cells or the oligomers in apoptotic cells in our cross-linking assay. There could be sequential events consisting in As_2O_3 inducing conformational changes of VDAC, which brings the different VDAC subunits in closer proximity to form a dimer to facilitate cyto *c* release. To the best of our knowledge, this is the first report indicating that VDAC forms a homodimer during As_2O_3-induced apoptosis in mammalian cells and that Bcl-2 prevents the homodimerization and apoptotic responses. Based on these data and our recent observation that Bcl-X_L interacts with VDAC via the putative loop region (Shi *et al*., 2003a), we propose that Bcl-2/Bcl-X_L may interact with VDAC to block its dimerization sterically, which may be a prerequisite for cyto *c* release. This may represent a novel mechanism for the inhibition of apoptosis by Bcl-2. Further studies are required to investigate the functional significance of VDAC homodimerization and its regulation by Bcl-2 family proteins.

Materials and methods

Chemicals

Bongkrekic acid (BA) was from BioMol Research Laboratories (Plymouth Meeting, PA, USA); 3,3′-dihexyloxacarbo-cyanineiodide [$DiOC_6(3)$] and anti-cytochrome *c* oxidase monoclonal antibody (COX-IV)(A-21348) were from Molecular Probes (Eugene, OR, USA). The Annexin V apoptosis detection kit and purified anti-cytochrome *c* antibody (65981A) were from PharMingen (San Jose, CA, USA). Disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL, USA). The VDAC1 (N-18) goat polyclonal antibody (sc-8828) was from Santa Cruz Biotechnology (CA, USA), the Bcl-x mouse monoclonal antibody (AHO0222) was from Biosource International (Camarillo, CA, USA). All other reagents were obtained from Sigma (St Louis, MO, USA).

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Cell culture

Human IM-9 multiple myeloma cells were routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin at 37°C and 5% CO_2. pSFFV-neo FLAG.Bcl-2 was used for stable transfection of IM-9 cells. Cell clones overexpressing Bcl-2 were selected in 1 mg/ml G418 as described previously (Chen *et al*., 2000). Exponentially growing cells were subjected to the various treatments as indicated.

M-3 and M22-2 yeast cells were grown in YPD medium containing 1% yeast extract, 2% peptone, and 2% dextrose (Gross *et al*., 2000). A human vdac1-expressing ΔVDAC *S. cerevisiae* strain [M22-2 (hVDAC1)] was produced by transfecting human *vdac1* cDNA using standard lithium acetate method with tryptophan selection. The transformed yeast cells were grown in the minimal SD base (yeast nitrogen base, dextrose, and ammonium sulfate) plus TRP DO (dropout) supplement (Clontech, Palo Alto, CA, USA).

Flow cytometric assay for Annexin V positivity

Apoptosis was measured using the Annexin V detection kit according to the manufacturer's instruction. Flow cytometric analysis was performed to monitor the green fluorescence of FITC-conjugated Annexin V (530 ± 30nm) and the red fluorescence of DNA bound propidium iodide (PI, 630 ± 22 nm) (Chen *et al*., 1998a). All data were analysed with a Cell Quest software (BD, CA, USA).

Measurement of mitochondrial membrane potential

This assay was performed as described previously (Chen *et al*., 1998a). Briefly, cells were collected after being treated with As_2O_3. $DiOC_6(3)$[2 μl of 2μM stock solution in dimethyl sulfoxide (DMSO)] was added to 0.4 ml cell suspension (4×10^5 cells/ml) in PBS (pH 7.2) and incubated at 37°C for 5 min. PI (5 μl of 500 μg/ml stock) was added 30 s before analysis. ΔΨm was analysed by flow cytometry with excitation at 488 nm. $DiOC_6(3)$ data were validated by addition of 1 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) after 5 min of $DiOC_6(3)$ loading.

Cell fractionation and immunoblot analysis

As_2O_3-treated cells were fractionated by differential centrifugation as previously described (Chen *et al*., 1997, 2000). Briefly, cells were homogenized with a Dounce homogenizer and the homogenate was centrifuged at 800 *g* for 5 min to remove unbroken cells and nuclei, and the cytosolic fractions were obtained by further centrifugation at 100 000 *g* for 30 min. For immunoblots, proteins were separated by SDS–PAGE, transferred onto nitrocellulose membranes (Schleicher and Schull), and probed with specific antibodies as indicated. Immunoreactive bands were visualized using enhanced chemiluminescence (Pierce).

Isolation of mouse liver mitochondria and measurement of PTP opening and cyto c release

Liver mitochondria from Balb/c mice were isolated by routine methods as previously described (Bernardi, 1984; Xia *et al*., 2002a, b). The quality of isolated mitochondria was examined by oxygen consumption. The protein content of mitochondria was determined by the micro biuret method using BSA as a standard. Isolated mitochondria (5 mg protein/ml) were kept in a PT buffer containing 250mM sucrose, 2mM HEPES (pH 7.4), 0.5mM KH_2PO_4, 2μM rotenone, and 4.2mM potassium succinate. The PTP opening was monitored by the decrease of 90° light scattering at 520nm using a Jobin-Yvon FluoroMax-2 spectrofluorimeter as previously described (Petronilli *et al*., 1993; Xia *et al*., 2002a, b). For cyto *c* release, different concentrations of As_2O_3 were added at the indicated time. Various PTP blockers, if needed, were usually added 5 min before As_2O_3 treatment. The samples

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were then centrifuged at 12 000 *g* at 4°C for 15 min. Cyto *c* in the supernatant and mitochondrial pellets was detected by Western blotting using anti-cyto *c* monoclonal antibody. Protein loading was verified by immunodetection of COX-IV in the mitochondrial pellets. In certain cases, the nonspecific bands that appeared in cyto *c* Western blotting were used as an internal loading control. In most cases, 1–5 μM As_2O_3 were used, but in certain experiments, whereas indicated, to avoid potential damage to the mitochondria due to the prolonged exposure, higher doses, and shorter duration of treatments were applied.

Isolation of yeast mitochondria and measurement of yeast mitochondria membrane potential

For isolation of yeast mitochondria (Gross *et al*., 2000), 500 ml yeast cells (OD_{600} = 1.0) were collected, washed, and incubated with 1.2M, sorbitol–K_2HPO_4–KH_2PO_4 (pH 7.4) buffer containing Zymolyase-20 T [20 000 U/g (ICN, CA, USA); 2mg per g of cells] for 1 h at 30°C. The spheroplasts were homogenized in a tight glass homogenizer with about 20 strokes. Yeast mitochondria were obtained by differential centrifugation and were resuspended in buffer containing 0.6M mannitol, 20mM HEPES (pH 7.4), and 0.1% fatty acid-free BSA before use.

Flow cytometric analysis of ΔΨm in yeast was performed as described previously (Gross *et al*., 2000). Cells (1×10^6) of different yeast strains were treated with As_2O_3, washed twice in ice-cold PBS, and stained with 40 nM $DiOC_6(3)$ at 30°C for 15 min in the dark. PI (10 μg/ml) was added 30 s before analysis for detecting the dead cells. ΔΨm of yeast mitochondria was analysed using FACS with excitation at 488nm and the data were validated by addition of 1 μM CCCP after 15 min of loading of $DiOC_6(3)$.

Expression and purification of recombinant proteins

Human Bcl-X_L was expressed as a GST fusion protein in *E. coli*, purified on a glutathione-Sepharose column, and concentrated by ultrafiltration to remove GSH. His-tagged human VDAC1 was purified by immobilized affinity chromatography (IMAC, Qiagen) to near homogeneity under denaturing conditions (Shi *et al*., 2003b).

Fluorescence measurement of cyto c release in VDAC liposomes

Liposomes were prepared by a standard method as described previously (Madesh and Hajnoczky, 2001; Shi *et al*., 2003a). Briefly, 500mg L-α-phosphatidyl choline was dissolved in 5ml chloroform, and the solvent was then evaporated under nitrogen. A phospholipid mixture was reconstructed in 10 ml liposome buffer containing 50mM KCl, 20mM KH_2PO_4, 20mM HEPES (pH 7.0) and 1mM EDTA. After sonication, purified VDAC (0.1 mg/ml, final concentration), and/or Bcl-X_L (0.1 mg/ml, final concentration) was then mixed with liposomes and incubated for 20 min at 25°C. The resulting proteoliposomes were dialysed overnight at 4°C to remove excessive detergent. FITC-conjugated cyto *c* was loaded into the proteoliposomes by three freeze–thaw cycles, and then the proteoliposomes were washed three times with the liposome buffer to remove the cyto *c*-FITC present outside of the vesicles. Aliquots of the three types of liposomes were mixed with 2 μM As_2O_3 and incubated for 1 h at 25°C and the reactions were terminated by centrifugation (18 000 *g*, 30 min at 4°C). The cyto *c*-FITC released in the supernatant was quantified by a fluorometric method (490nm excitation/510nm emission) and was also immunodetected using a specific cyto *c* monoclonal antibody. Equal loading of cyto *c* in the proteoliposomes was determined by both fluorometric and immunodetection of cyto *c* levels. The incorporation of proteins in proteoliposomes was verified by Western blotting using VDAC or Bcl-X_L-Specific antibodies.

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Cross-linking for VDAC

Following treatments with As_2O_3, cells or isolated mitochondria were washed with conjugating buffer containing 150 μM NaCl, 20mM HEPES (pH 7.2), 1.5mM $MgCl_2$, and 10mM glucose. DSS in DMSO was added to a final concentration of 2mM (Gross *et al*., 1998; Makin *et al*., 2001). After reaction at room temperature for 30 min, the cross-linker was quenched by the addition of 1M Tris-HCl (pH 7.5) to a final concentration of 20mM. Samples were then solubilized in 1% NP-40 and centrifuged at 12 000 *g* for 10 min. VDAC was detected by Western blotting using an anti-VDAC1 polyclonal antibody (sc-8828).

Statistical analysis

Significant differences between values under different experimental conditions were determined by paired Student's *t*-test analyses. A value of $P < 0.05$ was considered to be significant.

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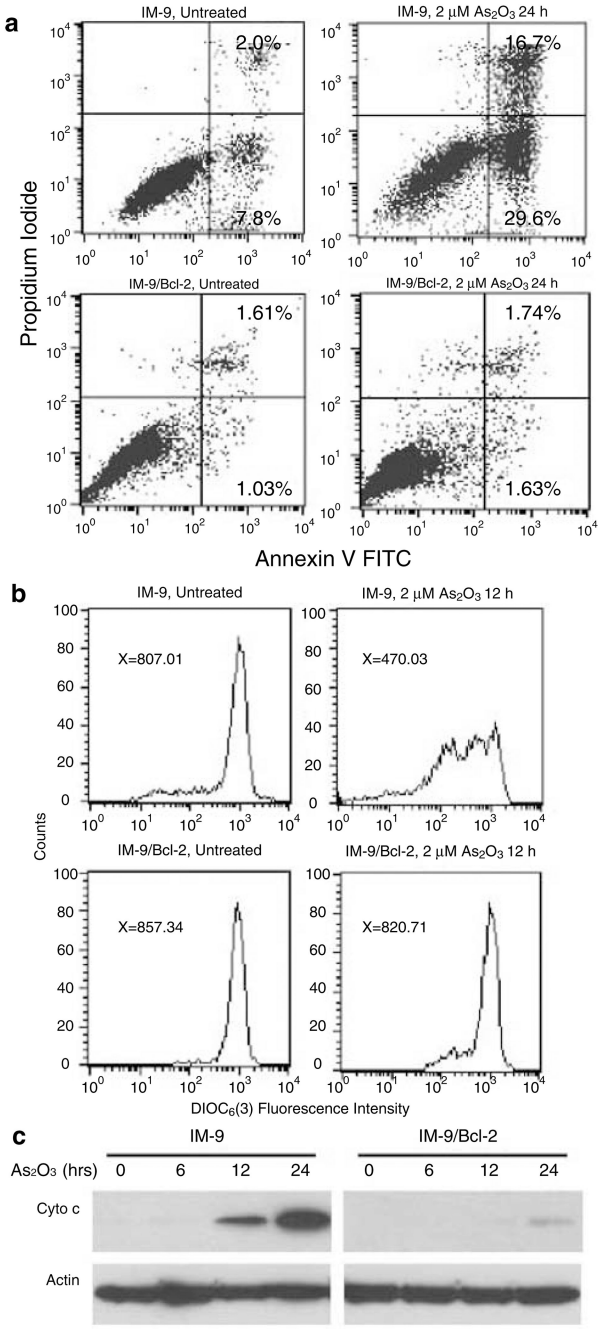

Figure 1.

As_2O_3 induces apoptosis, ΔΨm reduction, and cyto *c* release in IM-9 cells in a Bcl-2 inhibitable manner. (**a**) Flow cytometric analysis of apoptosis in IM-9 and IM-9/Bcl-2 cells treated in the absence or presence of 2μM As_2O_3 for 24 h as determined by binding of Annexin V and uptake of PI; (**b**) IM-9 and IM-9/Bcl-2 cells were treated with or without 2μM As_2O_3 for 12 h, and the ΔΨm was measured using $DiOC_6(3)$ by FACS analysis. X represents the mean value of green fluorescence [$DiOC_6(3)$] from the subpopulation of cells that were negative for red fluorescence (PI); (**c**) The levels of cyto *c* released into cytosol were determined by differential centrifugation followed by Western blotting as described in

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'Materials and methods' and actin was used as a loading control. All data shown are representative of three separate experiments

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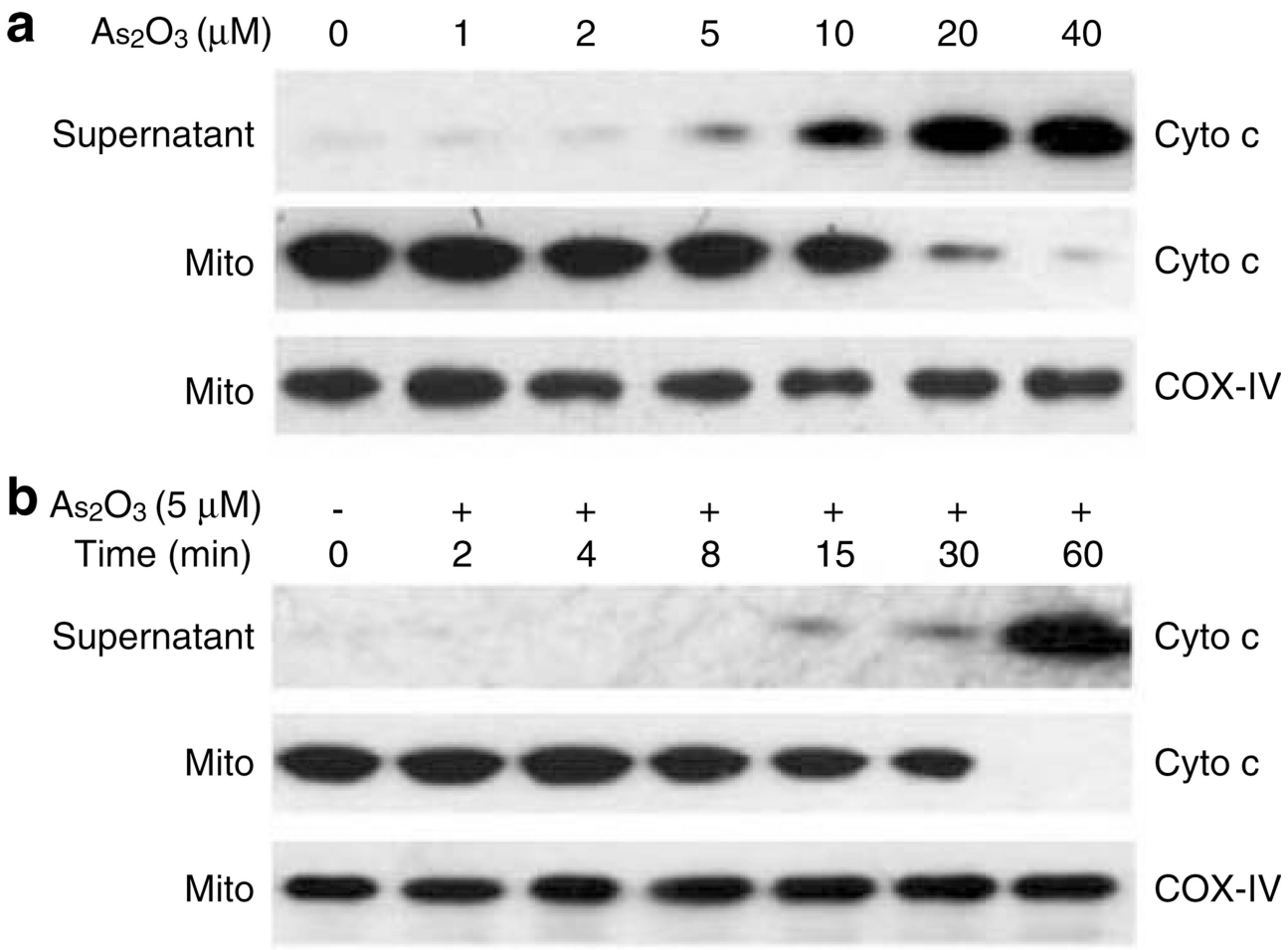

Figure 2.

As_2O_3 induces cyto *c* release from isolated mitochondria. Isolated mitochondria (5mg protein/ml) were incubated at 25°C for 30 min with different concentrations of As_2O_3 (**a**), or were incubated at 25°C with 5 μM As_2O_3 for different times (**b**). The levels of cyto *c* and COX-IV as a mitochondrial-specific marker were measured by Western blotting as described in 'Materials and methods'. Data are representative of at least three independent experiments

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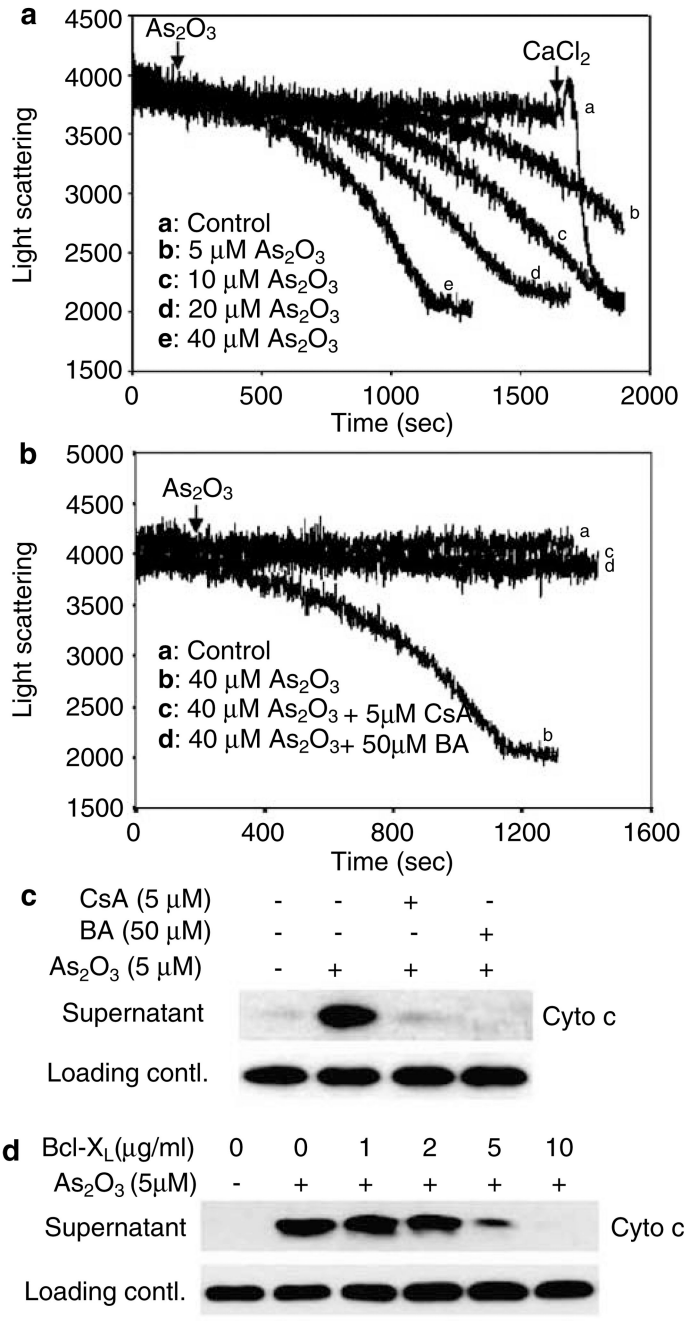

Figure 3.

PTP-specific inhibitors prevent As_2O_3-induced PTP opening and cyto *c* release, (**a**, **b**) PTP opening was monitored as described in 'Materials and methods'. Isolated mitochondria were pretreated with 5 μM CsA, or 50 μM BA (**c**) or preincubated with different concentrations of Bcl-X_L as indicated (**d**) in 50 μl PT buffer for 5 min before As_2O_3 (5 μM) being added, and further incubated at 25°C for 60 min. A volume of 100 μM $CaCl_2$ was added at the end of experiments as a positive control for PTP opening. Released cyto *c* was detected as described in 'Materials and methods'. Data are representative of at least three independent experiments

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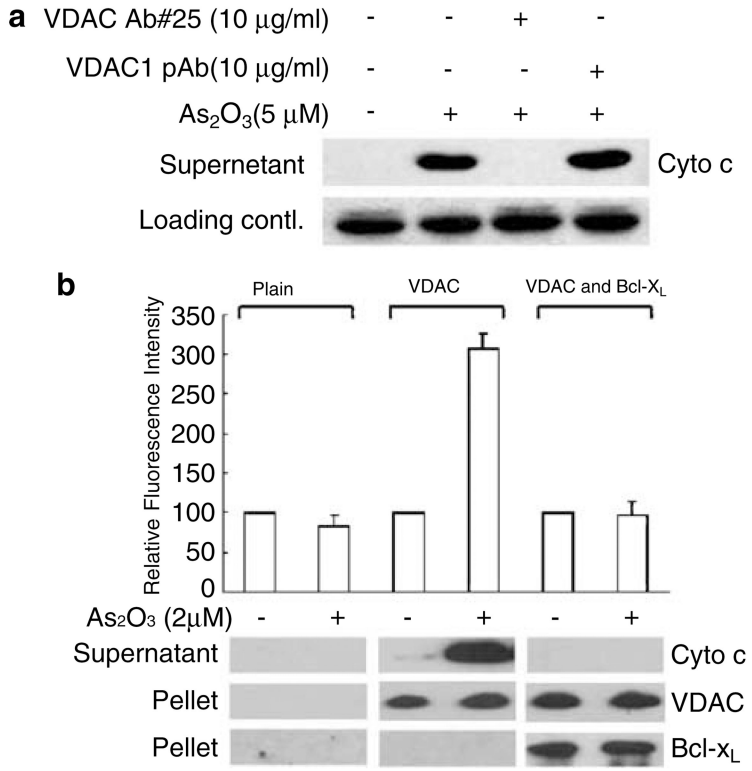

Figure 4.

VDAC is necessary and sufficient to mediate As_2O_3-induced cyto *c* release. (**a**) As $_2O_3$-induced cyto *c* release was specifically inhibited by the anti-VDAC antibody (#25). Isolated mitochondria (5mg protein/ml) were preincubated with 10 μg/ml anti-VDAC Ab#25 or a polyclonal antibody against the N terminus of VDAC1 for 15 min in 50 μl PT buffer before the addition of 5 μM As_2O_3. The cyto *c* released into the supernatant was measured by Western blotting as described in 'Materials and methods' (**b**) As $_2O_3$ induced hVDAC-mediated cyto *c* release from reconstituted liposomes, which was inhibited by Bcl-X_L. Liposomes were reconstituted as described in 'Materials and methods' and incubated in the presence or absence of 2 μM As_2O_3 for 1 h at 25°C. After centrifugation, the FITC fluorescence in the supernatants was determined using a spectrofluorimeter. The data, normalized against the fluorescence of the untreated group as a control, represent the mean values of three independent experiments. Cyto *c* released in the supernatants was also detected by Western blotting using an anti-cyto *c* monoclonal antibody. Incorporation of the VDAC and Bcl-X_L proteins in liposomes was confirmed by immunoblots with anti-VDAC1 and anti-Bcl-X_L polyclonal antibodies

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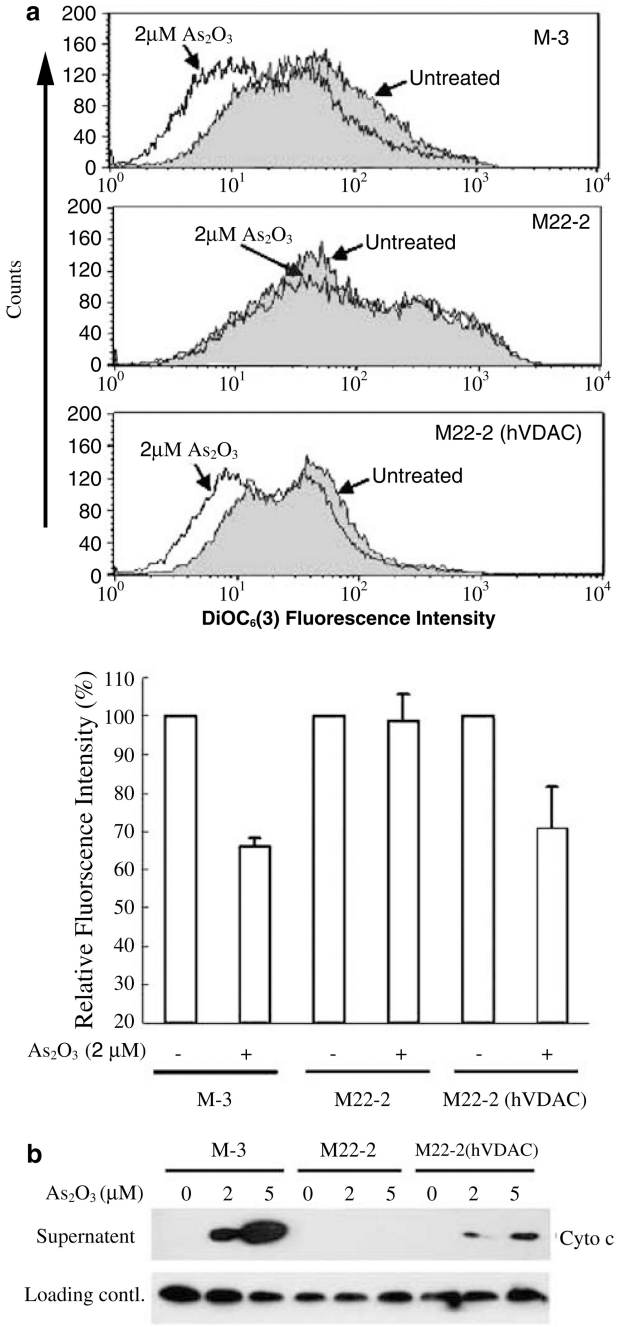

Figure 5.

VDAC is required for As_2O_3-induced ΔΨm reduction in yeast and cyto *c* release from isolated yeast mitochondria. (**a**) Wild-type (M-3), VDAC-1-deficient mutant (M22-2), or M22-2 mutant *S. cerevisiae* reconstituted with human VDAC-1, were incubated with or without 2 μM As_2O_3 at 30°C for 12 h before being subjected to ΔΨm analysis as described in 'Materials and methods'. PI-negative yeast cells were analysed with a Cell Quest software (BD, CA) and the data were normalized against the untreated control. The upper panel histogram data are representative of three independent experiments. (**b**) Isolated mitochondria (5 mg protein/ml) from the three yeast strains were incubated for 1 h at 30°C in 50 μl YPT buffer (0.6M mannitol, 2mM HEPES, pH 7.4, 0.5mM KH_2PO_4, and 4.2mM

potassium succinate) with different concentrations of As_2O_3, as indicated. The samples were then centrifuged at 12 000 *g* for 15 min at 4°C. The levels of cyto *c* in the supernatants were determined by Western blotting with an anti-cyto *c* antibody. The nonspecific bands that appeared in cyto *c* Western blotting were used as an internal loading control. Data are representative of three independent experiments

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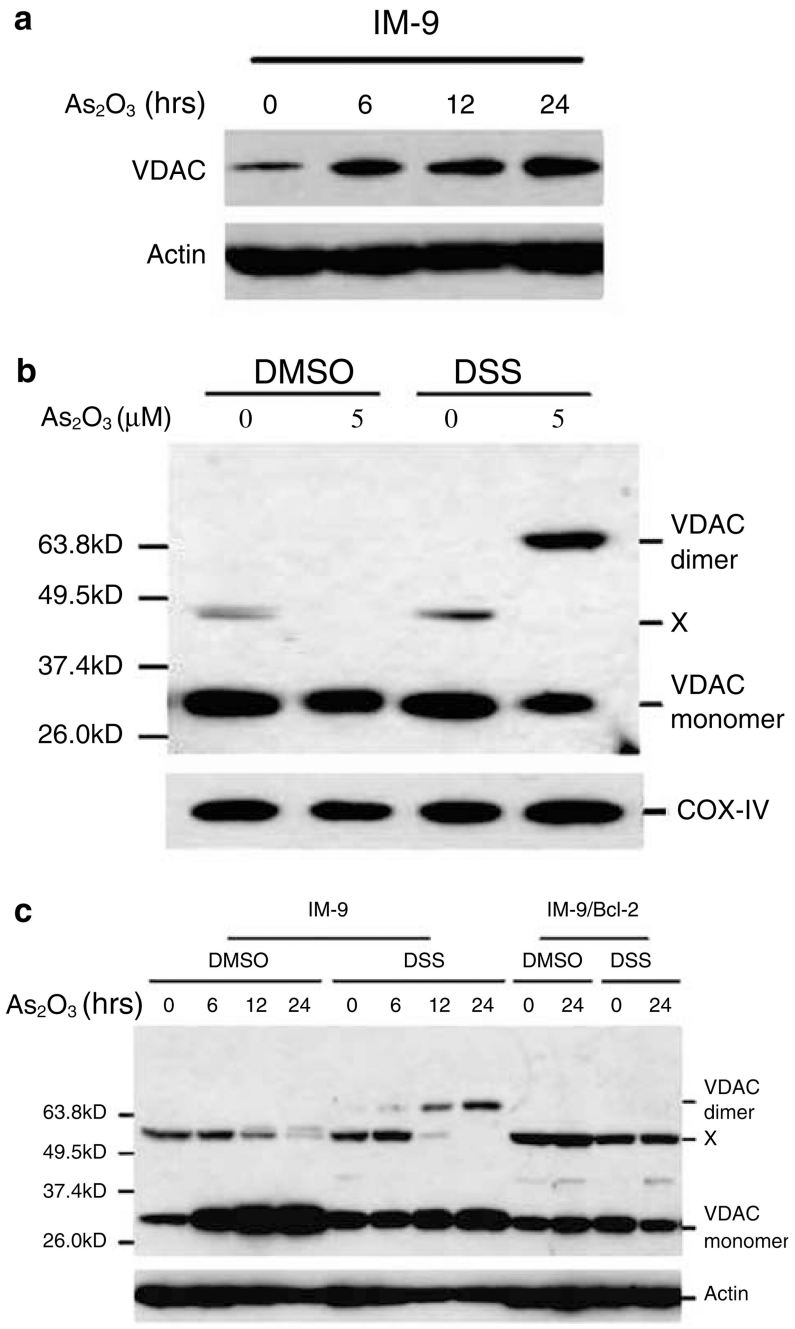

Figure 6.

As_2O_3 induces VDAC upregulation and homodimerization (**a**) IM-9 cells were treated with 2 μM As_2O_3 for various times and subjected to SDS–PAGE/immunoblot analysis with the indicated antibodies and β-actin was used as a loading control. (**b**) Isolated mitochondria were treated with 5 μM As_2O_3 for 1 h or (**c**) IM-9 cells were treated with 2 μM As_2O_3 for the indicated times before treatment with the cross-linking agent DSS. VDAC proteins were resolved by SDS–PAGE and detected by Western blotting using a VDAC1 polyclonal antibody. A 32-kDa band represents VDAC monomers, with bands at 64 kDa, detected only in the presence of DSS, representing VDAC homodimers. X represents a nonspecific band.

DMSO was used as the vehicle control. Results shown are representative of three independent experiments

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