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# $Ca^{2+}$-mediated regulation of VDAC1 expression levels is associated with cell death induction☆

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ABSTRACT

VDAC1, an outer mitochondrial membrane (OMM) protein, is crucial for regulating mitochondrial metabolic and energetic functions and acts as a convergence point for various cell survival and death signals. VDAC1 is also a key player in apoptosis, involved in cytochrome *c* (Cyto *c*) release and interactions with anti-apoptotic proteins. Recently, we demonstrated that various pro-apoptotic agents induce VDAC1 oligomerization and proposed that a channel formed by VDAC1 oligomers mediates cytochrome *c* release. As VDAC1 transports $Ca^{2+}$ across the OMM and because $Ca^{2+}$ has been implicated in apoptosis induction, we addressed the relationship between cytosolic $Ca^{2+}$ levels ($[Ca^{2+}]i$), VDAC1 oligomerization and apoptosis induction. We demonstrate that different apoptosis inducers elevate cytosolic $Ca^{2+}$ and induce VDAC1 over-expression. Direct elevation of $[Ca^{2+}]i$ by the $Ca^{2+}$-mobilizing agents A23187, ionomycin and thapsigargin also resulted in VDAC1 over-expression, VDAC1 oligomerization and apoptosis. In contrast, decreasing $[Ca^{2+}]i$ using the cell-permeable $Ca^{2+}$-chelating reagent BAPTA-AM inhibited VDAC1 over-expression, VDAC1 oligomerization and apoptosis. Correlation between the increase in VDAC1 levels and oligomerization, $[Ca^{2+}]i$ levels and apoptosis induction, as induced by $H_2O_2$ or $As_2O_3$, was also obtained. On the other hand, cells transfected to overexpress VDAC1 presented $Ca^{2+}$-independent VDAC1 oligomerization, cytochrome *c* release and apoptosis, suggesting that $[Ca^{2+}]i$ elevation is not a pre-requisite for apoptosis induction when VDAC1 is over-expressed. The results suggest that $Ca^{2+}$ promotes VDAC1 over-expression by an as yet unknown signaling pathway, leading to VDAC1 oligomerization, ultimately resulting in apoptosis. These findings provide a new insight into the mechanism of action of existing anti-cancer drugs involving induction of VDAC1 over-expression as a mechanism for inducing apoptosis. This article is part of a Special Issue entitled: Calcium Signaling in Health and Disease. Guest Editors: Geert Bultynck, Jacques Haiech, Claus W. Heizmann, Joachim Krebs, and Marc Moreau

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## 1. Introduction

Various studies have demonstrated that components of $Ca^{2+}$ signaling pathways are remodeled or de-regulated in cancer so as to support cancer cell proliferation and survival [1]. Furthermore, accumulated evidence has demonstrated the importance of intracellular $Ca^{2+}$ ($[Ca^{2+}]i$) in both the activation and the execution of cell death [2,3]. Indeed, several studies have shown that $Ca^{2+}$ is a mediator of cell death signaling and that different pro-apoptotic agents, such as thapsigargin, staurosporin, selenite, cisplatin, as well as $Ca^{2+}$ ionophores, induce apoptotic cell death by interfering with $Ca^{2+}$ homeostasis [4]. $[Ca^{2+}]i$ increases have been observed in the apoptotic cell and have been shown to be required for apoptosis to even occur [5–7].

In addition to free cytoplasmic $Ca^{2+}$, mitochondrial $Ca^{2+}$ has also been implicated in apoptosis induction [8]. Indeed, mitochondria are a major hub of cellular $Ca^{2+}$ homeostasis, controlling energy metabolism, oxidative phosphorylation, secretion and cell death [9,10]. $Ca^{2+}$ transport across the mitochondria requires negotiating both the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). $Ca^{2+}$ transport across the IMM is mediated by the $Ca^{2+}$ uniporter protein (MCU), catalyzing the uptake of $Ca^{2+}$, while the $Na^{+}/Ca^{2+}$ exchanger is considered the major agent of $Ca^{2+}$ efflux [11–14]. At the OMM, $Ca^{2+}$ transport is mediated by the voltage-dependent anion channel 1 (VDAC1) [15–17]. VDAC1 also possesses $Ca^{2+}$-binding sites, allowing it to function as the $Ca^{2+}$-sensitive barrier of the OMM [15,18–21]. VDAC1, moreover, provides the main interface between the mitochondria and the cytosol and plays a critical role in apoptosis [22,23] through its function in the release of apoptotic proteins located in the inter-membrane space (IMS) [24–26] and via its interaction with the anti-apoptotic proteins Bcl-2, Bcl-xL [27–30] and hexokinase (HK) [31–34].

*Abbreviations:* Cyto *c*, cytochrome *c*; EGS, ethylene glycol bis[succinimidylsuccinate]; OMM, outer mitochondrial membrane; VDAC, voltage-dependent anion channel

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Studies from our lab and others have revealed that VDAC1 can exist in a dynamic equilibrium between monomers and oligomers [35–39]. We proposed that upon apoptosis induction, VDAC1 oligomerizes to form a large flexible pore that allows Cyto *c* to cross the OMM [24,27,37,39,40]. This concept has gained experimental support with the demonstration that apoptosis induction in cultured cells leads to an up to 20-fold increase in VDAC1 oligomerization, as revealed by chemical cross-linking and Bioluminescence Resonance Energy Transfer (BRET) assays [37]. Apoptosis-mediated increase in VDAC1 oligomerization was observed regardless of the cell type or apoptosis inducer employed, including STS, curcumin, arsenic trioxide, etoposide, cisplatin, selenite, TNF-α, hydrogen peroxide, and UV light, all affecting mitochondria yet acting via different mechanisms. Conversely, the apoptosis inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) prevented STS-induced VDAC1 oligomerization and apoptosis [25,37].

At the same time, several studies demonstrated an increase in VDAC1 expression levels following apoptosis induction. For example, up-regulation of VDAC1 expression was observed in acute lymphoblastic leukemia cell lines following prednisolone treatment [41] and in cisplatin-sensitive cervix squamous carcinoma cells exposed to cisplatin, while in a cisplatin-resistant cell line, such treatment resulted in down-regulation of VDAC1 [42]. Furthermore, in human malignant melanoma cells, arbutin (hydroquinone-O-beta-D-glucopyranoside), a tyrosinase inhibitor and a potential anti-cancer agent, was found to induce apoptosis by causing VDAC1 over-expression [43,44]. Somatostatin, reported to be useful in the treatment of advanced prostate cancer, was found to up-regulate the expression of VDAC1 and VDAC2 in the LNCaP prostate cancer cell line [45]. In addition, both UV irradiation and reactive oxygen species (ROS) were shown to up-regulate VDAC1 expression [46,47]. Importantly, the causal relationship between VDAC1 levels and drug sensitivity was emphasized in several studies. Prostate cancer cell lines that were relatively resistant to apoptosis induction by oblimersen sodium (G3139) were found to express lower levels of VDAC1 than G3139-sensitive prostate cancer cells [48]. The anti-cancer activity of furanonaphthoquinones was increased upon VDAC1 over-expression and decreased upon silencing of VDAC1 expression [49,50]. Recently, it was also shown that over-expression of VDAC1 sensitized carcinoma cells to apoptosis induced by cisplatin, mechlorethamine, and its derivative, melphalan [51]. Thus, VDAC1 over-expression following apoptosis induction by various agents, as well as the correlation between drug efficacy and VDAC1 expression level, suggests that the activity of numerous anti-cancer drugs and treatments is mediated via regulating VDAC1 expression levels.

$Ca^{2+}$ has been shown to regulate gene transcription, with several steps in the process of activity-dependent gene expression being $Ca^{2+}$-dependent [52,53]. As $Ca^{2+}$ enhances gene expression by inducing different nuclear events according to the nature of the $Ca^{2+}$-activated transcription factor involved [54], apoptosis stimuli may increase VDAC1 expression levels by regulating $[Ca^{2+}]$i. Here, we report that $Ca^{2+}$ plays a major role in promoting the expression of VDAC1, thereby serving as an important mediator of VDAC1-dependent apoptotic cell death. We further show that many pro-apoptotic agents and stimuli, including several anti-cancer drugs, increase $[Ca^{2+}]$i and promote VDAC1 over-expression and oligomerization. Based on these findings, we propose a new concept according to which several apoptosis-inducing agents and conditions act by up-regulating VDAC1 expression in a $Ca^{2+}$-dependent manner, leading to VDAC1 assembly into high oligomeric structures that mediate Cyto *c* release and subsequent cell death.

## 2. Materials and methods

### 2.1. Materials

A23187, acridine orange, arsenic (III) oxide ($As_2O_3$), dimethyl sulfoxide (DMSO), etoposide, ethidium bromide, hepes, hydrazine, ionomycin, lactic acid, leupeptin, nicotinamide adenine dinucleotide (NAD+), phenylmethylsulfonyl fluoride (PMSF), propidium iodide, thapsigargin (TG) and Tris were purchased from Sigma (St. Louis, MO). Ethylene glycolbis (succinimidylsuccinate) (EGS) was obtained from Pierce. Rabbit monoclonal antibodies against VDAC1 (ab154856) and Cy-2-conjugated anti-mouse antibodies came from Abcam (Cambridge, UK). Monoclonal antibodies against actin were obtained from Millipore (Billerica, MA) and anti-Cyto *c* antibodies (556432 and 556433) were obtained from BD Bioscience (San Jose, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were obtained from Promega (Madison, WI). Annexin V (FITC) was from Enzo Life Sciences (Lausen, Switzerland), BAPTA-AM was obtained from Tocris Bioscience (Bristol, UK), while Fluo-4-AM came from Invitrogene (Grand Island, NY). Hank's balanced salts solution (HBSS) without calcium, magnesium or phenol red, Dulbecco's modified Eagle's medium (DMEM) growth media, and the supplements fetal calf serum (FCS), L-glutamine and penicillin-streptomycin were all obtained from Biological Industries (Beit Haemek, Israel).

### 2.2. Cell lines and transfection

HeLa (human cervical adenocarcinoma), A549 (non-small human lung carcinoma) and SKOV3 (human ovarian carcinoma) cells were maintained in DMEM supplemented with 10% FCS, (except during treatment with ionomycin (1%) and cisplatin and etoposide (no serum)), 2 mM L-glutamine, 1000 U/ml penicillin, and 1 μg/ml streptomycin and maintained in a humidified atmosphere at 37 °C with 5% $CO_2$.

### 2.3. Cell transfection with VDAC1

A549 cells were transiently transfected with plasmid pcDNA4/TO (1–2 μg DNA) encoding native rVDAC1, using the JetPRIME transfection agent (Illkirch, France) according to the manufacturer's instructions.

### 2.4. Cellular $Ca^{2+}$ imaging and analysis

Fluo-4-AM was used to monitor changes in cytosolic $Ca^{2+}$ levels. HeLa cells ($6 \times 10^5$ cells/ml) were harvested after the appropriate treatment, collected (1500 ×*g* for 10 min), washed with HBSS buffer (5.33 mM KCl, 0.44 mM $KH_2PO_4$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM $Na_2HPO_4$, 5.6 mM glucose, 0.03 mM phenol red) supplemented with 1.8 mM $CaCl_2$ (HBSS(+)) and incubated with 2.5 μM Fluo-4 in 200 μl HBSS(+) buffer for 30 min at 37 °C and protected from light. After washing the remaining dye, the cells were incubated with 200 μl HBSS(+) buffer and changes in the cellular free $Ca^{2+}$ concentration were measured immediately by flow cytometer analysis. At least 10,000 events were recorded on the FL1 detector, represented as a histogram, and analyzed by the FACSCalibur flow cytometer software (BD Biosciences, Franklin Lakes, NJ). Positive cells showed a shift to an enhanced level of green fluorescence (FL1). Changes in cellular $Ca^{2+}$ were monitored in live cells using the high content Operetta screening system (Perkin–Elmer, Hamburg, Germany). In each well, ten fields were imaged using a 20× wide field objective, a 520–550 nm excitation filter and a 560–630 nm emission filter.

### 2.5. Chemical cross-linking

HeLa cells were harvested after the appropriate treatment and incubated (1.5 to 3 mg/ml) in PBS, pH 8.2) with the cross-linking reagent EGS (150–300 μM) for 15 min at 30 °C. Samples (60 to 90 μg) were subjected to SDS-PAGE and immunoblotting using anti-VDAC1 antibodies, as described below. Quantitative analysis of immuno-reactive VDAC1 dimer bands was performed using Image Gauge software (version 4.0; Science Lab 2001) provided by Fujifilm.

### *2.6. Apoptosis analysis*

Following treatment with the indicated reagents, HeLa cells ($2 \times 10^5$) were analyzed for apoptotic cell death upon staining with propidium iodide (PI) and annexin V-fluorescein isothiocyanate (FITC), followed by flow cytometer analysis. Cells were collected (1500 ×g for 10 min), washed, and resuspended in 200 μl binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Annexin V–FITC was added according to the recommended protocol, and the cells were incubated for 15 min, protected from light. Cells were then washed once with binding buffer and resuspended in 200 μl binding buffer, to which PI was added immediately before flow cytometry measurements. At least 10,000 events were collected, recorded on a dot plot, and analyzed by FACSCalibur flow cytometer software. Apoptotic cell death was also visualized by acridine orange and ethidium bromide staining as described previously [55].

### *2.7. Cytochrome c release*

A549 cells were seeded on coverslips and were transfected 24 h later with an empty or rVDAC1-encoding plasmid (2 μg). 48 h post-transfection, cells were paraformaldehyde-fixed (4%, 15 min), incubated for 1 h with blocking solution (5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS) and incubated with anti-Cyto *c* (1:300, Cat no. 556432, BD Bioscience) antibodies for 1 h. After washing with PBS, the cells were incubated for 1 h with Cy2-conjugated antibodies (1:300) and washed with PBS. To stain nuclei, cells were incubated with DAPI for 5 min at room temperature and washed with PBS. Coverslips were mounted on glass slides with mounting medium and sealed with nail polish. Cells were visualized by confocal microscopy.

For Cyto *c* release analysis by immunoblot, cells were harvested, washed twice with PBS and gently resuspended at a concentration of 6 mg/ml in ice-cold buffer (100 mM KCl, 2.5 mM $MgCl_2$, 250 mM sucrose, 20 mM HEPES/KOH pH 7.5, 0.2 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, 5 μg/ml cytochalasin B and 0.1 mM phenylmethylsulfonyl fluoride) containing 0.03% digitonin and incubated 10 min on ice. Samples were centrifuged at 10,000 ×g at 4 °C for 5 min to obtain supernatants corresponding to cytosolic extracts free of mitochondria and pellets corresponding to a fraction that contains mitochondria. Equivalent amounts of supernatants and pellets were analyzed by SDS-PAGE and immuno-probed using anti-Cyto *c* antibodies (1:2000, Cat no. 556433, BD Bioscience).

### *2.8. Gel electrophoresis and immunoblotting*

SDS-PAGE was performed according to the Laemmli method [56]. Gels were electro-transferred onto nitrocellulose membranes for immunostaining. Membranes containing the transferred proteins were blocked with 5% non-fat dry milk and 0.1% Tween-20 in Tris-buffered saline and incubated with monoclonal anti-VDAC1 antibodies, followed by incubation with HRP-conjugated anti-mouse IgG secondary antibodies. After treatment with the appropriate primary and secondary antibodies, bands were visualized using an enhanced chemiluminescence kit (Biological Industries). For VDAC1 over-expression and cross-linked product analysis, 15–30 μg protein of cell lysate was subjected to SDS-PAGE and immunoblotting using anti-VDAC1 antibodies. Quantitative analysis of immuno-reactive VDAC1 bands was performed using Image Gauge software (version 4.0; Science Lab 2001), provided by Fujifilm.

### *2.9. Quantification of hydrogen peroxide*

HeLa cells were incubated with hydrogen peroxide (0.8 mM) and at different time points (0–4 h), aliquots of the medium were analyzed for $H_2O_2$ levels by spectrophotometric measurement at 454 nm, using the vanadium pentoxide/sulfuric acid method [57].

### *2.10. Lactate dehydrogenase levels*

HeLa cells were treated as described above and aliquots of the culture medium and total cell lysates were analyzed for lactate dehydrogenase (LDH) activity by monitoring NADH reduction at 340 nm. The assay buffer contained (final concentrations) glycine 160 mM, hydrazine 160 mM, $NAD^+$ 0.29 mM, lactate 5 mM and equivalent amounts of medium or cell lysates. Rates of LDH activity were measured at 340 nm using a plate reader.

## **3. Results**

### *3.1. Apoptosis stimuli increase VDAC1 expression levels*

To test our hypothesis that apoptosis stimuli act via enhancing VDAC1 expression, leading to VDAC1 oligomerization and apoptosis, we evaluated the effects of four different apoptosis inducers on VDAC1 expression levels (Fig. 1A). The effects of $As_2O_3$, etoposide, cisplatin and $H_2O_2$ on VDAC1 expression were analyzed in HeLa (human cervical cancer), SKOV-3 (human ovarian carcinoma) and A549 (human lung adenocarcinoma alveolar basal epithelium) cells following their incubation with escalating concentrations of these reagents. All apoptosis inducers increased the VDAC1 expression level in each of the three cell lines tested in a concentration-dependent manner, as monitored by immunoblotting (Fig. 1A). Drug-induced VDAC1 over-expression was time-dependent, with the time for maximal increase in VDAC1 level (1–16 h) being dependent on the reagent used and its concentration (Fig. 1B). Specifically, VDAC1 expression was increased by over 4-fold, depending on the agent concentration and the cell type tested (Fig. 1C).

As these apoptosis agents are also known to disrupt $Ca^{2+}$ homeostasis, we analyzed whether they also elevated cytosolic $Ca^{2+}$ levels under those conditions leading to VDAC1 over-expression (Fig. 1D). Incubation of HeLa cells with $H_2O_2$, cisplatin, etoposide or $As_2O_3$ resulted in elevated $[Ca^{2+}]i$ levels, as visualized by Fluo-4 fluorescence and monitored by cell imaging with the Operetta system (Fig. 1D). Together, these results indicate that different apoptosis-inducing compounds, regardless of their mechanism of action, elevated intracellular $Ca^{2+}$ levels and induced VDAC1 over-expression.

### *3.2. Apoptosis inducers lead to VDAC1 over-expression, intra-cellular $[Ca^{2+}]$ elevation, VDAC1 oligomerization and apoptosis*

Next, we examined the relationship between VDAC1 over-expression, elevation of $[Ca^{2+}]i$, VDAC1 oligomerization, and apoptosis induction (Fig. 2). $As_2O_3$ induced VDAC1 over-expression (Fig. 2A) and VDAC1 oligomerization (Fig. 2B) in a concentration-dependent manner, as revealed using chemical cross-linking with EGS and Western blotting with anti-VDAC1 antibodies. Several anti-VDAC1 antibody-labeled protein bands corresponding to VDAC1 dimers, trimers, tetramers and multimers were obtained upon exposure to $As_2O_3$. In addition, monomeric VDAC1 with higher mobility was obtained upon apoptosis induction and EGS treatment (asterisk in Fig. 2B), representing a monomer that underwent conformational changes that were fixed upon inter-molecular cross-linking, thereby modifying its mobility in SDS-PAGE. In parallel to the increase in VDAC1 expression, elevation of $[Ca^{2+}]i$ (Fig. 2C) and apoptotic cell death as analyzed by annexin V–FITC and PI staining followed by flow cytometer analysis, were also obtained (Fig. 2D). All these parameters were similarly increased as a function of $As_2O_3$ concentration (Fig. 2E), suggesting association between VDAC1 over-expression, increased cytosolic $Ca^{2+}$, VDAC1 oligomerization and apoptosis.

Next, to verify the sequence of events, we analyzed the time course of the increase in VDAC1 expression (Fig. 3A and C), VDAC1 oligomerization (Fig. 3B and C), elevation in $[Ca^{2+}]i$ levels and apoptosis, all as induced by $H_2O_2$ (Fig. 3C). The results show that the increase in $[Ca^{2+}]i$ induced by $H_2O_2$ is transient and proceeds enhanced VDAC1 over-

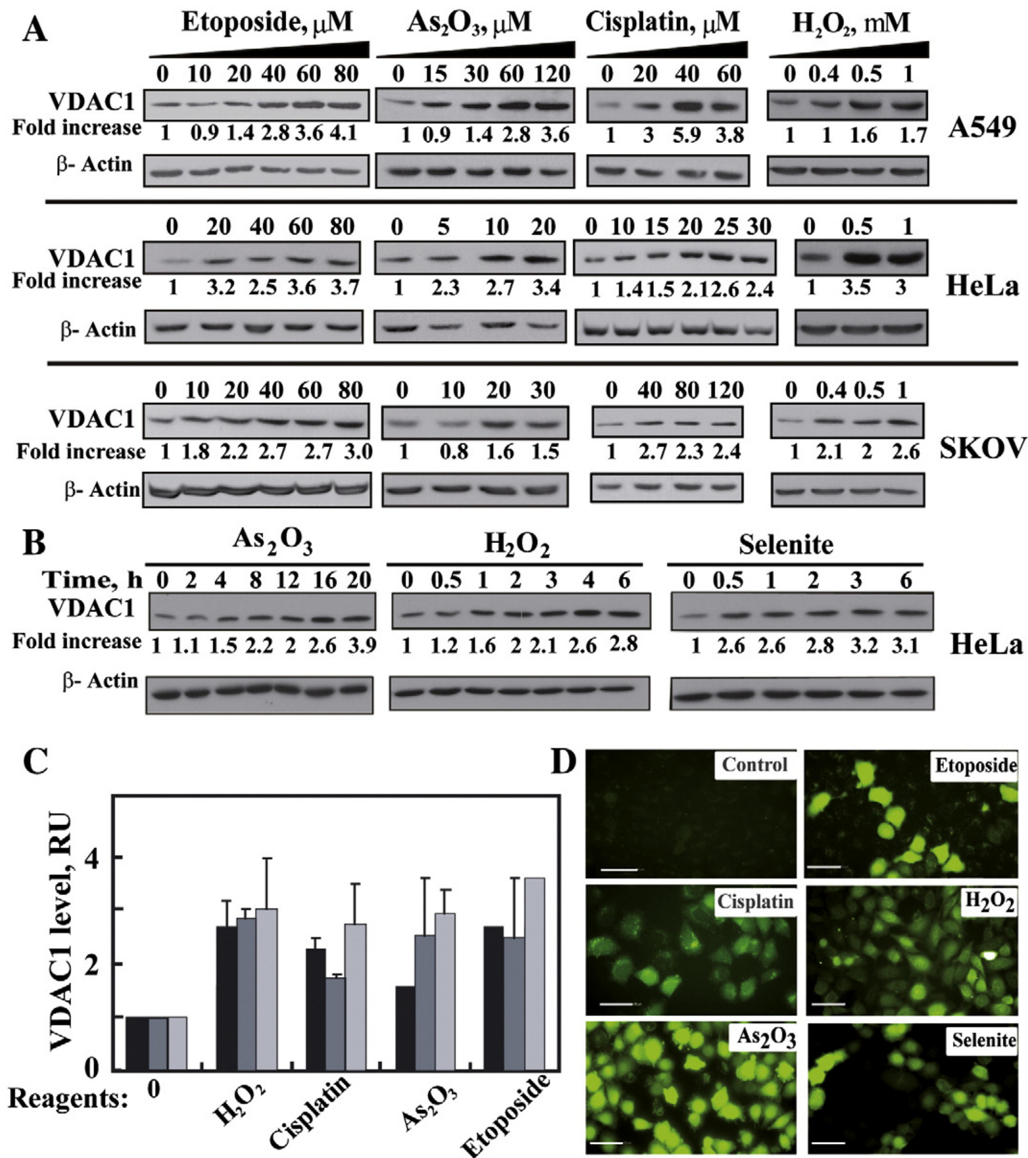


**Fig. 1.** Apoptosis inducers up-regulate VDAC1 expression and elevated cytosolic $Ca^{2+}$. (A) SKOV-3, A549 and HeLa cells were incubated with the indicated concentrations of cisplatin (24 h), $H_2O_2$ (6 h), etoposide (24 h) or $As_2O_3$ (12 h). VDAC1 expression levels were analyzed by immunoblotting followed by quantitative analysis of VDAC1 expression presented in the bottom of each blot as fold increase. (B) For the time course of selenite (8 μM)-, $H_2O_2$ (0.5 mM)- and $As_2O_3$ (20 μM)-induced VDAC1 over-expression, HeLa cells were incubated with the indicated reagent for the indicated time and the VDAC1 expression level was analyzed as in A. (C) The increase in VDAC1 levels obtained in the HeLa (grey bars), SKOV-3 (black bars) and A549 (dark grey bars) cells following treatment with $H_2O_2$ (0.4, 0.5, 1 mM, for SKOV-3, HeLa and A549, respectively), $As_2O_3$ (20, 20, 120 μM, for HeLa, SKOV-3 and A549, respectively), cisplatin (20 μM) or etopoide (60 μM) is presented. (D) For analysis of changes in cytosolic $Ca^{2+}$ upon apoptosis induction, HeLa cells were plated on black CellCarrier 96-well plates overnight and then treated for 8 h with $As_2O_3$ (10 μM), etoposide (10 μM), cisplatin (25 μM), $As_2O_3$ (10 μM) or $H_2O_2$ (0.5 mM for 6 h), followed by incubation with Fluo-4. Cell fluorescence was recorded using the Operetta. The results shown correspond to either the mean ± SD or a representative of 2–3 similar experiments, as appropriate.

expression, VDAC1 oligomerization and apoptosis. However, when analyzing the concentration of $H_2O_2$ in the medium as function of time, it was clear that $H_2O_2$ concentration decreased with time with a similar time course as the decrease in $[Ca^{2+}]i$ (Fig. 3C). A correlation between the increase in VDAC1 expression level, VDAC1 oligomerization and apoptosis was observed, suggesting a strong association between the three processes that followed the increase in cytosolic $[Ca^{2+}]i$. The $H_2O_2$-induced elevation in $[Ca^{2+}]i$ is required for enhanced VDAC1 expression, oligomerization and apoptosis, as all were inhibited by BAPTA (see Fig. 5).

### *3.3. Increased intracellular $Ca^{2+}$ encodes information that up-regulates VDAC1 expression*

Next, to test the direct effect of elevating $[Ca^{2+}]i$ on the levels of VDAC1 expression and oligomerization, we employed $Ca^{2+}$-mobilizing agents, namely the ionophores ionomycin and A23187 and the inhibitor of the $Ca^{2+}$ pump SERCA (sarco/endoplasmic reticulum calcium-ATPase), thapsigargin. All allowed for an increase in $[Ca^{2+}]i$ in both cytosol and mitochondria. Incubation of HeLa cells with these $[Ca^{2+}]i$ level-elevating reagents resulted in a several-fold increase in VDAC1 expression levels, in a concentration-dependent manner (Fig. 4A), and as expected, increased $[Ca^{+}]i$ (Fig. 4B). Cells treated with thapsigargin or A23187 showed intense punctuated Fluo-4 fluorescence, which may represent $Ca^{2+}$ accumulated in the mitochondria or in other intracellular organelles (Fig. 4B, arrows). Incubation of cells with ionomycin also resulted in a marked increase in VDAC1 over-expression (Fig. 4C) and oligomerization (Fig. 4D), as well as in apoptotic cell death, as analyzed by annexin V–FITC and PI staining (Fig. 4E) and as visualized upon staining with acridine orange/ethidium bromide (Fig. 4F). As expected, ionomycin increased $[Ca^{2+}]i$ levels, as monitored using Fluo-4 and flow cytometer analysis, at all tested concentrations, except at the highest concentration used,

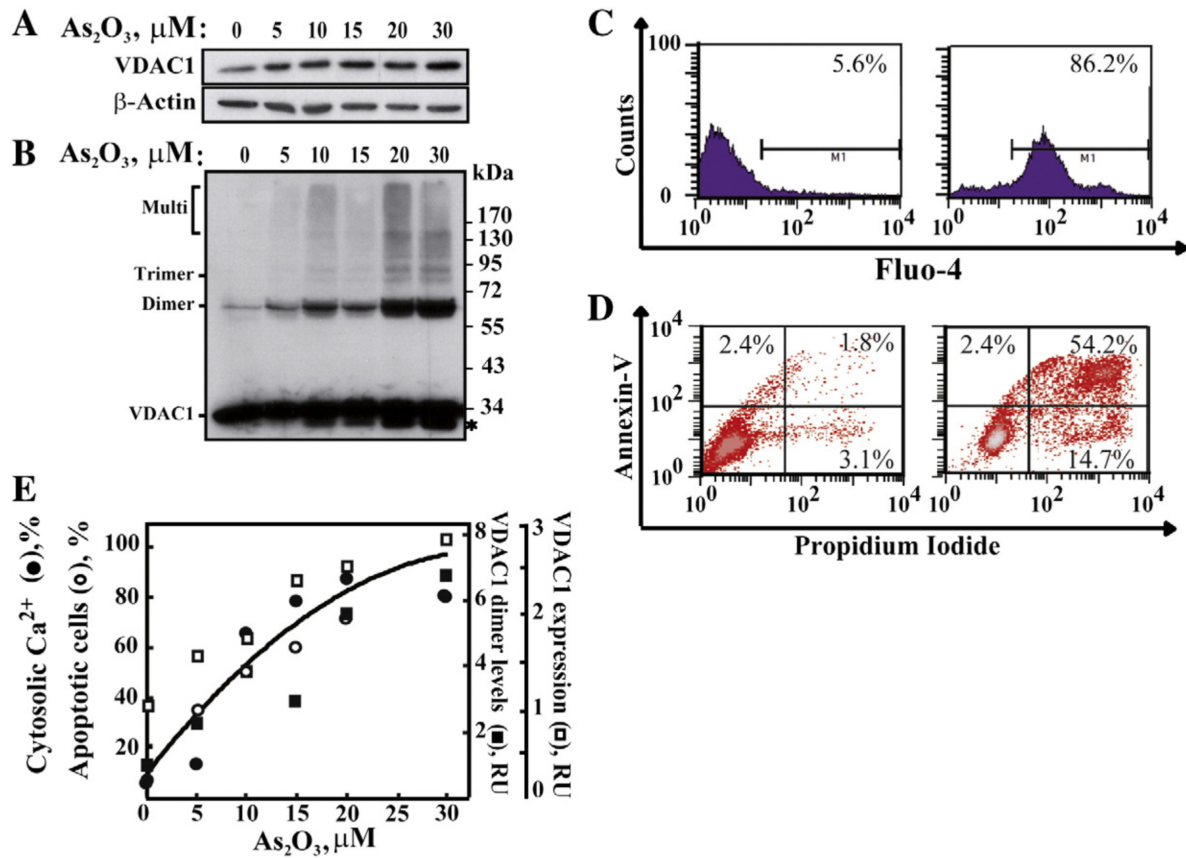


**Fig. 2.** $As_2O_3$ elevates interacellular $Ca^{2+}$ levels, up-regulates VDAC1 expression and induces VDAC1 oligomerization and apoptosis. HeLa cells were incubated (14 h) with various concentrations of $As_2O_3$, harvested and VDAC1 expression (A), VDAC1 oligomerization (B), intracellular $Ca^{2+}$ levels (C) and apoptotic cell death (D) were analyzed. VDAC1 oligomerization was revealed using EGS-based cross-linking. Cells (2.5 mg/ml) were washed with PBS, pH 8.3 and incubated with EGS (250 μM) at 30 °C for 15 min and then subjected to SDS-PAGE and immunoblotting using anti-VDAC1 antibodies. The positions of VDAC1 monomers to multimers are indicated. (C) Representative flow cytometer analysis of $[Ca^{2+}]_i$ levels in control cells and in cells treated with $As_2O_3$ (20 μM, 14 h), as monitored using Fluo-4. The values shown indicate the increase in $[Ca^{2+}]_i$. (D) Apoptosis was determined using annexin V–FITC and PI staining and flow cytometer analysis. (E) Summary of the quantitative analysis of VDAC1 expression, VDAC1 dimer and $[Ca^{2+}]_i$ levels and apoptosis. The results shown correspond to either the mean ± SD or a representative of 2–4 similar experiments, as appropriate.

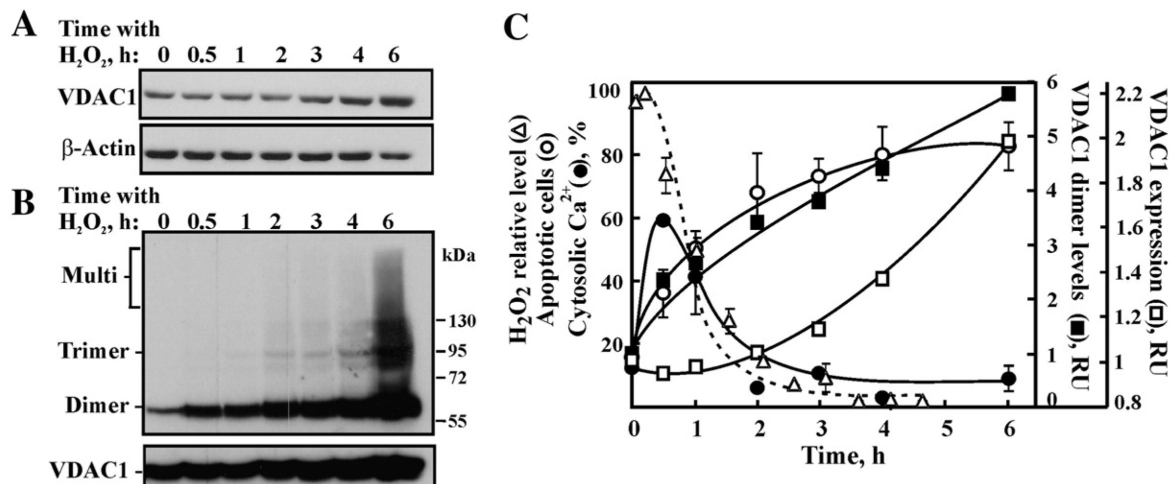


**Fig. 3.** $H_2O_2$ elevates interacellular $Ca^{2+}$ levels, up-regulates VDAC1 expression and induces VDAC1 oligomerization and apoptosis. HeLa cells were incubated with 0.8 mM $H_2O_2$, harvested and VDAC1 expression (A), VDAC1 oligomerization (B), intracellular $Ca^{2+}$ levels and apoptotic cell death (C) were measured as a function of incubation time with $H_2O_2$. VDAC1 expression levels were analyzed by immunoblotting, using anti-VDAC1 antibodies (A). VDAC1 oligomerization was revealed using EGS-based cross-linking. Cells (2.5 mg/ml) were washed with PBS, pH 8.3 and incubated with EGS (250 μM) at 30 °C for 15 min and then subjected to SDS-PAGE and immunoblotting using anti-VDAC1 antibodies (B). The positions of VDAC1 monomers to multimers are indicated. Apoptotic cell death and $[Ca^{2+}]_i$ levels were monitored as a function of time by flow cytometer analysis using annexin-V FITC and propidium iodide, and Fluo-4, respectively (C). $H_2O_2$ levels as a function of time were analyzed and are presented as a dashed line. The results shown correspond to either the mean ± SD or a representative of 2 similar experiments, as appropriate.

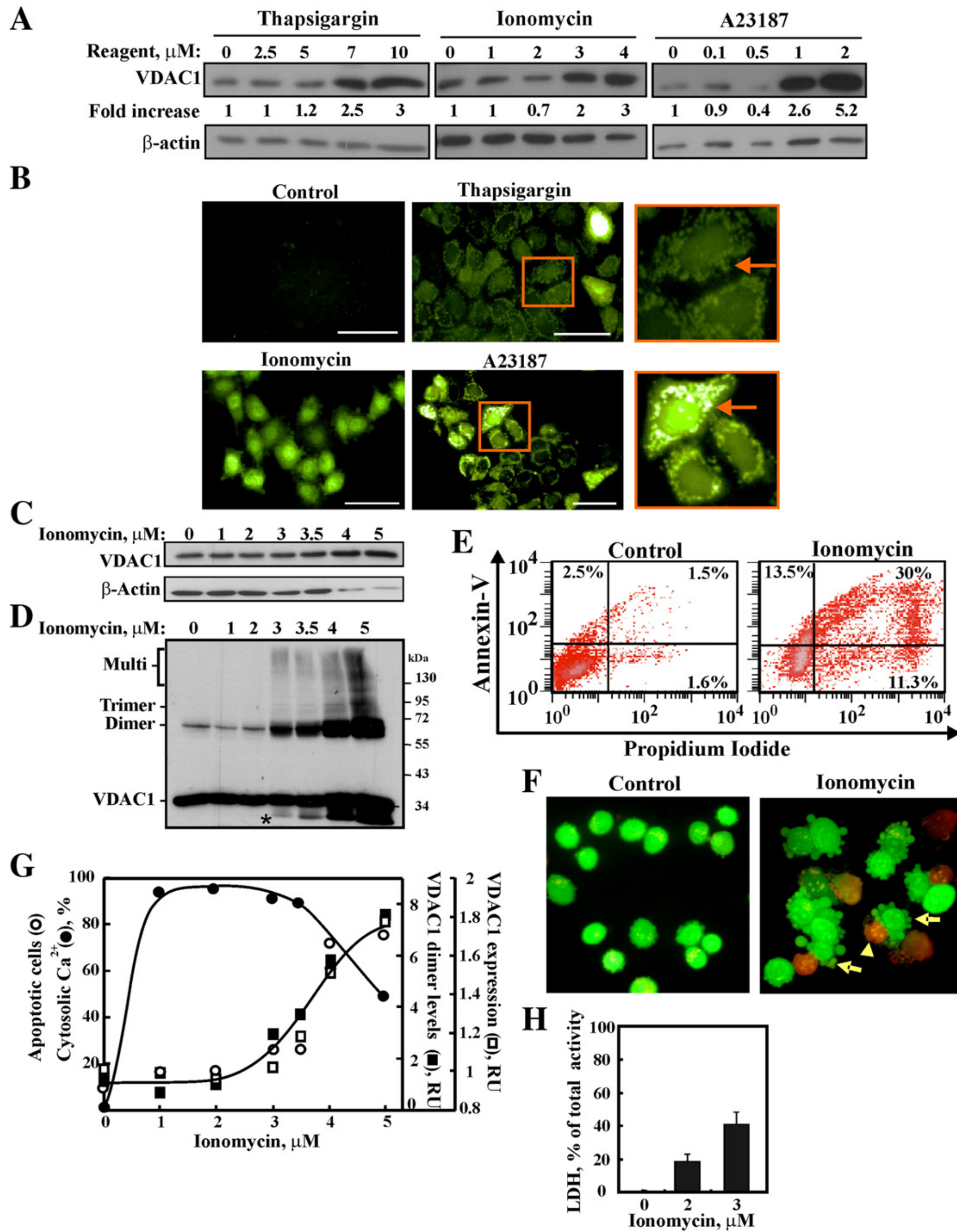


**Fig. 4.** Increased intracellular $Ca^{2+}$ induces up-regulation of VDAC1 expression that is correlated with increased VDAC1 oligomerization and apoptosis. (A) HeLa cells were incubated with thapsigargin (10 μM, 24 h), ionomycin (4 μM, 45 min) or A23187 (2 μM, 24 h), harvested and VDAC1 expression levels were analyzed by immunoblotting followed by quantitative analysis of VDAC1 expression. Relative amounts (RU) of VDAC1 expression are given at the bottom. (B) For monitoring $[Ca^{2+}]_i$ levels, HeLa cells were seeded on black CellCarrier 96-well plates overnight and then treated as described in (A), followed by incubation with Fluo-4. Cell fluorescence was recorded using the Operetta. Representative images are shown. (C,D) HeLa cells were incubated with the indicated concentrations of ionomycin (1–5 μM, 45 min), followed by analysis of VDAC1 expression levels (C) and oligomerization (D). Apoptotic cell death was analyzed using annexin-V FITC/propidium iodide staining and flow cytometer analysis (E) and acridine orange/ethidium bromide staining (F). In F, arrows indicate cells at an early apoptotic state, reflected by degraded nuclei (stained with acridine orange) and membrane blebbing. Arrowheads indicate cells in the late apoptotic state (stained with both acridine orange and ethidium bromide, orange color). (G) Quantitative analysis of VDAC1 expression levels (C) and oligomerization (D), apoptotic cell death (E), as well as of $[Ca^{2+}]_i$ levels, as determined by Fluo-4 and flow cytometer analysis is presented in (G) as function of ionomycin concentration. The results shown correspond to either the mean ± SD or a representative of 2–4 similar experiments, as appropriate. LDH levels in the medium from control and ionomycin-treated cells were analyzed as described in the Methods section (Fig. 4H). The total cellular LDH in control cells is considered as 100%.

when the $[Ca^{2+}]$i level started to decline, most probably due to intensive cell death (Fig. 4E).

Several studies, particularly those involving cardiomyocytes or neurons, suggest that A23187 induces the necrotic form of cell death [58,59]. Yet, A23187 was shown to induce a rise in cytoplasmic $Ca^{2+}$ at both early and late stages of the apoptotic process [60] and to induce mitochondria-associated apoptotic events [61]. Similarly, ionomycin was also shown to induce either necrosis or apoptosis [62,63]. Thus, although our results (Fig. 4E and F) clearly indicate that ionomycin induced apoptotic cell death, we tested whether under the conditions used it also caused necrosis. Release of LDH to the medium is considered to be a necrotic event. Therefore, the level of LDH in the medium from

control and ionomycin-treated cells was analyzed. The results clearly indicate that under the condition used, the level of LDH in the medium was increased, although this activity represents only 20–40% of the total cellular LDH activity (Fig. 4H). It should be noted, however, that LDH release may reflect primary necrosis but also secondary necrosis as a consequence of apoptotic cell death (see [64]).

The integrated results (Fig. 4G) indicate that the increase in $[Ca^{2+}]i$ precedes VDAC1 over-expression and oligomerization and apoptosis, with all showing similar augmentation as a function of ionomycin concentration. These results suggest that the increase in $[Ca^{2+}]i$ is prerequisite for activation of the other tested processes, and demonstrate that strong association exists between the increase in $[Ca^{2+}]i$, VDAC1 expression levels, VDAC1 oligomerization and apoptosis.

### 3.4. $Ca^{2+}$ is required for pro-apoptotic agent-induced VDAC1 over-expression, VDAC1 oligomerization and apoptosis

To assess the requirement of $Ca^{2+}$ for VDAC1 over-expression, VDAC1 oligomerization and apoptosis induction, the effects of the cell-permeable $Ca^{2+}$ chelating reagent BAPTA-AM (acetoxymethyl ester form of bis(aminophenoxy)ethan -N,N,N′,N′-tetraacetic acid) on these processes were evaluated. Pre-incubation of HeLa or A549 cells with BAPTA-AM resulted in prevention of cisplatin- (data not shown), $H_2O_2$- and $As_2O_3$-induced VDAC1 over-expression (Fig. 5A–F). BAPTA also prevented the apoptotic cell death induced by $H_2O_2$ (Fig. 5D).

The relationship between the effects of BAPTA on VDAC1 expression, VDAC1 oligomerization and apoptotic cell death was studied by monitoring these activities in parallel, as all induced by $As_2O_3$ (Fig. 6). Apoptotic cell death (Fig. 6A), VDAC1 over-expression (Fig. 6B) and VDAC1 oligomerization (Fig. 6C) were all strongly inhibited by BAPTA-AM with a clear association between these activities (Fig. 6D), indicating a major role for $[Ca^{2+}]i$ in inducing VDAC1 over-expression, oligomerization and apoptotic cell death.

### 3.5. VDAC1 over-expression-induced VDAC1 oligomerization and apoptosis can occur with no increase in cytosolic $Ca^{2+}$ levels

To demonstrate that increased VDAC1 levels encourage VDAC1 oligomerization and apoptosis, as well as the requirement for $[Ca^{2+}]i$ when VDAC1 is over-expressed, cells were transfected to over-express VDAC1 and the above activities were analyzed (Fig. 7). Cells transfected with empty or VDAC1-expressing vectors were subjected to cross-linking with different EGS concentrations and the formation of VDAC1 homo-oligomers, comprising dimers to higher molecular mass complexes, was analyzed and found to be highly enhanced in cells over-expressing VDAC1 (Fig. 7A). Almost no oligomerization was observed in control cells, unless apoptosis was induced, such as with $H_2O_2$ (Fig. 7B). In VDAC1-over-expressing cells, the concentration of EGS required to attain 50% of the maximal level of dimers (or oligomers) was over 16-fold lower than in $H_2O_2$-treated cells, respectively

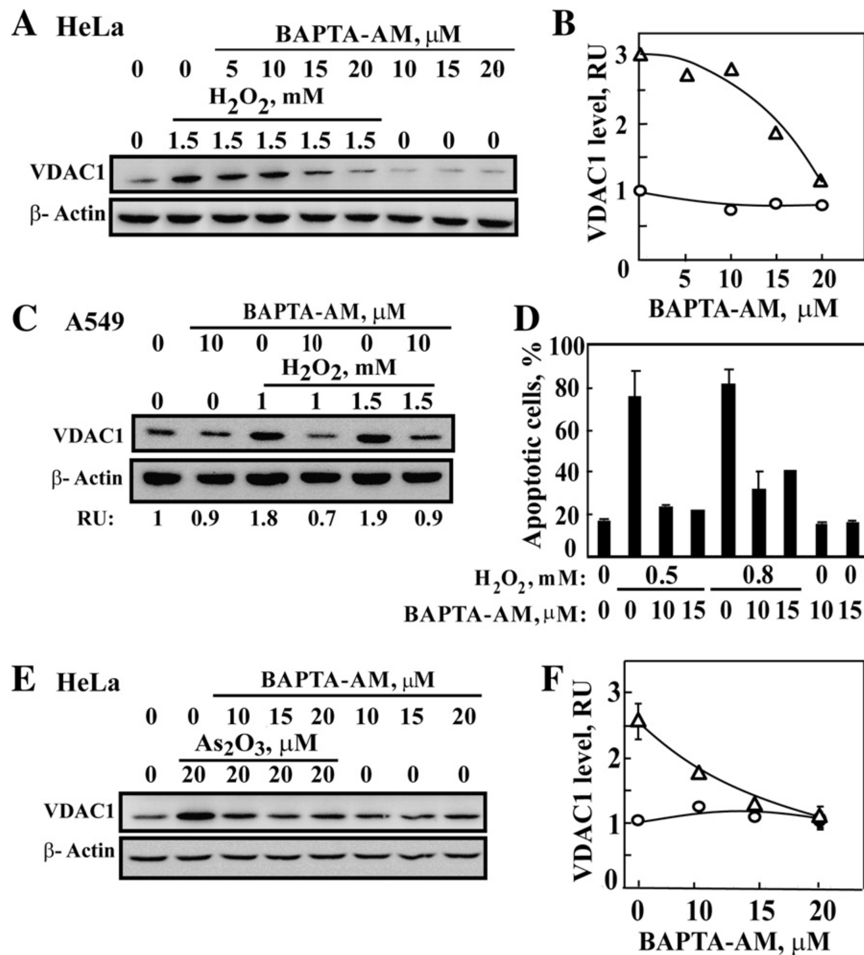


**Fig. 5.** Up-regulation of VDAC1 expression induced by $H_2O_2$ or $As_2O_3$ is inhibited by BAPTA-AM. HeLa or A549 cells were incubated with the indicated concentration of BAPTA-AM for 1 h and then without or with the indicated concentrations of $H_2O_2$ (A, B, 5 h) or $As_2O_3$, (E, F, 12 h), harvested, and VDAC1 expression levels were analyzed by immunoblotting (A, C, E). Quantitative analysis of VDAC1 expression levels of HeLa cells treated with $As_2O_3$ (20 μM, 12 h, Δ) (B) or with $H_2O_2$ (1.5 mM, 5 h, Δ) (F), as a function of BAPTA-AM concentration is presented. (o) Indicates untreated cells. The effect of BAPTA-AM on apoptosis induced by $H_2O_2$ was analyzed using annexin V–FITC and PI staining, followed by FACS analysis (D). The results shown correspond to either the mean ± SD or a representative of 2–4 similar experiments, as appropriate.

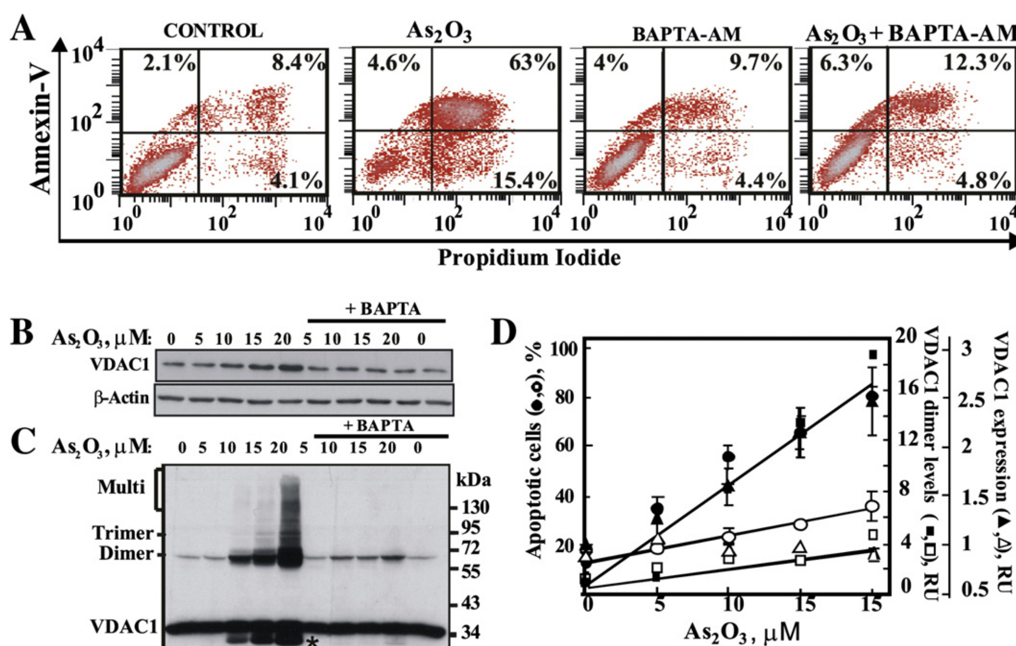


**Fig. 6.** BAPTA-AM prevents VDAC1 over-expression, VDAC1 oligomerization and apoptotic cell death induced by $As_2O_3$. HeLa cells were incubated with BAPTA-AM (20 μM, 1 h) and then with or without $As_2O_3$ (5–20 μM, 14 h). Cells were harvested, cross-linked with EGS (250 μM, 15 min), and analyzed by immunoblotting using anti-VDAC1 antibodies for VDAC1 expression levels (A), VDAC1 oligomerization (B) and apoptotic cell death (C), as described in the legend to Fig. 2. Results are representative of 2–3 similar experiments. Quantitative analysis of VDAC1 over-expression (▲,Δ) and VDAC1 oligomerization (dimers) (■,□) and apoptosis (●,○), with the empty symbols representing the presence of BAPTA, are presented as a function of $As_2O_3$ concentration (D) ($n = 2$–3).

(Fig. 7C). This suggests that upon VDAC1 over-expression (Fig. 7D), the equilibrium shifted from the monomeric to the oligomeric state, leading to Cyto *c* release as analyzed by both immunocytochemistry and immunoblotting (Fig. 7E and F). Indeed, as reported previously [34,65–69], cells over-expressing VDAC1 underwent apoptotic cell death, as visualized upon staining with acridine orange/ethidium bromide (Fig. 7G). These cells showed membrane blebbing, chromatin condensation and orange-staining, reflecting a late apoptotic stage. The apoptotic cell death induced by VDAC1 over-expression was also analyzed by Annexin V/PI staining (Fig. 7H).

To verify whether an increase in $[Ca^{2+}]$i is required for apoptosis induction when VDAC1 is over-expressed, we analyzed changes in $[Ca^{2+}]$i and apoptosis and the effects of BAPTA-AM on both parameters in cells over-expressing VDAC1. Upon VDAC1 over-expression (Fig. 7D), about 50% of the cells underwent apoptosis, as analyzed by Annexin V/PI staining (Fig. 7H). In contrast, no change in the $[Ca^{2+}]$i level was observed (Fig. 7H), with BAPTA-AM having no effect on apoptosis as induced by VDAC1 over-expression, as opposed to the inhibition of $As_2O_3$- or $H_2O_2$-induced apoptosis (Figs. 5 and 6). These findings suggest that changes in $[Ca^{2+}]$i levels are not pre-requisite for apoptosis induction when VDAC1 is over-expressed.

## 4. Discussion

### *4.1. Apoptosis inducers induce VDAC1 over-expression and increase intracellular $Ca^{2+}$ levels*

While accepted modes of actions have been described for the majority of apoptosis inducers, various studies indicate that most of these agents act via multiple pathways. In the present report, an additional mode of action for apoptosis stimulus involving increased expression of VDAC1 is proposed. Indeed, several cancer treatments and apoptosis inducers were found to increase VDAC1 expression levels [41–45,47]. We further argue that apoptosis-inducing agents act by increasing $[Ca^{2+}]$i and that this in turn leads to an up-regulation of VDAC1 expression. This is followed by VDAC1 oligomerization, Cyto *c* release and, finally, cell death (Fig. 8B). Hence, based on our findings, we offer a new view of the mechanism of apoptosis induction by chemotherapeutic agents and other apoptosis-inducing treatments.

Although the novel mechanism we propose, whereby apoptosis inducers act by initially increasing VDAC1 expression, is supported by the results presented in this study (Figs. 1–7), previously published findings also can be explained by the mode of action we propose. For example, the commonly accepted mechanism of cisplatin action involves the cross-linking of nuclear DNA [70]. However, cisplatin-mediated cell death was also proposed to involve mitochondria dysfunction and VDAC1 [71,72]. Here we have shown that cisplatin induces over-expression of VDAC1 in the three cell lines tested (Fig. 1). Likewise, cisplatin was shown to induce VDAC1 over-expression in a cisplatin-sensitive cervix squamous cell carcinoma cell line (A431) but not in a cisplatin-resistant cell line (A431/Pt) [42]. The requirement of VDAC1 for cisplatin-induced cell death is also reflected in the inhibition of cisplatin-induced apoptosis and Bax activation in non-small cell lung cancer when the VDAC1 expression level was reduced by siRNA [73]. Similarly, etoposide, targeting DNA topoisomerase II [74], was found to affect cell metabolism and gene expression at different levels [75]. Here, we have demonstrated that etoposide induces VDAC1 over-expression (Fig. 1). In the case of $H_2O_2$, induction of apoptosis was proposed to occur via oxidative stress [76]. In the present study, we find that $H_2O_2$ also induced VDAC1 over-expression (Figs. 1, 3 and 7). Indeed, ROS were previously shown to induce VDAC1 up-regulation in a manner that was prevented by the ROS chelator epigallocatechin [46]. Finally, selenite was proposed to induced cell death via multiple modes of action [77,78], albeit via poorly defined mechanism(s) [78, 79]. Here, we showed that selenite induced VDAC1 over-expression (Fig. 1B). The decrease in selenite-induced apoptosis seen upon silencing VDAC1 expression [78] suggests that VDAC1 is involved in the mode of action of this compound. Interestingly, selenite was found to induce Bax over-expression [80]. This, with the increase in VDAC1

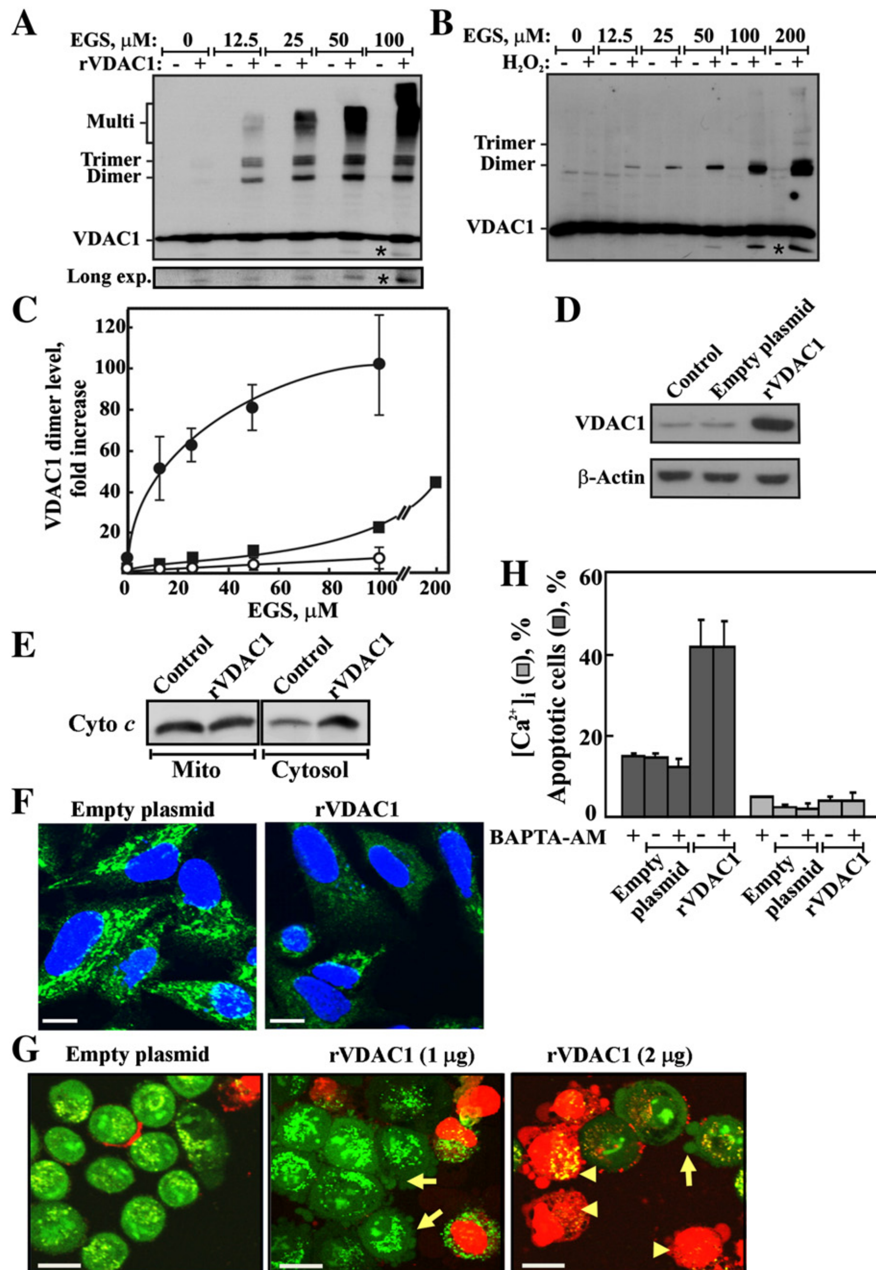


**Fig. 7.** VDAC1 over-expression results in VDAC1 oligomerization and apoptosis, without affecting cytosolic $Ca^{2+}$ levels. In (A), A549 cells were transfected with an empty vector or rVDAC1-encoding pcDNA 4/To (2 μg of DNA). After 48 h, the cells were harvested and subjected to cross-linking with the indicated EGS concentration, followed by immunostaining using anti-VDAC1 antibodies. In (B), cells were exposed to $H_2O_2$ (0.8 mM, 6 h) and then subjected to cross-linking with the indicated concentration of EGS. Quantitative analysis of VDAC1 dimer formation in cells transfected with the empty plasmid, control (□), over-expressing rVDAC1 (●) or exposed to $H_2O_2$ (■) is presented as a function of EGS concentration (C). A representative of three similar experiments is shown. For the assay of apoptosis and Cyto *c* release, cells were transfected with an empty vector or rVDAC1-encoding pcDNA 4/To (as indicated, 1 or 2 μg of DNA) and samples were analyzed for VDAC1 expression levels (D), Cyto *c* release (E, F), as analyzed by immunoblotting or immunocytochemical staining using anti-Cyto *c* antibodies (scale bars, 10 μm) or for apoptotic cell death, visualized by acridine orange and ethidium bromide staining (G). In G, arrows indicate cells at an early apoptotic state, reflected by degraded nuclei (stained with acridine orange) and membrane blebbing. Arrowheads indicate cells in the late apoptotic state (stained with both acridine orange and ethidium bromide, orange color). Scale bars, 30 μm. In F, about 70% of the cells showed diffused and weak fluorescence representing Cyto *c* released from the mitochondria. In H, cells were transfected with the empty vector or rVDAC1-encoding pcDNA 4/To (2 μg of DNA) and 48 h post-transfection, the cells were incubated with or without BAPTA-AM (20 μM, 12 h) and analyzed for apoptotic cell death using Annexin V/PI staining (grey bars). Changes in $Ca^{2+}$ levels were monitored using Fluo-4 and flow cytometer analysis (black bars). The results shown correspond to either the mean ± SD or a representative of 2–4 similar experiments, as appropriate.

expression (Fig. 1), suggests that selenite may act by affecting protein expression levels.

In summary, although these different apoptosis inducers elicit cell death via different mechanisms, the present study shows that all not only induce VDAC1 over-expression but also increase $[Ca^{2+}]$i levels (Figs. 1–6). Thus, it is possible that elevating $[Ca^{2+}]$i represents a common mechanism for the various apoptosis stimuli considered here, and that elevated $[Ca^{2+}]$i leads to elevation in VDAC1 expression levels. Indeed, $Ca^{2+}$ was found to be essential for VDAC1 over-expression, since BAPTA-AM, which abrogates intracellular $Ca^{2+}$ levels, significantly decreased VDAC1 over-expression as induced by various agents (Figs. 5 and 6). Furthermore, the direct increase in $[Ca^{2+}]$i generated by the $Ca^{2+}$ ionophores A23187, ionomycin or thapsigargin induced VDAC1 over-expression (Fig. 4). These findings suggest that $Ca^{2+}$

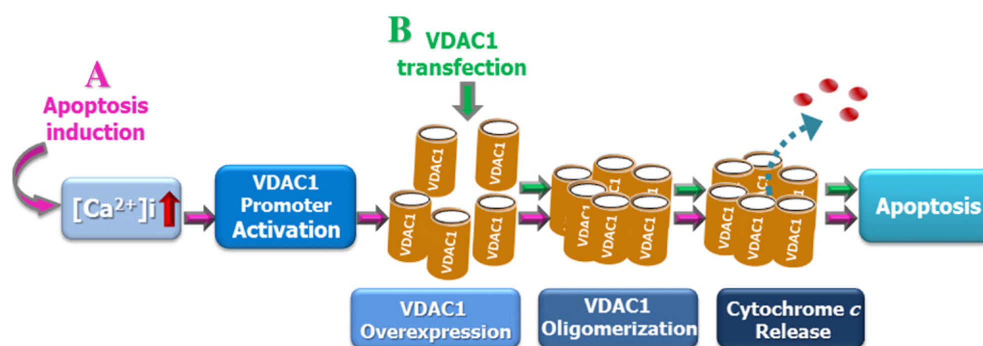


**Fig. 8.** Proposed model for the role of increasing [$Ca^{2+}$]i in VDAC1 expression and subsequent cell death induction. The model presented in (A) suggests that apoptosis induction by a given stimuli causes an increase in intracellular $Ca^{2+}$ levels, leading to enhanced VDAC1 expression, most probably activating the VDAC1 promoter directly or by stimulating the activity of transcription factors. The increase in VDAC1 expression shifts the equilibrium towards the VDAC1 oligomeric state, allowing for Cyto *c* release from the inner mitochondria space, leading to apoptotic cell death. When VDAC1 is over-expressed by transfection, no change in the intracellular $Ca^{2+}$ level was observed (Fig. 7G), suggesting that $Ca^{2+}$ is required for induction of VDAC1 over-expression and not for the subsequent steps leading to VDAC1 oligomerization, Cyto *c* release and finally, apoptotic cell death (B).

directly or indirectly regulates VDAC1 expression level, with the enhanced VDAC1 expression being subsequently associated with an induction of cell death (Fig. 8).

*4.2. Apoptosis-activating stimuli act by inducing VDAC1 over-expression: a proposed mechanism*

The cellular expression level of VDAC1 is a crucial factor for the process of mitochondria-mediated apoptosis. As presented here (Fig. 7) and in previous studies [34,65–69], exogenous over-expression of VDAC1 results in cell death, regardless of the cell type considered or the origin of VDAC used [34,67–69]. Cell death, as induced by exogenous VDAC1 over-expression, presents the classical hallmarks of apoptosis, including those morphologic changes associated with apoptosis, such as cell shrinkage, membrane blebbing, phosphatidylserine surface exposure, and nuclear condensation and fragmentation, as well as release of Cyto *c* (Fig. 7). Such cell death can be blocked by HK [34] or DIDS [68], both shown to interact with VDAC1 [32,81].

It was shown previously [16,27,37–39] and here (Fig. 7) that over-expression of VDAC1 dramatically enhanced VDAC1 oligomerization and apoptosis in the absence of any apoptotic stimuli. These reports further argued that VDAC1 oligomerization is involved in the release of Cyto *c* from mitochondria, thereby contributing to subsequent cell death [37,38].

In the present study, we demonstrated the tight correlation that exists between the increase in VDAC1 expression levels, VDAC1 oligomerization and apoptosis (Figs. 2–6). This is further reflected in the constant VDAC1 monomer to dimer ratio of 2.3 to 2.6 obtained when apoptosis was induced chemically or upon VDAC1 over-expression (Figs. 2–7). Accordingly, we suggest that apoptotic stimuli act by inducing VDAC1 over-expression, leading to the increased formation of VDAC1 oligomers mediating Cyto *c* release, and as such, apoptosis (Fig. 8).

We further propose that changes in [$Ca^{2+}$]i provide the link between the actions of apoptosis inducers and the elevation of VDAC1 expression levels. Accumulated evidence has demonstrated the importance of $Ca^{2+}$ in the activation and execution of cell death [5–7,82]. Indeed, [$Ca^{2+}$]i increases have been observed during apoptotic cell death [5–7,82] and have been shown to be required for apoptosis to transpire [6,7,83]. We have now shown that VDAC1 over-expression, VDAC1 oligomerization and apoptosis, as induced by various apoptosis stimuli, are not only all associated with an increase in [$Ca^{2+}$]i (Figs. 1–3) but can also be induced by a direct increase in [$Ca^{2+}$]i (Fig. 4). On the other hand, the same events are blocked by BAPTA, again suggesting a link between the increase in [$Ca^{2+}$]i, VDAC1 over-expression, VDAC1 oligomerization and apoptotic cell death. While the sequence of these events was not fully resolved in this study, the presented results indicate that the increase in [$Ca^{2+}$]i occurs prior to VDAC1 over-expression, VDAC1 oligomerization or apoptosis induction (Figs. 3–6). The proposed order of reactions is presented in Fig. 8. Still, while $Ca^{2+}$ is required for drug-induced VDAC1 over-expression and apoptosis induction (Figs. 5 and 6), it is not required for apoptosis as induced by plasmid-mediated VDAC1 over-expression, as no increase [$Ca^{2+}$]i was observed, and BAPTA did not prevent such apoptotic cell death (Fig. 7). This suggests that under the conditions used here, [$Ca^{2+}$]i is required for inducing VDAC1 over-expression but not for the subsequent steps leading to apoptosis (Fig. 8B).

Several potential $Ca^{2+}$-dependent steps could contribute to the enhanced expression of proteins. These steps include mRNA transcription, elongation, splicing, stability, and/or translation [84–86]. In addition, $Ca^{2+}$-responsive elements (CaRE) or $Ca^{2+}$-dependent transcription factors could be involved, as could be the activation of calcineurin, leading to the expression of hypertrophic response genes [87–89], or $Ca^{2+}$-mediated activation of promoter-bound transcription factor complexes [90]. The mechanism(s) by which $Ca^{2+}$ induces VDAC1 over-expression could be at the transcriptional level, via $Ca^{2+}$-dependent transcription factors or by other mechanisms. This suggestion, however, does not rule out the possibility that $Ca^{2+}$ assumes other roles in cell death, as induced by apoptosis stimuli.

In conclusion, the findings presented in this study, together with the observed induction of VDAC1 over-expression by various drugs [41–47], offer support for a new mechanism of pro-apoptotic drug action, namely enhancement of VDAC1 expression. Further study of this novel mechanism may thus provide a platform for developing a new class of anti-cancer drugs.

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