A BAX/BAK-INDEPENDENT MECHANISM OF CYTOCHROME C RELEASE
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Bax and Bak are multidomain pro-apoptotic members of the Bcl-2 family of proteins that regulate mitochondria-mediated apoptosis by direct modulation of mitochondrial membrane permeability. Since mouse embryonic fibroblasts with deficiency of Bax and Bak (DKO MEFs) are resistant to multiple apoptotic stimuli, Bax and Bak are considered to be an essential gateway for various apoptotic signals. Here we showed that the combination of calcium ionophore A23187 and arachidonic acid (ArA) induced cytochrome c release and caspase-dependent death of DKO MEFs, indicating that other mechanisms of cytochrome c release exist. Furthermore, A23187/ArA induced caspase-dependent death was significantly suppressed by the treatment of several serine protease inhibitors including 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), but not the overexpression of anti-apoptotic Bcl-2 family of proteins or the inhibition of mitochondrial membrane permeability transition. These results indicate that there are at least two mechanisms of cytochrome c release leading to caspase activation, a Bax/Bak-dependent mechanism and a Bax/Bak-independent, but serine protease(s)-dependent mechanism.

Apoptosis plays a critical role in the regulation of development processes, tissue homeostasis, and elimination of damaged cells. It has been shown that the mitochondria play a crucial role in apoptosis by releasing several apoptogenic molecules, such as cytochrome c, Smac/Diablo, and HtrA2/Omi (1,2). After release into the cytosol, cytochrome c binds to Apaf-1 to cause recruitment of caspase-9, which leads to the initiation of a caspase cascade that includes caspase-3 and results in the occurrence of apoptotic cell death. Smac/Diablo and HtrA2/Omi bind directly with members of the IAP family, which are endogenous caspase inhibitory proteins, and thus contribute to caspase activation (1,2).

The best characterized regulators of apoptosis are the Bcl-2 family of proteins, which directly modulate outer mitochondrial membrane permeability during apoptosis. This family of proteins can be categorized into anti-apoptotic members (such as Bcl-2, Bcl-xL and Mcl-1) and pro-apoptotic members, which consist of multidomain proteins (such as Bax and Bak) and BH3-only proteins (including Bid, Bim, Bik, Bad, Noxa, and Puma) (3). The multidomain pro-apoptotic proteins Bax/Bak are essential and redundant regulators of a diverse intrinsic mitochondrial cell death pathway: Bax/Bak double-deficient murine embryonic fibroblasts (MEFs) are resistant to multiple apoptotic stimuli that increase outer mitochondrial membrane permeability, including staurosporine, ultraviolet radiation, growth factor deprivation, and etoposide (4).

It was recently reported that DKO MEFs undergo death in a caspase-dependent manner, although at a lower rate compared with WT MEFs, after exposure to agents such as arachidonic acid and Ca^{2+} ionophore (5), raising the possibility that there is a Bax/Bak-independent mechanism which regulates mitochondrial membrane permeability. In this present study, we found that the treatment of the combination of arachidonic acid and A23187 caused cytochrome c release and subsequently caspase activation succeeding the cell death in DKO MEFs. These events were inhibited by the
treatment of AEBSF, serine protease inhibitor, but not the treatment of mitochondrial permeability transition (mPT) inhibitors, deletion of cyclophilin D (mPT component) and overexpression Bcl-2 or Bcl-xL.

Experimental Procedures

Antibodies and chemicals  The following antibodies were used. Numbers in parenthesis indicate dilutions used. Anti-caspase-3 (1:250) and anti-caspase-9 (1:1000) monoclonal antibodies were purchased from Transduction Lab. (Lexington, KY) and MBL (Nagoya, Japan), respectively. Anti-lamin B1 (1:1000) and anti-GAPDH (1:1000) monoclonal antibodies were obtained from Zymed Inc. (South San Francisco, CA) and BD Biosciences (San Jose, CA), respectively. Anti-cytochrome c monoclonal antibodies for Western blotting (clone 7H8.2C12, 1:1000) and immuno-staining (clone 6H2.B4, 1:500) were purchased from Pharmingen (San Diego, CA). A23187 and arachidonic acid were obtained from Calbiochem (La Jolla, CA) and Sigma (St. Louis, MI), respectively. 4-(2-aminoethyl)benzenesulfonamide (AEBSA) were purchased from Aldrich Chemical Co. (Steinheim, Germany). DFP and other chemicals were purchased from Wako Co. (Osaka, Japan).

Cell culture and DNA transfection  SV40 T antigen-immortalized WT MEFs, Bax/Bak DKO MEFs (kindly provided by Dr. S.J. Korsmeyer) and Bax/Bak/CypD TKO MEFs were grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Apaf-1-deficient MEFs and control MEFs were kindly provided by Dr. X. Wang, and were also grown in the same medium. DNAs encoding human Bax and human Bcl-xL were used in the pUC-CAGGS expression vector. Cells (1 x 10^6) were transfected with plasmid DNA using the Amaxa electroporation system according to the supplier’s protocol (Kit V, program U-20). The transfection efficiency was more than 75% as assessed by co-transfection with DNA expressing green fluorescence protein (GFP). All of the siRNAs were produced by Dharmacon Research. The sequences used were as follows (numbers in parentheses indicate nucleotide positions within the respective open reading frames): mouse cytochrome c siRNA; 5'-GGGAGAAGGGCGACGACCUGA-3’ (267-285), mouse HtrA2/Omi siRNA; 5’-GGGGAGUUUGUUGUUGCGCA-3’ (760-778), and GFP siRNA, 5’-GGCUACGUCCAGGAGCGCA-3’ (274-292). Mouse caspase-9 siRNA SMARTpool™ was also purchased from Dharmacon Research. Cells (1 x 10^6) were transfected twice on alternate days with 10 μg of siRNA using the Amaxa electroporation system. Twenty four hours after the 2nd transfection with siRNA, cells were used for experiments.

Cell viability and DEVDase activity assay  Cells (2 x 10^5 per well) were seeded into 6-well dishes. After 24 hours, the cells were treated with 10 μM A23187/100 μM arachidonic acid, or 10 μM etoposide in the presence or absence of 100 μM zVAD-fmk or 100 μg/ml AEBSF. Cells were harvested and stained with 1 μM PI, 1 μM Cy3-conjugated Annexin-V, or 1 μM Hoechst 33342 for 5 min at room temperature, and were analyzed with a Flow Cytometer (Becton-Dickinson, FACS Caliber) or under a fluorescence microscope (Olympus, BX50). For DEVDase assay, cells were washed three times with phosphate buffered-saline (PBS) and suspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 10 mM EGTA. After addition of Triton-X to 0.1%, cells were incubated for 30 min at on ice. Lysates were clarified by centrifugation at 8000 rpm for 3 min, and cleared lysates containing 50 μg protein were incubated with 100 μM of enzyme substrate Ac-DEVD-MCA at 37°C for 1 hour. Levels of released 7-amino-4-methlycoumarin (AMC) were measured using a spectrofluorometer (Hitachi F-3000) with excitation at 380 nm and emission at 460 nm.

Preparation of the cytosolic fraction and total cell lysate  For the detection of released cytochrome c, the cytosolic fraction was collected from MEFs after incubation with 0.1 mg/ml digitonin for 5 min at 37°C in isotonic buffer (20 mM potassium-Hepes [pH 7.4], 10 mM KCl, 1.5 mM MgCl2, 250 mM sucrose, and 1 mM Na^2+EDTA). After
centrifugation at 8000 rpm for 5 min, aliquots of the supernatant (cytosolic fraction) and the pellet (mitochondrial fraction) were analyzed by Western blotting with an anti-cytochrome c antibody. In some experiments, cells were lysed with RIPA buffer (50 mM Tris-HCl [pH 8.0], 0.1% SDS, 1% NP40, 0.5% deoxycholate [sodium salt] and 150 mM NaCl).

**Immunofluorescence staining** Cells were fixed in 4% paraformaldehyde for 30 min, and then permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After incubation with 2% FBS in PBS for 1 hour, the cells were incubated with anti-cytochrome c for 1 hour. After washing three times with PBS, the cells were incubated with the secondary antibody (Alexa 488-conjugated anti-mouse IgG, 1:1000) for 1 hour. Then fluorescence was detected under a confocal microscope (Zeiss, LSM 510).

**Microinjection** DKO MEFs (1.5 x 10^4) were plated on 35 mm dishes 1 day before use. PBS or PBS containing 400 μM AEBSF, 400 μM AEBSA, 100 μM DFP, 100 μM TLCK or 200 μM TPCK was microinjected through a glass capillary into the cytoplasm with a Narishige micromanipulator. For identification of injected cells, samples were mixed with green fluorescent protein. Cells were then treated with A23187/ArA at 1 hour. Cell morphology was examined 20 hours after A23187/ArA treatment under a fluorescence microscope.

**RESULTS**

We added various apoptotic reagents to Bax/Bak double-knockout (DKO) MEFs, Apaf-1-/- MEFs and their control MEFs, all of which were immortalized, and then measured DEVDase activity. In both DKO MEFs (Fig. 1A) and Apaf-1-/- MEFs (Fig. 1B), activation of DEVDase was not observed after exposure to etoposide that induced mitochondria-mediated apoptosis, whereas in the control MEFs, activation of DEVDase was readily observed, confirming previous observations that Bax/Bak is essential for such apoptosis (4,6). Time-dependent decrease of DEVDase activities was due to disruption of plasma membrane. Similar results were also obtained when these cells were treated with STS, UV, and X-rays (data not shown). In contrast, when the cells were treated with A23187 plus arachidonic acid (ArA), activation of DEVDase was observed in DKO MEFs, but not in Apaf-1-/- MEFs (Fig. 1C, D). It has previously been reported that ionomycin (another Ca^{2+} ionophore) plus ArA could induce DEVDase activity in DKO MEFs (5). Consistent with the elevation of caspase activation, A23187/ArA treatment induced cleavage of caspase-9, caspase-3, and lamin B1 in WT and DKO MEFs, but not in Apaf-1-/- MEFs (Fig. 1E, F). Furthermore, A23187/ArA induced the death of DKO MEFs, as assessed by staining with Annexin-V (Fig. 1G) and propidium iodide (PI) (data not shown), while such cell death was not completely inhibited by zVAD-fmk, a pan-caspase inhibitor (Fig. 1G and data not shown), indicating that A23187/ArA induced both caspase-dependent and caspase-independent death of DKO MEFs. It is known that not few reagents induce both apoptosis and necrosis, such as oxidative stress (7) and Ca^{2+} overload (8). Staining with Hoechst 33342 revealed that A23187/ArA induced nuclear pyknosis, which was partially inhibited by zVAD-fmk (Fig. 1H). A23187/ArA-induced caspase activation and death of DKO MEFs were slightly delayed compared with these processes in WT MEFs (Fig. 1C, G). Primary cultures of DKO MEFs tended to mainly undergo caspase-independent death (data not shown). These results indicated that A23187/ArA induced caspase-dependent death of immortalized MEFs in both Bax/Bak-dependent and Bax/Bak-independent manner.

To examine whether mitochondria were involved in the A23187/ArA-induced caspase-dependent death of DKO MEFs, we used gene silencing with siRNA to down-regulate caspase-9 and cytochrome c. Caspase-9 is activated by forming a complex called the apoptosome with Apaf-1 and cytochrome c in the presence of (d)ATP (9). Proteolytic activation of caspase-9 leads to activation of caspase-3 (9). Endogenous caspase-9 and cytochrome c were silenced in DKO MEFs by the respective siRNAs (Fig. 2A, C). Silencing of caspase-9 and cytochrome c in DKO MEFs markedly reduced A23187/ArA-induced death, as assessed by Annexin-V staining, but did not completely abolish it (Fig. 2B, D). Although silencing of caspase-9 only partially inhibited the activation of DEVDase.
(Fig. 2B), probably due to incomplete silencing (Fig. 2A), silencing of cytochrome c inhibited more strongly the activation of DEVDase (Fig. 2D). These results indicated that A23187/ArA-induced caspase activation required the presence of Apaf-1/cytochrome c. Incomplete inhibition of cell death after silencing of cytochrome c was mainly due to the simultaneous occurrence of caspase-independent death, because addition of zVAD-fmk did not further reduce A23187/ArA-induced death, as shown in Fig. 2D. To avoid the influence of mitochondrial respiration in the experiment with silencing of cytochrome c, we also performed the experiment in the presence of antimycin A (an inhibitor of respiration) and, although antimycin A enhanced cell death, virtually identical results were obtained (Fig. 2E). These results indicated that A23187/ArA-induced caspase-dependent death required the presence of Apaf-1/cytochrome c, but not Bax/Bak.

We next examined whether cytochrome c was released from the mitochondria in A23187/ArA-treated DKO MEFs. Subcellular fractionation revealed that etoposide induced cytochrome c release in WT MEFs, but not DKO MEFs (data not shown), whereas A23187/ArA caused cytochrome c release from both WT and DKO MEFs in the presence of zVAD-fmk (Fig. 3A). Consistent with this result, cytochrome c localization was altered in A23187/ArA-treated DKO MEFs, but not etoposide-treated DKO MEFs, as assessed by immunofluorescence microscopy (Fig. 3B). All these findings indicated that A23187/ArA induced cytochrome c release in a Bax/Bak-independent manner, and subsequently activated caspasas to induce cell death.

How was cytochrome c released from the mitochondria in a Bax/Bak-independent manner after treatment with A23187/ArA? First, we examined whether Bcl-xL could inhibit this Bax/Bak-independent process of cytochrome c release. As shown in Fig. 4A, a pan-caspase inhibitor, but not overexpression of Bcl-xL, inhibited A23187/ArA-induced death of DKO MEFs. Consistent with this result, both activation of caspasas and cytochrome c release were not influenced by Bcl-xL (Fig. 4B, C). As expected, the expression of Bcl-xL markedly inhibited etoposide-induced apoptosis in Bax-transfected DKO MEFs (Fig. 4D).

ArA and Ca$^{2+}$ ionophore A23187 are thought to induce the mitochondrial permeability transition (mPT) (8,10,11), which is a Ca$^{2+}$-dependent, cyclosporin A (CsA)-sensitive increase of mitochondrial membrane permeability that allows various solutes to equilibrate across the membranes (12). Since the CsA-sensitive mPT has been suggested to have a role in apoptotic cytochrome c release, we investigated whether it was also involved in A23187/ArA-induced Bax/Bak-independent cytochrome c release and cell death. Accordingly, cyclosporin A (CsA), bongkrekic acid (BKA) and l-carnitine, all of which are known to inhibit the mPT (12,13), were added to cultures of A23187/ArA-treated DKO MEFs. As shown in Fig. 4E, none of these reagents inhibited A23187/ArA-induced death, while zVAD-fmk caused significant inhibition, suggesting that the mPT was not involved in this mode of cell death. To confirm this possibility, we employed mice lacking cyclophilin D (Cyp D), in which the CsA-sensitive mPT is completely blocked (14). Cross-breeding produced Bax$^{-/-}$Bak$^{-/-}$Cyp D$^{-/-}$ (TKO) mice, from which immortalized MEFs were obtained. As shown in Fig. 4F, A23187/ArA equally induced the death of TKO and DKO MEFs, with cell death being partially inhibited by zVAD-fmk. Consistent with these findings, cytochrome c release was also observed in A23187/ArA-treated TKO MEFs (Fig. 4G), indicating that the CsA-sensitive mPT was not involved in Bax/Bak-independent cytochrome c release and cell death induced by A23187/ArA.

To obtain some insight into the molecular mechanisms of A23187/ArA-induced cytochrome c release, we tested the effects of various drugs, such as protease inhibitors and lipase inhibitors, on A23187/ArA-treated DKO MEFs, and found that 4-(2-aminoethyl)benzenesulfonfluoride (AEBSF), which inhibits serine proteases through sulfonylation of the active site serine residue (15), and has been described to inhibit some forms of cell death (16-19), could prevent A23187/ArA-induced caspase activation and cell death (Fig. 5A, B, C). AEBSF inhibited A23187/ArA-induced cell death in a dose dependent manner, whereas 4-(2-aminoethyl)benzenesulfonamide (AEBSA), an inactive analog of AEBSF, did not inhibit it at any doses tested (Fig. 5D). To confirm the involvement of a serine protease(s), we examined...
other serine protease inhibitors, DFP, TLCK, and TPCK. Addition of DFP, TLCK, and TPCK into culture medium did not inhibit A23187/ArA-induced death (data not shown), which could be due to their membrane impermeability, therefore, we microinjected them into cells. As shown in Fig. 5E, all the serine protease inhibitors inhibited A23187/ArA-induced cell death. Since inhibitors are diluted to 2.5 x 10^2-10^3 folds in the cytoplasm (20), the estimated intracellular concentrations of AEBSF are calculated to 40-400 nM. AEBSF not only inhibited A23187/ArA-induced cell death, but also blocked cytochrome c release (Fig. 5F, G). These results indicated that a serine protease(s) is involved in the process of A23187/ArA-induced cell death upstream of cytochrome c release. AEBSF was a more effective inhibitor of A23187/ArA-induced cell death than zVAD-fmk (Fig. 5A), suggesting that it acted at a common step further upstream in the death-signaling pathways or that it inhibited independently both caspase-dependent and caspase-independent cell death. We examined the influence of Omi/HtrA2, a serine protease located in the mitochondria that is known to be involved in apoptosis (21,22). As shown in Fig. 5H-J, silencing of Omi/HtrA2 had no effect on A23187/ArA-induced death, although it significantly reduced Fas-mediated apoptosis of DKO MEFs (data not shown).

**DISCUSSION**

This study revealed the following findings. 1) the combination of Ca^{2+} ionophore A23187 and arachidonic acid causes caspase-dependent cell death following cytochrome c release as well as caspase-independent death in Bax/Bak double-knockout MEFs. 2) The Bcl-2 family of proteins and the CsA-sensitive mPT are not involved in this mode of cell death. 3) Instead, this mode of death is mediated by an unknown serine protease(s). These findings demonstrate the existence of a novel mechanism of cytochrome c release that is regulated by serine proteases, but not by the Bcl-2 family.

Bax/Bak-dependent and Bax/Bak-independent mechanisms seem to respond to different stimuli. After treatment with etoposide, UV, or staurosporine, cytochrome c release occurred in a Bcl-2 family-dependent manner, whereas A23187/ArA not only induced cytochrome c release in a Bcl-2 family-dependent manner, but also in a Bcl-2 family-independent, a serine protease(s)-dependent manner. This difference might be related to the influence of molecule(s) acting upstream of the mitochondria. Since apoptosis induced by BH3-only proteins occurs in a Bax/Bak-dependent manner (23,24), A23187/ArA probably activates unidentified apoptotic molecule(s) in addition to BH-3 only proteins. A23187/ArA interferes with intracellular Ca^{2+} homeostasis (5), so Ca^{2+} itself or Ca^{2+}-regulated proteins might be involved in the mechanism of A23187/ArA-induced cell death. If Ca^{2+} ion was a major signal mediator, it seemed likely that cytochrome c release would occur via the mitochondrial permeability transition (mPT). However, we excluded this possibility by employing MEFs deficient in Bax, Bak, and Cyp D (Fig. 4F, G). This result was consistent with our findings obtained in Cyp D-deficient MEFs and mice (14) that cell death mediated via the mPT is necrosis but not apoptosis, probably due to the decline of ATP caused by Δψ loss. Therefore, a Ca^{2+}-regulated proteins, rather than Ca^{2+} itself, may be the most suitable candidate as an inducer of Bax/Bak-independent cytochrome c release. We could not formally exclude the possibility that A23187/ArA-induced cytochrome c release was mediated by the CsA-insensitive mPT.

Since A23187/ArA-induced death of DKO MEFs was inhibited by the treatment of serine protease inhibitors including AEBSF and TPCK, proteolytic activity of a serine protease(s) seems to be involved this form of cell death. Although it is still to be determined how serine protease inhibitors suppressed A23187/ArA-induced death, the possibility was excluded that AEBSF inhibited A23187/ArA-induced death by blocking mitochondrial Ca^{2+} uptake, by using Rhod2-AM as an indicator of mitochondrial Ca^{2+} uptake (unpublished results). A serine protease(s) may cleave one or more unidentified substrates in the mitochondria that are involved in the regulation of death due to A23187/ArA. For example, a mitochondrial channel protein might be cleaved by a serine protease(s), resulting in conformational changes that allow cytochrome c release, but elucidation of the actual mechanism will require further investigation.
A serine protease(s)-mediated, but not Bax/Bak-mediated, cytochrome c release and caspase-dependent cell death were observed in immortalized MEFs. Dose other normal cells also have the potential to undergo these forms of death? It has previously been described that the brains of bax<sup>-/-</sup>bak<sup>-/-</sup> mice displayed a normal gross anatomy, regardless of the neuronal progenitor cells derived from these mice show strongly resistant to apoptosis (25,26). Note that mature neurons present in cerebellar granule cell cultures from bax<sup>-/-</sup>bak<sup>-/-</sup> mice are sensitive to excitotoxic cell death as wild type cells (26). The excitotoxic cell death has both necrotic as well as apoptotic features (27), and some excitotoxins induce nitric oxide production which can cause cell death with apoptotic features (28). In excitotoxic death of cerebellar granule cells, novel apoptotic mechanisms which are Bax/Bak-independent, might be activated.

In conclusion, we demonstrated the existence of a novel mechanism of cytochrome c release that involves a serine protease(s), but not the mPT or Bcl-2 family proteins.

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FIGURE LEGENDS

Fig. 1. Induction of caspase-dependent death of DKO MEFs by A23187/ArA. A-D, Activation of DEVDase in MEFs exposed to death stimuli. WT MEFs and DKO MEFs (A, C) or Apaf-1+/+ and Apaf-1-/- MEFs (B, D) were treated with 10 μM etoposide (A, B), and 10 μM A23187 plus 100 μM arachidonic acid (C, D), and DEVDase activity was measured. Representative results from three independent experiments are shown. E-F, Activation of caspasas in DKO MEFs, but not in Apaf-1-/- MEFs, by A23187/ArA. The indicated MEFs were incubated with A23187/ArA for the indicated times. Cleavage of pro-caspase-9, pro-caspase-3, and lamin B1 was analyzed by Western blotting. GAPDH was also analyzed as a loading control. (*) nonspecific band. G-H, Induction of death in A23187/ArA-treated DKO MEFs. WT and DKO MEFs were not treated (NT) or were treated with A23187/ArA in the presence or absence of 100 μM zVAD-fmk (G, H) or 10 μM etoposide (H) for 36 hours. The extent of cell death was assessed by Annexin-V staining (G). Data are shown as the mean ± SD (n=4). Representative nuclear morphology is shown (H). Bars, 50 μm.

Fig. 2. Inhibition of A23187/ArA-induced death of DKO MEFs by silencing of caspase-9 and cytochrome c. A, DKO MEFs were treated with 10 μg of caspase-9 siRNA as described in Methods, after which the expression of caspase-9 and GAPDH (loading control) was analyzed by Western blotting. B, DKO MEFs with silencing of caspase-9 were treated with 10 μM A23187 plus 100 μM ArA for 24 hours, and then the extent of cell death (defined by Annexin-V staining) and DEVDase activity was measured (n=4). C, DKO MEFs were treated with 5 μg of cytochrome c siRNA. Expression of cytochrome c and GAPDH was analyzed. D-E, DKO MEFs with silencing of cytochrome c were treated with A23187/ArA in the absence or presence of 100 μM zVAD-fmk for 24 hours. (E) The same experiments as shown in (D) were performed, except in the presence of 1 μM antimycin A for 12 hours. Cell death (defined by annexin-V staining) and DEVDase activity were measured (n=4).

Fig. 3. Cytochrome c release in A23187/ArA-treated MEFs. WT and DKO MEFs were not treated or were treated with 10 μM A23187 plus 100 μM ArA in the presence of zVAD-fmk (100 μM). In (B), MEFs were also treated with 10 μM etoposide plus 100 μM zVAD-fmk. A, After 18 hours, the cytoplasmic fraction was recovered, and samples were subjected to Western blotting for detection of cytochrome c. B, After 12 hours, MEFs were fixed and immunostained with an anti-cytochrome c monoclonal antibody. Bars, 50 μm.
Fig. 4. No effect of Bcl-xL or inhibition of the permeability transition on A23187/ArA-induced death of DKO MEFs. A-B, Overexpression of Bcl-xL did not affect A23187/ArA-induced death of DKO MEFs. Cells were transfected with the indicated plasmids (0.5 μg). After 24 hours, the cells were incubated with 10 μM A23187 plus 100 μM ArA in the presence or absence of 100 μM zVAD-fmk. Cell death (defined by Annexin-V staining) (A) and DEVDase activity at 24 hours (B) were measured (n=4). C, Overexpression of Bcl-xL did not affect A23187/ArA-induced cytochrome c release in DKO MEFs. DKO MEFs with or without Bcl-xL overexpression were incubated with A23187/ArA in the presence of 100 μM zVAD-fmk for 18 hours. Then the cytoplasmic and mitochondrial fractions were recovered, and samples were subjected to Western blotting for detection of cytochrome c. D, Bcl-xL inhibited Bax-induced apoptosis. DKO MEFs were transfected with plasmids expressing Bax, Bcl-xL, or control vector together with a GFP-expressing plasmid. After 24 hours, cells were treated with 100 μM etposide in the presence or absence of 100 μM zVAD-fmk for another 24 hours. Cell death was determined by counting the propidium iodide (PI)-positive cells among GFP-positive cells. E, mPT inhibitors failed to inhibit A23187/ArA-induced death of DKO MEFs. Cells were treated with A23187/ArA in the presence of 100 μM zVAD-fmk or several mPT inhibitors (1 μM cyclosporin A (CsA), 10 μM bongkrekic acid (BKA), or 2 mM 1-carnitine). After 24 hours, cell death was assessed by PI staining. F, Lack of Cyp D did not influence A23187/ArA-induced death of DKO MEFs. DKO MEFs (Bax/Bak-deficient) and TKO MEFs (Bax/Bak/Cyp D-deficient) were incubated with A23187/ArA in the presence or absence of 100 μM zVAD-fmk. Then cell death was assessed by Annexin-V staining (n=4). G, Lack of Cyp D did not affect A23187/ArA-induced cytochrome c release. The same experiment as in (C) was performed, except for the use of TKO MEFs and DKO MEFs.

Fig. 5. Inhibition of A23187/ArA-induced cell death of DKO MEFs by the treatment of serine protease inhibitors. A-C, Inhibition of A23187/ArA-induced cell death of DKO MEFs by AEBSF. Cells were incubated with 10 μM A23187 plus 100 μM ArA in the presence or absence of 100 μM zVAD-fmk or 100 μg/ml AEBSF. Then cell death (defined by Annexin-V staining) (A) and DEVDase activity (B) were measured (n=4). Representative nuclear morphology of A23187/ArA-treated DKO MEFs in the presence or absence of AEBSF at 24 hours is shown in (C). Bars, 50 μm. D, Inhibition of A23187/ArA-induced cell death by AEBSF but not AEBSA, an inactive analog. DKO MEFs were incubated with A23187/ArA in the presence of 40, 200, and 400 μg/ml AEBSF or AEBSA. After 30 hours, the extent of cell death was assessed by Annexin-V staining. Data are shown as the mean ± SD (n=3). E, Inhibition of A23187/ArA-induced cell death by various serine protease inhibitors. Serine protease inhibitors, AEBSF, DFP, TLCK, and TPCK or the negative control (AEBSA and PBS) were microinjected into the DKO MEFs with GFP as described in Methods. After 1 hour, cells were treated with A23187/ArA, and the cell death was assessed after 20 hours. Cell viability was determined by counting GFP-positive cells attached on the dish among total injected GFP-positive cells. Data are shown as the mean ± SD (n=3). F-G, Inhibition of A23187/ArA-induced cytochrome c release by AEBSF. DKO MEFs were not treated or were treated with A23187/ArA in the presence of zVAD-fmk (100 μM) or AEBSF (100 μg/ml) for 18 hours (F) or 12 hours (G). In (F), the cytoplasmic fraction was recovered, and samples were subjected to Western blotting for detection of cytochrome c. In (G), MEFs were fixed and immunostained with an anti-cytochrome c monoclonal antibody. H-J, No inhibition of A23187/ArA-induced cell death by silencing of HtrA2/Omi, a mitochondrial serine protease. H, DKO MEFs were treated with 10 μg of HtrA2/Omi siRNA as described in Methods, after which the expression of HtrA2/Omi and GAPDH (loading control) was analysed by Western blotting. DKO MEFs with silencing of HtrA2/Omi or GFP (control) were treated with A23187/ArA, after which cell death (defined by annexin-V staining) (I) and DEVDase activity (J) were measured (n=4).
Fig. 1

A

WT
DKO

DEVDase activity (AFU/min)

0 12 24 36 (hr)

B

Apaf-1 ++/
Apaf-1 +/-

DEVDase activity (AFU/min)

0 12 24 36 (hr)

C

WT
DKO

DEVDase activity (AFU/min)

0 12 24 36 (hr)

D

Apaf-1 ++/
Apaf-1 +/-

DEVDase activity (AFU/min)

0 12 24 36 (hr)

E

WT
DKO

pro-caspase-9
pro-caspase-3
active-caspase-3
full length lamin B1

caspase-cleaved lamin B1

GAPDH

F

Apaf-1++/
Apaf-1-/-

pro-caspase-9
pro-caspase-3
active-caspase-3
full length lamin B1

caspase-cleaved lamin B1

GAPDH

H

WT
DKO

NT

A23187/ArA

Annexin-V positive cells (%)

0 12 24 36 (hr)
Fig. 2

A

GFP siRNA, caspase-9 siRNA

pro-caspase-9

GAPDH

B

Annexin-V positive cells (%)

siCaspase-9

siGFP

A23187/ArA

DEVDase activity (AFU/min)

A23187/ArA

C

GFP siRNA, cyt c siRNA

cyt c

GAPDH

D

Annexin-V positive cells (%)

cyt c siRNA

GFP siRNA

NT, A23187 ArA, A23187 ArA +zVAD

DEVDase activity (AFU/min)

NT, A23187 ArA, A23187 ArA +zVAD

E

Annexin-V positive cells (%)

cyt c siRNA

GFP siRNA

NT, A23187 ArA

DEVDase activity (AFU/min)

NT, A23187 ArA
**Fig. 3**

A

| Treatment          | WT | DKO |
|--------------------|----|-----|
| No treatment       | ![WT WT] | ![WT DKO] |
| A23187/ArA + zVAD  | ![WT WT] | ![WT DKO] |

B

| Condition            | WT | DKO |
|----------------------|----|-----|
| NT                   | ![WT WT] | ![WT DKO] |
| A23187/ArA + zVAD    | ![WT WT] | ![WT DKO] |
| Etoposide + zVAD     | ![WT WT] | ![WT DKO] |
Fig. 4

A

A23187/ArA

- zVAD
- zVAD
+ zVAD
+ zVAD

vector transfection
Bcl-xL transfection

Annexin-V positive cells (%)

0 24 36 (hr)

vector Bcl-xL

B

DEVDase activity (AFU/min)

vector transfection
Bcl-xL transfection

0 10 20 30 40

C

vector transfection
Bcl-xL transfection

A23187/ArA+etoposide+zVAD
etoposide+zVAD

PI positive cells (%)

0 25 50 75 100

D

etoposide etoposide+zVAD

PI positive/GFP positive cells (%)

0 25 50 75 100

E

etoposide etoposide+zVAD

PI positive cells (%)

0 25 50 75 100

F

A23187/ArA

- DKO
- TKO
DKO + zVAD
TKO + zVAD

Annexin-V positive cells (%)

0 25 50 75 100

G

DKO

NT
A23187/ArA+etoposide+zVAD
etoposide+zVAD

TKO

NT
A23187/ArA+etoposide+zVAD
etoposide+zVAD

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Fig. 5

A23187/ArA

A

no inhibitor
+zVAD
+AEBSF

DEVDase activity (AFU/min)

0 12 24 36 (hr)

A23187/ArA

B

no inhibitor
+zVAD
+AEBSF

DEVDase activity (AFU/min)

0 12 24 36 (hr)

Annexin-V positive cells (%)

A23187/ArA

C

A23187/ArA

+AEBSF

NT

Annexin-V positive cells (%)

A23187/ArA

D

AEBSF
+AEBSA

Annexin-V positive cells (%)

A23187/ArA

E

PBS
AEBSA
AEBSF
DFP
TLCK
TPCK

Cell death (%)

F

cytosol
mitochondria

G

HtrA2/Omi
GFP siRNA
Omi siRNA

Annexin-V positive cells (%)

H

HtrA2/Omi
GAPDH

13
A BAX/BAK-independent mechanism of cytochrome C release
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Yoshihide Tsujimoto

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