



Original Contribution

Oxidative modification sensitizes mitochondrial apoptosis-inducing factor to calpain-mediated processing

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ABSTRACT

Although processing of mitochondrial apoptosis-inducing factor (AIF) is essential for its function during apoptosis in most cell types, the detailed mechanisms of AIF cleavage remain elusive. Recent findings indicate that the proteolytic process is Ca^{2+} -dependent and that it is mediated by a calpain located in the mitochondrial intermembrane space. We can now report that, in addition to a sustained intracellular Ca^{2+} elevation, enhanced formation of reactive oxygen species (ROS) is a prerequisite step for AIF to be cleaved and released from mitochondria in staurosporine-treated cells. These events occurred independent of the redox state of the mitochondria and were not influenced by binding of pyridine nucleotides to AIF. Chelation of cytosolic Ca^{2+} by BAPTA/AM suppressed the elevation of both Ca^{2+} and ROS, suggesting that the Ca^{2+} rise was the most upstream signal required for AIF processing. We could further show that the stimulated ROS production leads to oxidative modification (carbonylation) of AIF, which markedly increases its rate of cleavage by calpain. Accordingly, pretreatment of the cells with antioxidants blocked AIF carbonylation, as well as its subsequent cleavage and release from the mitochondria. Combined, our data provide evidence that ROS-mediated, posttranslational modification of AIF is critical for its cleavage by calpain and thus for AIF-mediated cell death.

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Apoptosis-inducing factor (AIF) is a flavoprotein anchored to the mitochondrial inner membrane. Under physiological conditions, AIF exhibits NADH oxidase activity and has been shown to be important for mitochondrial respiration and oxidative phosphorylation [1]. However, during apoptosis AIF can be processed and released from the mitochondria into the cytosol for further translocation to the nucleus. In fact, AIF was the first mitochondrial protein shown to be involved in apoptotic cell death [2]. AIF-mediated cell death seems to be particularly important for neuronal cells and for non-small-cell lung carcinoma (NSCLC) [3,4].

AIF contains a nuclear localization signal and translocates to the nucleus, where it participates in chromatin condensation and large-scale DNA fragmentation. The mechanism by which AIF is processed is not fully understood, although it has been studied extensively during recent years. Hence, it has been demonstrated that AIF is synthesized as a 67-kDa protein in the cytosol. Upon import into the mitochondria it matures to a 62-kDa protein, which is N-terminally integrated into the membrane. To be released from mitochondria, AIF needs to be cleaved at the N-terminus to free it from its membrane anchor [5]. It has been reported that such cleavage can be catalyzed by Ca^{2+} -

dependent calpain and by Ca^{2+} -independent cathepsins B, L, and S [6,7]. Moreover, based on studies using recombinant mouse AIF it was proposed that the cleavage site is not exposed under normal conditions because of its binding of NADP^+ and that its interaction with pyridine nucleotides needs to be disrupted before AIF can be cleaved [8].

As a result of tissue-specific alternative splicing, there are several isoforms of AIF [9]. Furthermore, in brain mitochondria there seems to be a minor pool of full-length AIF that is loosely bound to the cytosolic surface of the mitochondria from where it can be released into the cytosol without proteolytic processing [10]. Whether this is true also for some other cell types remains to be investigated.

We have previously reported that, for AIF to be cleaved and released from the mitochondria in NSCLC cells, a prolonged elevation (>10 min) of the intracellular Ca^{2+} level was required [11]. Treatment of the cells with staurosporine (STS) triggered Ca^{2+} entry into the cells and caused activation of calpain in the intermembrane space of mitochondria, which mediated the proteolysis of AIF [11]. However, it is also known that antioxidants can suppress AIF-mediated cell death in neurons and in cancer cells of various origins, such as pancreatic and breast cancer [12–14]. Hence, the aim of this study was to investigate whether AIF processing is influenced by reactive oxygen species (ROS) and, if so, by what mechanism. Our results indicate that STS-induced Ca^{2+} import into cells stimulates mitochondrial ROS accumulation and leads to oxidative modification (carbonylation) of AIF, making it more prone to proteolysis by calpain.

Abbreviations: AIF, apoptosis-inducing factor; ROS, reactive oxygen species; STS, staurosporine; NSCLC, non-small-cell lung carcinoma; MPT, mitochondrial permeability transition.

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Materials and methods

Cell culture

U1810 cells were grown in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2% (w/v) glutamine, 100 units/ml penicillin and 100 units/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C and were maintained at a cell density allowing exponential growth. HeLa cells and neuroblastoma SH-SY5Y cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin.

Experiments using isolated rat liver mitochondria and U1810 cells

Mitochondria were isolated as described previously [11] and were incubated in MSH buffer (210 mM mannitol, 70 mM sucrose, and 5 mM HEPES, pH 7.4) supplemented with 0.5 mM CaCl₂, 2 μM ruthenium red, and 1 μM cyclosporin A. Oxidation of mitochondrial pyridine nucleotides was achieved by adding H₂O₂ (400 μM) to nonrespiring mitochondria. Reduction of NAD(P)⁺ was achieved by adding 5 mM succinate, which can reduce pyridine nucleotides through reversed electron flow. ROS production was triggered by adding antimycin A (2.5 μg/ml) or paraquat (100 μM) and was suppressed by addition of *N*-acetylcysteine (NAC; 10 mM). To test whether NADP⁺ could block the cleavage of AIF, isolated liver mitochondria were incubated for 10 min in a medium (125 mM KCl, 0.5 mM KH₂PO₄, 20 mM HEPES (pH 7.4), 1 mM MgCl₂, 1 μM ruthenium red) containing either 5 mM succinate/2 μM rotenone or 5 mM malate/5 mM pyruvate. Subsequently, 0.5 mM CaCl₂, 2 mM NADP⁺, and 100 μM calpain inhibitor I were added to selected samples (see Fig. 1) and incubated for an additional 70 min at 30°C. The reaction was stopped by the addition of Laemmli's buffer.

Immunoblotting

Proteins from each sample were mixed with Laemmli's loading buffer, boiled for 5 min, and subjected to 12% SDS-PAGE at 40 mA followed by transfer to nitrocellulose membranes for 90 min at 120 V. Membranes were blocked for 30 min with 5% nonfat milk in Tris-buffered saline (TBS) at room temperature and subsequently probed with the desired primary antibody. Blots were visualized by ECL (Amersham Biosciences). The following primary antibodies were used: goat anti-AIF and rabbit anti-AIF (Santa Cruz Biotechnology), rabbit anti-actin (Sigma), rabbit anti-Atg5 (Abgent), and mouse anti-poly(ADP ribose) polymerase (PARP) (BD Pharmingen). The primary antibodies were diluted in TBS containing 1% bovine serum albumin, 0.05% Tween 20, and 0.1% Na₂S₂O₃. Secondary antibodies were diluted in blocking buffer. Horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce Chemical Co.

Flow cytometry

Phosphatidylserine exposure on the outer leaflet of the plasma membrane was detected using the Annexin V-FLUOS staining kit (Roche) according to the manufacturer's instructions. In brief, 10⁶ cells were pelleted after the treatments (as indicated in Fig. 2) and washed in PBS. Next, the cell pellets were resuspended in 100 μl of Annexin V-FLUOS labeling solution, incubated for 15 min at room temperature, and analyzed by FACS.

Live cell imaging

For Ca²⁺ measurements, the cells were incubated (30 min at 37°C in 5% CO₂) in Hepes medium (130 mM NaCl, 4.7 mM KCl, 1.3 mM

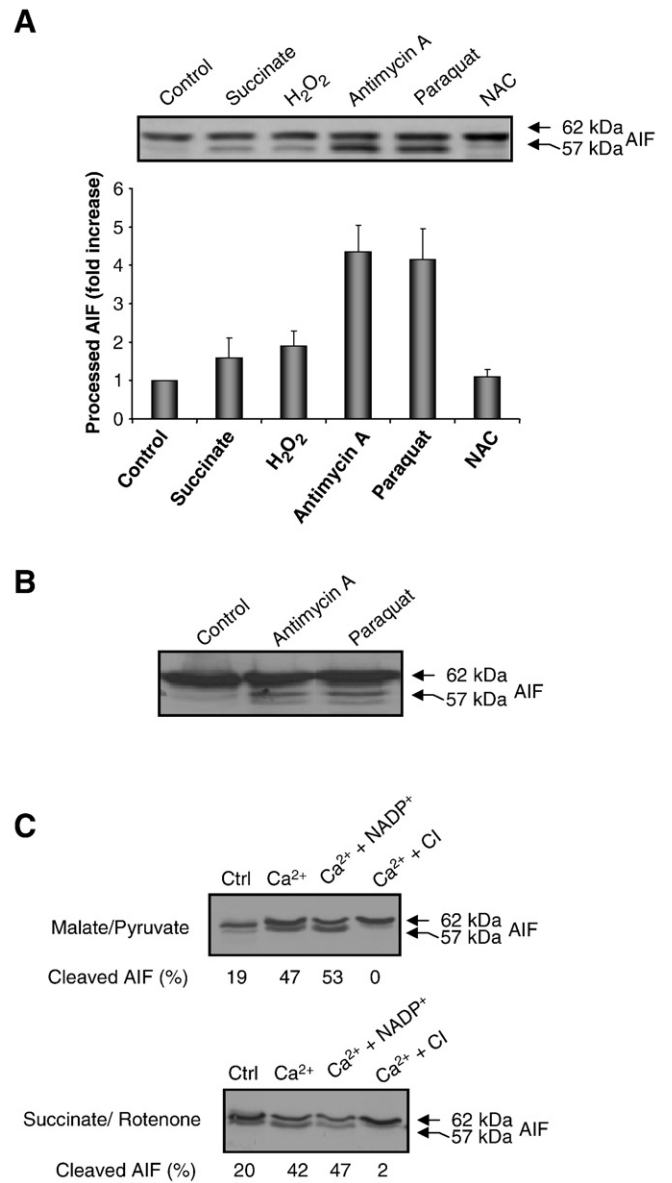


Fig. 1. ROS formation promotes AIF cleavage. (A) Liver mitochondria were incubated in the presence of 0.5 mM CaCl₂ with 5 mM succinate, 400 μM H₂O₂, 2.5 μg/μl antimycin A, 100 μM paraquat, or 10 mM NAC, and AIF cleavage was monitored by Western blot. A quantification of the cleaved form of AIF is presented in the graph as the mean ± SD. (B) Permeabilized U1810 cells were incubated in the presence of 0.5 mM CaCl₂ with 2.5 μg/μl antimycin A or 100 μM paraquat, and AIF processing was assessed by Western blot. (C) Isolated mitochondria were incubated in respiration medium containing 5 mM succinate/2 μM rotenone or 5 mM malate/5 mM pyruvate in the absence or presence of 0.5 mM CaCl₂, 2 mM NADP⁺, and 100 μM calpain inhibitor I (Cl). The full-length and the truncated forms of AIF were quantified, and truncated AIF is presented as percentage of full-length AIF.

CaCl₂, 1 mM MgSO₄, 1.2 mM KH₂PO₄, 20 mM HEPES (pH 7.4), and 5 mM dextrose) containing 5 μM Fluo-4/AM (Molecular Probes) together with 0.1% Pluronic F-127 (Molecular Probes). The cells were seeded on coverslips and, on the following day, transiently transfected with either pHyPer-dMito (Evrogen) or pEGFP-AIF using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. All drugs were bath-applied 5 min after start of time lapse. All experiments were performed using a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss). The drugs used were 1 μM STS, 2.5 μg/ml antimycin A, 10 μM BAPTA/AM, 100 μM Trolox, 100 μM MnTBAP, or 10 mM NAC.

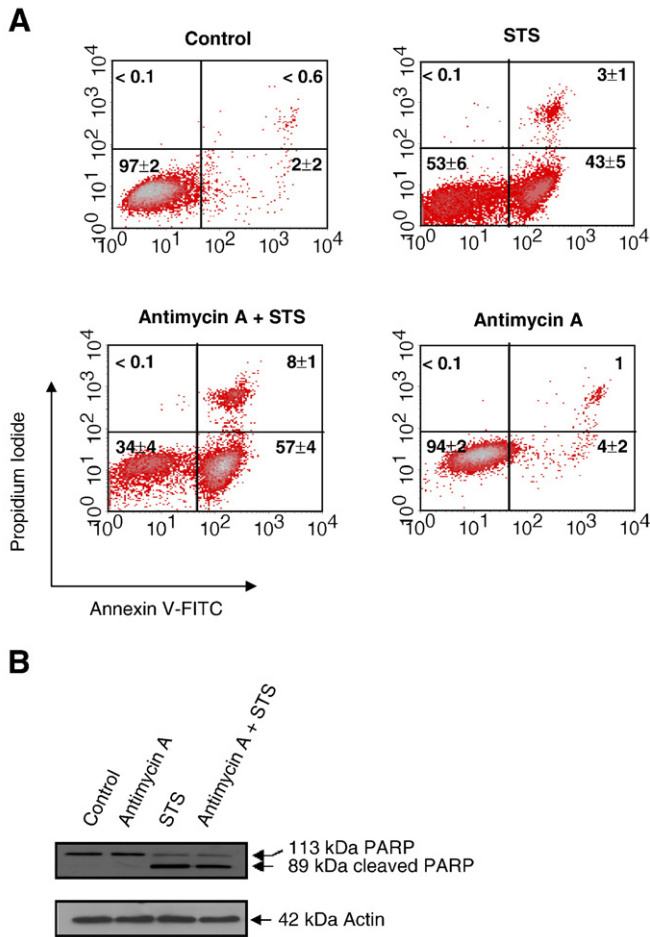


Fig. 2. Antimycin A promotes STS-induced AIF-mediated cell death. (A) Cells were treated with 0.2 μ M STS for 20 h (upper right), or pretreated with 2.5 μ g/ μ l antimycin A before STS treatment for 20 h (lower left), or treated with 2.5 μ g/ μ l antimycin A alone for 20 h (lower right) and analyzed by annexin V-FITC and propidium iodide costaining using FACS. The percentage of cells in each field is given in an upper corner as the mean \pm SD. (B) Western blot of PARP cleavage. The membrane was reprobed for actin to confirm equal sample loading.

Membrane insertion of EGFP-AIF

Analysis of membrane insertion was performed as described previously [15]. Briefly, the mitochondrial pellet was resuspended in 100 mM Na_2CO_3 and incubated with continuous shaking for 30 min at 4°C. The membranes were recovered by centrifugation (30 min, 100,000g, 4°C) and dissolved in loading buffer.

Immunoprecipitation

Cells were treated with 1 μ M STS for various time periods and harvested. The immunoprecipitation (IP) lysis buffer contained 1% Triton X-100, 140 mM KCl, 10 mM HEPES, 1 mM EDTA, and 1 \times Complete inhibitor cocktail. Protein lysate (700 μ g) was mixed with rabbit anti-AIF antibodies and incubated at 4°C overnight. Then, the mixture was incubated with protein G-Sepharose beads and washed three times in 0.1% IP lysis buffer, before the bound protein was eluted with 1% SDS.

Detection of protein carbonylation

Protein carbonyls were detected using the OxyBlot protein oxidation detection kit (Chemicon International) according to the manufacturer's instructions. Briefly, the immunoprecipitated AIF was

used and carbonyls were derivatized with 2,4-dinitrophenylhydrazine (DNPH). The mixture was subjected to 12% SDS-PAGE and Western blotting. Membranes were stained with Ponceau red to confirm equal loading. Immunoblotting was performed using anti-DNP antibody.

Calpain cleavage of AIF

Immunoprecipitated AIF, from control cells (noncarbonylated AIF) or STS-treated cells (carbonylated AIF), was mixed with various concentrations of recombinant calpain-1 and 1 mM CaCl_2 in MSH buffer. The mixture was incubated for 3 h at 37°C and the reaction was stopped by the addition of Laemmli's buffer. Samples were then subjected to 12% SDS-PAGE.

Results

AIF processing is dependent on ROS production but independent of the redox state of mitochondrial pyridine nucleotides

A previous report suggested that AIF can undergo redox-dependent conformational changes leading to the exposure of the calpain cleavage site [8]. To investigate whether AIF cleavage is, in fact, influenced by the redox state of mitochondrial $\text{NAD(P)}^+/\text{NAD(P)H}$, or by enhanced ROS production, the electron flux through the respiratory chain in isolated rat liver mitochondria was modulated. Mitochondria were incubated in MSH buffer containing CaCl_2 (to activate calpain in the intermembrane space), ruthenium red (to prevent Ca^{2+} accumulation in the mitochondrial matrix), and cyclosporin A (to block accidental induction of the mitochondrial permeability transition (MPT)). The redox state of the mitochondrial pyridine nucleotides was affected by administration of H_2O_2 (oxidation) or succinate (reduction). ROS generation at Complex III of the respiratory chain was stimulated by the addition of antimycin A to the respiring mitochondria and was suppressed by a ROS scavenger, NAC. We found that treatment with antimycin A or paraquat promoted Ca^{2+} -mediated AIF processing, whereas AIF cleavage was significantly diminished in the presence of NAC (Fig. 1A). To confirm this, we performed a similar experiment using U1810 cells. As expected, both generators of ROS, antimycin A and paraquat, caused similar stimulation of AIF processing in this model system also (Fig. 1B). Thus, AIF processing was enhanced by ROS produced either at Complex III (antimycin A) or at Complex I (paraquat) of the electron transport chain.

To examine whether an interaction of AIF with pyridine nucleotides might influence the proteolysis of AIF, respiring rat liver mitochondria were incubated in the absence or presence of calpain inhibitor I and added NADP^+ . As shown in Fig. 1C, addition of Ca^{2+} stimulated AIF processing, which was blocked by calpain inhibitor I. In contrast, added NADP^+ was unable to prevent AIF processing (Fig. 1C). Neither did addition of other pyridine nucleotides (NAD(H) , NADPH) affect AIF cleavage by mitochondrial calpain (data not shown).

Antimycin A promotes STS-induced caspase-independent apoptosis

We demonstrated previously that the AIF-mediated pathway is essential for the killing of NSCLC cells by anticancer agents [3,4]. To investigate whether increased ROS production promotes cell death in this experimental model, U1810 cells were exposed to STS or antimycin A, or to the combination of both drugs, and analyzed for apoptosis by FACS using annexin V-FITC and propidium iodide costaining. Whereas we observed 43% apoptotic cells upon treatment with STS alone, the apoptotic cell population increased to 57% when the cells were exposed to antimycin A before STS (Fig. 2A).

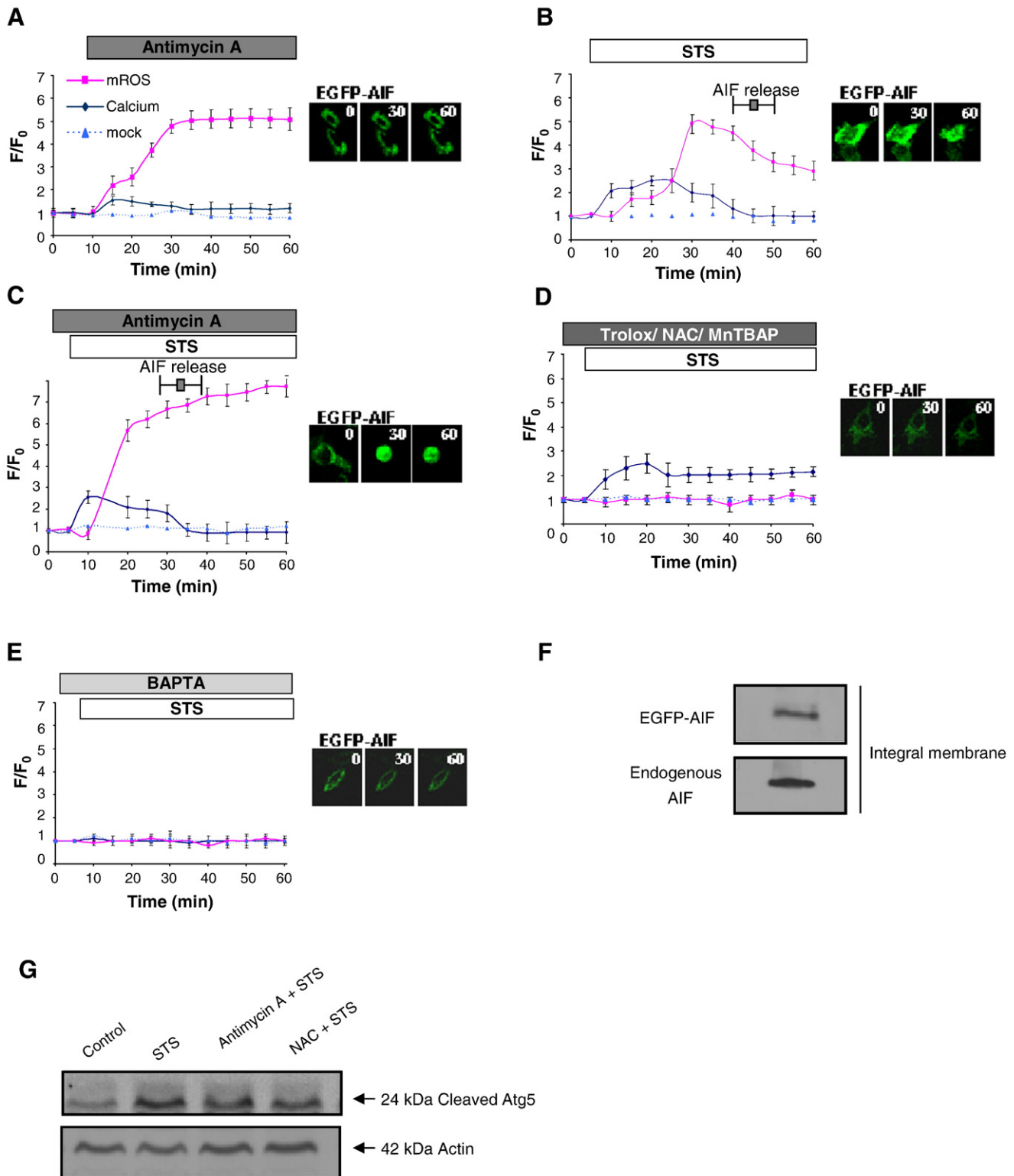


Fig. 3. Ca^{2+} -induced ROS formation promotes AIF cleavage and release. HeLa cells were either transfected with pHyPer-dMito or AIF-EGFP or loaded with the Ca^{2+} -sensitive dye Fluo-4/AM and subsequently exposed to (A) 2.5 $\mu\text{g}/\text{ml}$ antimycin A, (B) 1 μM STS, or (C) 2.5 $\mu\text{g}/\text{ml}$ antimycin A for 5 min followed by 1 μM STS. (D) Cells were preexposed to 100 μM Trolox, 10 mM NAC, or 100 μM MnTBAP before STS treatment. (E) Cells were preincubated with 10 μM BAPTA/AM for 30 min before treatment with 1 μM STS. Representative single-cell recordings of Ca^{2+} or mitochondrial H_2O_2 are shown as the means \pm SD from at least four independent experiments for each of the two parameters analyzed. Mock was either ethanol or DMSO. In (B) and (C), where AIF was released, this is presented with a gray box as start time of release (mean \pm SD). The ratio F/F_0 represents fluorescence of baseline. Snapshot pictures from the time lapse are shown to the right. (F) Membrane insertion of EGFP-AIF into mitochondria was monitored by alkaline extraction. (G) U1810 cells were treated with STS for 2 h in the presence or absence of 2.5 $\mu\text{g}/\text{ml}$ antimycin A or 10 mM NAC, and Atg5 cleavage was analyzed using Western blotting. The membrane was re probed for actin to confirm equal loading of the samples.

To investigate whether antimycin A-induced ROS generation also promoted caspase-dependent cell death, PARP cleavage was analyzed. As shown in Fig. 2B, enhanced ROS production by antimycin A did not increase the level of cleaved PARP. Thus, we can conclude that the increased cell death observed in Fig. 2A was indeed a result of the activation of the caspase-independent, AIF-mediated pathway. Combined with the results shown in Fig. 1, these findings indicate that increased ROS formation promoted both AIF processing and the apoptotic outcome thereof.

Ca²⁺-induced ROS formation stimulates AIF cleavage and release from mitochondria

To further substantiate the finding that ROS promotes AIF cleavage, we monitored: (a) mitochondrial H₂O₂ accumulation using a genetically encoded fluorescent sensor [16]; (b) the cytosolic Ca²⁺ level using the Ca²⁺-sensitive dye Fluo-4/AM; and (c) AIF release using an EGFP-AIF construct, in HeLa cells, which were treated with either antimycin A or STS or pretreated with antimycin A before the addition of STS. Antimycin A treatment caused an increase in mitochondrial H₂O₂ level, but no Ca²⁺ accumulation or AIF release was observed (Fig. 3A). When cells were treated with staurosporine, the cytosolic Ca²⁺ elevation was followed by a rise in H₂O₂ level, and AIF release started after 46 ± 5 min (Fig. 3B). However, when cells were pretreated with antimycin A before the addition of STS, the release of AIF was markedly accelerated and was detected already after 33 ± 4 min (Fig. 3C). This finding is in accordance with the observation that antimycin A stimulated both AIF processing in rat liver mitochondria (Fig. 1A) and caspase-independent cell death in STS-treated U1810 cells (Fig. 2). Importantly, the majority of the cells did not release AIF when they were preexposed to various antioxidants, including NAC and Trolox (a water-soluble derivative of α-tocopherol, vitamin E) and MnTBAP (a cell-permeable MnSOD mimetic), although an STS-induced Ca²⁺ increase was observed (Fig. 3D). This suggests that Ca²⁺ accumulation alone was insufficient for AIF processing and release from mitochondria into the cytosol. Instead, it implies that, in addition to the Ca²⁺ signal, generation of ROS, triggered by STS, antimycin A, or paraquat, was essential for proteolysis and release of AIF. To exclude the possibility that the observed effects of ROS formation were due to stimulation of calpain activity, we monitored the cleavage of another known calpain substrate, Atg5, in the presence of pro- or antioxidants and found that there was no difference in the processing of Atg5 (Fig. 3G).

We next examined whether the Ca²⁺ and ROS responses to STS treatment were interrelated. Chelation of cytosolic Ca²⁺ with BAPTA/AM completely suppressed the ROS response as well as the AIF release (Fig. 3E), suggesting that ROS were indeed produced in a Ca²⁺-dependent manner. Combined, these findings show that AIF cleavage and release were sequentially regulated by Ca²⁺ and ROS and that the Ca²⁺ elevation was the most upstream signal required for AIF processing in STS-treated cells.

Oxidative modification of AIF increases its sensitivity to calpain-mediated cleavage

Proteins are major targets for ROS, which can trigger multiple modifications of the protein structure. For instance, posttranslational modification by carbonylation might result in either partial unfolding, inactivation, or proteasomal degradation [17,18]. Therefore, we addressed the question if processing of AIF might involve its oxidative modification by assessing AIF carbonylation. An increase in overall protein carbonylation was, in fact, observed in STS-treated cells (Fig. 4A), and when AIF was immunoprecipitated we could detect selective carbonylation of AIF in STS-treated cells (Fig. 4B).

It was also of interest to examine whether carbonylation of AIF preceded its cleavage and release and whether inhibition of

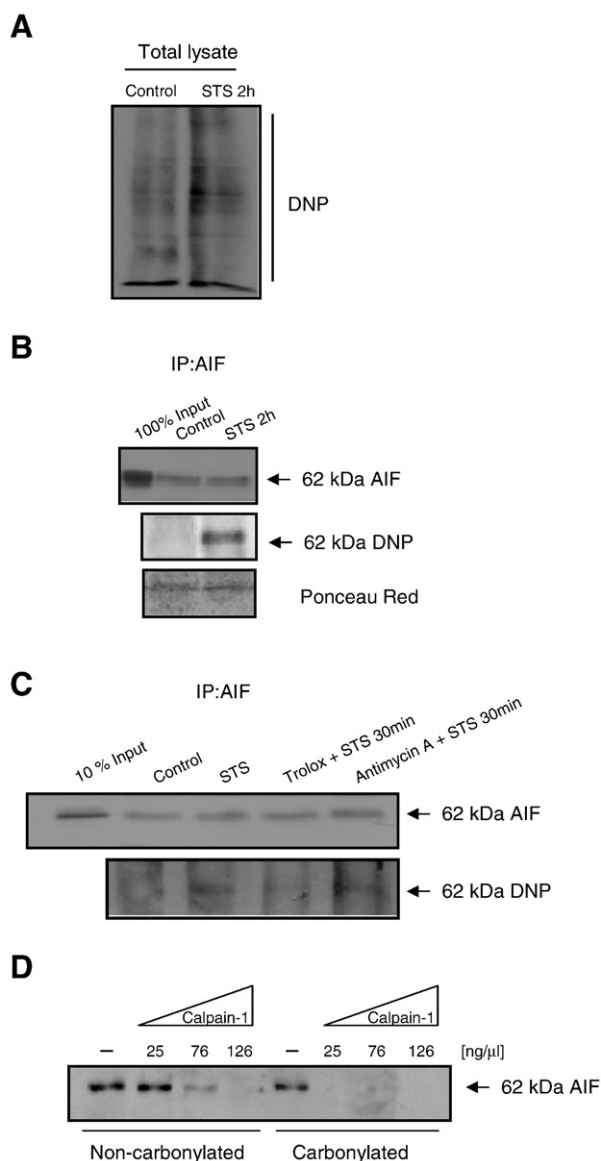


Fig. 4. Carbonylation of AIF increases its susceptibility to calpain-mediated cleavage. (A) U1810 cells were treated with 1 μM STS for 2 h, and total protein lysate was derivatized with DNPH and analyzed by Western blot using anti-DNP antibody. (B) Cells were treated as in (A), and AIF was subsequently subjected to immunoprecipitation from the cell lysate, and protein carbonyls on AIF were detected using anti-DNP antibody. Ponceau red staining was used to confirm equal loading of the samples. (C) Cells were pretreated with 100 μM Trolox or 2.5 μg/ml antimycin A before incubation with 1 μM STS for 30 min. AIF was then immunoprecipitated and analyzed for carbonyls. (D) Cells were exposed to STS for 30 min before immunoprecipitation of AIF. The precipitated AIF was incubated with 0.5 mM CaCl₂ and various concentrations of recombinant calpain-1 as indicated.

carbonylation by antioxidants prevented AIF cleavage. Hence, HeLa cells were treated with STS for 30 min, in the presence or absence of Trolox, before AIF immunoprecipitation. The results revealed that, before being released, AIF underwent carbonylation, which could be prevented by Trolox (Fig. 4C). Importantly, similar results were obtained with the neuroblastoma cell line SH-SY5Y (data not shown).

Finally, we compared the ability of calpain to cleave normal (noncarbonylated) vs oxidatively modified (carbonylated) AIF. The results revealed that calpain could cleave carbonylated AIF approximately fivefold more efficiently than the noncarbonylated protein (Fig. 4D). This demonstrates that calpain-mediated proteolysis of AIF requires its oxidative modification, given that antioxidant treatment

inhibited the carbonylation (Fig. 4C) as well as the cleavage (Fig. 1A) and release of AIF (Fig. 3D).

Discussion

Recent findings have indicated that AIF cleavage and release during apoptosis are governed by multiple regulatory steps. For example, based on in vitro experiments using recombinant AIF protein, it has been suggested that both the respiratory and the proapoptotic functions of AIF are controlled by its interaction with NAD(P)H [8]. More recently, we presented evidence for a pivotal role for a sustained intracellular Ca^{2+} increase leading to the activation of a mitochondrial calpain responsible for the cleavage of AIF in STS-treated cells [11]. Other studies have indicated that antioxidants could restrain cell death in experimental systems in which the AIF-mediated pathway is important [12–14]. The latter findings suggested that ROS formation might influence AIF processing during apoptosis.

In this study we found that, for AIF to be processed during STS-induced apoptotic signaling, there are sequential requirements for both a sustained elevation of the intracellular Ca^{2+} level and a Ca^{2+} -stimulated ROS formation. Indeed, it is well known that a cross talk between intracellular Ca^{2+} and ROS exists [19]. Hence, mitochondrial Ca^{2+} import activates the mitochondrial dehydrogenases and, indirectly, the activity of the respiratory chain. Moreover, accumulation of Ca^{2+} by mitochondria might trigger the MPT, causing the release of cytochrome *c* and other proapoptotic proteins from these organelles. The loss of cytochrome *c* from the respiratory chain can also lead to enhanced electron leakage and thereby stimulate the production of superoxide anion radicals [17,20]. In addition, the MPT results in the suppression of mitochondrial antioxidant capacity because of the loss of GSH and NAD(P)H—important cofactors for the ROS-detoxifying enzyme systems in the mitochondria [21]. Stimulation of ROS in a subpopulation of mitochondria can, in turn, stimulate MPT induction and subsequent ROS production in neighboring mitochondria, causing so-called ROS-induced ROS release [22].

Our findings indicate that AIF becomes oxidatively modified (carbonylated) as a consequence of enhanced mitochondrial ROS generation during STS-induced apoptosis and that this significantly promotes its cleavage by calpain. Suppression of ROS accumulation by pretreatment of the cells with antioxidants prevented carbonylation, as well as the cleavage and release of AIF. This seemed to be independent of the redox state of the mitochondria, and in our study AIF cleavage was not influenced by the addition of pyridine nucleotides to respiring mitochondria. In fact, several calpain substrates have been reported to require oxidation, or oxidative modification, to increase their sensitivity to proteolysis by calpain, including Na^+/K^+ -ATPase [23], dihydropyrimidinase-like 3 protein [24], protein tyrosine phosphatases [25], and neurofilaments [26–29]. This is probably because carbonylation often causes partial unfolding of proteins, leading to the exposure of normally hidden residues, which might serve as calpain cleavage sites [17,18]. Presumably, calpain cleavage site(s) on AIF could also become exposed because of a similar modification of AIF conformation. Indeed, we found that oxidative modification (carbonylation) preceded the cleavage and release of AIF and that it was also associated with a marked increase in cleavage rate. However, at present we cannot exclude that effects of oxidative modification other than carbonylation might have contributed to the ROS-mediated enhancement of AIF processing seen in our study. Hence experiments aiming to identify the site(s) of carbonylation in the AIF protein, and the effects of carbonylation on the 3-D structure of AIF, are ongoing in our laboratory. Eventually, we would also like to mutate the potential carbonylation site(s) in AIF to conclusively demonstrate the importance of carbonylation for AIF processing during apoptosis.

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