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Calcium Dysregulation Induces Apoptosis-inducing Factor Release: Cross-talk Between PARP-1- and Calpain- Signaling Pathways

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Abstract

Recent discoveries show that caspase-independent cell death pathways are a pervasive mechanism in neurodegenerative diseases, and apoptosis-inducing factor (AIF) is an important effector of this mode of neuronal death. There are currently two known mechanisms underlying AIF release following excitotoxic stress, PARP-1 and calpain. To test whether there is an interaction between PARP-1 and calpain in triggering AIF release, we used the NMDA toxicity model in rat primary cortical neurons. Exposure to NMDA resulted in AIF truncation and nuclear translocation, and shRNA-mediated knock down of AIF resulted in neuroprotection. Both calpain and PARP-1 are involved with AIF processing as AIF truncation, nuclear translocation and neuronal death were attenuated by calpain inhibition using adeno-associated virus-mediated overexpression of the endogenous calpain inhibitor, calpastatin, or treatment with the PARP-1 inhibitor 3-ABA. Activation of PARP-1 is necessary for calpain activation as PARP-1 inhibition blocked mitochondrial calpain activation. Finally, NMDA toxicity induces mitochondrial Ca^{2+} dysregulation in a PARP-1 dependent manner. Thus, PARP-1 and mitochondrial calpain activation are linked via PARP-1-induced alterations in mitochondrial Ca^{2+} homeostasis. Collectively, these findings link the two seemingly independent mechanisms triggering AIF-induced neuronal death.

Keywords

NMDA toxicity; calpain; PARP-1; apoptosis-inducing factor; ischemia; mitochondria; calcium homeostasis

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INTRODUCTION

Over the past decade, increasing evidence suggests that caspase-independent pathways play a critical role in neuronal death, and apoptosis-inducing factor (AIF) is emerging as a predominate mediator (Culmsee and Landshamer, 2006; Boujrad et al., 2007). Apoptosis-inducing factor is a mitochondrial flavoprotein that functions as an oxidoreductase within the inner membrane (Miramar et al., 2001). Cell death stimuli that disrupt the mitochondrial membrane, such as excitotoxicity, oxidative stress, DNA damage and cerebral ischemia cause AIF release from the mitochondria and subsequent translocation to the nucleus (Susin et al., 1999; Cregan et al., 2002; Yu et al., 2002; Cao et al., 2003; Zhu et al., 2003; Plesnila et al., 2004). The presence of AIF in the nucleus is necessary for AIF-induced cell death (Cheung et al., 2006), where AIF binds directly to DNA and causes chromatin condensation and high molecular weight (~50kb) DNA fragmentation (Susin et al., 1999; Ye et al., 2002).

There are two hypotheses regarding the mechanism of AIF release from mitochondria. The first involves a byproduct of the activation of the DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1), the poly(ADP-ribose) (PAR) polymer. Activation of PARP-1 is essential for AIF release (Wang et al., 2004; Culmsee et al., 2005; Moubarak et al., 2007), and results in the production of high levels of neurotoxic PAR polymer (Yu et al., 2006). Moreover, PAR polymer has been directly linked to the induction of AIF translocation (Andrabi et al., 2006), thus providing a death signaling mechanism that links the nucleus and mitochondria in neurons.

The second hypothesis governing AIF release and translocation entails activation of the calcium-dependent cysteine protease calpain (Cao et al., 2007). Calcium dysregulation is nearly a ubiquitous facet of neuronal injury and death (Vosler et al., 2008). Accordingly, calpain activation has been implicated in the pathology of most neurodegenerative diseases [for review see (Vosler et al., 2008)]. Stemming from this fact, it was discovered that calpain was necessary for AIF truncation and release from isolated brain and liver mitochondria (Polster et al., 2005). We have demonstrated that calpain activation and N-terminus cleavage of AIF is also essential for AIF translocation and ischemia-induced neuronal death (Cao et al., 2007).

The purpose of the present study was to determine whether PARP-1-mediated AIF release is sequentially linked to calpain-dependent AIF release. To this end, we studied the potential interaction between PARP-1 and calpain signaling pathways using the excitotoxic NMDA exposure model in primary neurons. Our results suggest that PARP-1 and calpain work in concert following calcium dysregulation to induce AIF release.

METHODS

Gene transfection in primary neurons by AAV vectors

Construction and production of adeno-associated virus (AAV) vectors carrying either the short hairpin siRNA AIF targeting sequence (AAV-AIFt), its scrambled control sequence (AAV-AIFs) or the calpastatin cDNA (AAV-Cps) have been described elsewhere (Cao et al., 2007). The neuronal cultures were infected with the AAV-AIFt, AAV-AIFs, or AAV-Cps vector or the control vector [AAV-green fluorescent protein (GFP) or empty AAV] at the particle/cell ratio of 1×10^5 /liter for 6 h in serum-free media, and then incubated in vector-free normal media for 72 h. The overexpression of calpastatin in neurons was confirmed by Western blot using the anti-hemagglutinin (HA) antibody. The AIF knockdown effect by AAV-AIFt in neurons was examined using Western blot with the anti-AIF antibody.

Primary neuronal culture and NMDA neurotoxicity

Primary cultures of cortical neurons were prepared from 17 d Sprague Dawley rat embryos. Experiments were conducted at 12-14 d *in vitro* (DIV), when cultures consisted primarily of neurons (97%) as determined using cell phenotype-specific immunocytochemistry (Cao et al., 2001). To induce NMDA neurotoxicity, cultures were incubated with NMDA at the concentration of 100 μ M for 30 min and then returned to normal culture media.

Fluorescence of Alamar blue (Accumed International, Westlake, OH), an indicator that changes from blue to red and fluoresces when reduced by cellular metabolic activity, was used to measure the viability of the cultured neuron at 24 h after induction of NMDA neurotoxicity. One-half of the culture medium was replaced with MEM-Pak containing 10% Alamar blue (v/v), and cultures were incubated for 1.5 h at 37°C in humidified 95% air and 5% CO₂. Fluorescence was determined in a Millipore (Billerica, MA) CytoFluor 2300 automated plate-reading fluorometer, with excitation at 530 nm and emission at 590 nm.

NMDA-induced cell death was quantified by measuring lactate dehydrogenase (LDH) release from damaged cells into the culture medium (Cao et al., 2007). In brief, 10 μ l aliquots of medium taken from the cell culture wells were added to 200 μ l of LDH reagent (Sigma, St. Louis, MO). Using a spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA), the emission was measured at 340 nm, which is proportional to the amount of LDH in the medium. The data is expressed as the percentage change of LDH release and we used NMDA toxicity alone as maximum toxicity (100%).

Subcellular fractionation and Western blot analysis

Nuclear and mitochondrial protein extracts were prepared from cultured neurons at 0.5, 2, 6, and 24 h after NMDA exposure. The cells were first suspended in a hypotonic buffer containing 50 mM Tris-HCl (pH 8.0), 25 mM MgCl₂, and 0.1 mM phenylmethylsulfonyl fluoride and kept on ice for 15 minutes. The nuclear and mitochondrial fractions of protein were separately isolated by centrifugation as previously described (Cao et al., 2003). The nuclear protein samples were then immunoreacted with the rabbit monoclonal anti-AIF antibody at a dilution of 1:1000, and immunoblotting of Histone-1 served as the protein loading control.

Western blotting was performed using standard methods and the enhanced chemiluminescence detection reagents (GE Healthcare). The following antibodies were used: rabbit monoclonal anti-AIF antibody (clone E20, 1:1000) was purchased from Epitomics (Burlingame, CA); mouse monoclonal anti-cytochrome *c* oxidase IV antibody (1:1000) was from Invitrogen (Carlsbad, CA); rabbit polyclonal antibody histone-1 (1:500) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Calpain activity assay

Calpain activity assay was performed using a fluorescent calpain I substrate as described previously (Cuerrier et al., 2005). In brief, mitochondrial proteins (30 μ g) were incubated with calpain reaction buffer [20 mM HEPES, pH 7.6, 1 mM EDTA, 50 mM NaCl, and 0.1% (v/v) 2-mercaptoethanol] containing 10 μ M calpain I fluorescent substrate HE(EDANS)PLF AERK (DABCYL)-OH (Calbiochem, La Jolla, CA). The reaction was initiated by addition of CaCl₂ (final concentration of 5 μ M) and incubated at 37°C for 30 min. The activity of calpain was measured by detecting the increase in fluorescence using excitation/emission wavelengths of 335/500 nm. Calpain activity was calculated quantitatively based on the standard curve generated using recombinant calpain I (Calbiochem) and expressed as units per milligram protein.

Mitochondrial Ca^{2+} (Ca^{2+}_m)

Neurons on coverslips were incubated at 37°C for 60 min with 9 μM Rhod2-AM (Invitrogen, Carlsbad, CA) which was reduced with a minimum of sodium borohydride and 3 mM sodium succinate in an HCO_3^- -MEM buffer solution as described before (Marks et al., 2005; Kintner et al., 2007). Cells were then loaded with 200 nM MitoTracker green (Invitrogen, Carlsbad, CA) in HEPES-MEM buffer for 30 min at 37°C. The coverslip was placed in a perfusion chamber (Warner Instruments, Hamden, CT) on the stage of a Leica DMIRE2 confocal microscope (Exton, PA). Cells were visualized with a 100X oil-immersion objective. Cells (2-3 in the field) were scanned sequentially for MitoTracker green (ex. 488 nm argon laser line, em 500-545 nm) and Rhod-2 (ex. 543 HeNe laser, em 544-677). The MitoTracker green signal was used to maintain focus before each sequential scan. Sequential scans were analyzed using Leica confocal software. Average grayscale values were collected from regions of interest (ROI) around perinuclear mitochondrial clusters which exhibit colocalization of MitoTracker green and Rhod-2 signals (typically, 5-6 ROIs were selected in each cell). Ca^{2+}_m values were expressed as relative change of Rhod-2 signals from the baseline values and summarized data represents the average of the calculated values from 4-5 randomly selected cells. Rhod-2 AM ($K_d \sim 580$ nM) was taken up into the mitochondria electrophoretically where it was de-esterified and trapped. At the end of each experiment, 1 μM FCCP was applied in order to depolarize mitochondria and the subsequent loss of Rhod-2 fluorescence signal verified its specific localization in mitochondria.

Statistical Analysis

Data are presented as mean $\pm$ SE. Statistical assessment was performed using analysis of variance (ANOVA) with *post hoc* Scheffe's tests. A level of $p < 0.05$ was considered statistically significant.

RESULTS

NMDA toxicity induces AIF truncation and AIF-dependent neuronal death

Previously we have established that calpain mediates AIF truncation as a prerequisite for release from mitochondria (Cao et al., 2007). However, it is known that both full length and truncated AIF can bind to DNA to cause DNA damage (Yu et al., 2002; Cao et al., 2003). Therefore, we wanted to confirm that NMDA-mediated toxicity induced the release of the truncated form of AIF. In rat primary cortical neurons, 30 minutes of NMDA exposure resulted in progressive appearance of truncated AIF in whole-cell extracts beginning at 2 h after exposure (Figure 1A). In parallel with increases in truncated AIF in whole-cell extracts, there was an increased presence of truncated but not full length AIF in the nuclear fraction of NMDA exposed neurons (Figure 1B). These results support previous findings that truncated AIF is released from the mitochondria and translocates to the nucleus following excitotoxic stress.

In order to confirm that AIF translocation is neurotoxic (Wang et al., 2004), we infected neurons using an adeno-associated viral (AAV) vector expressing AIF-targeting shRNA (shRNAt) to inhibit AIF expression. Figure 2A demonstrates a nearly complete loss of AIF expression in shRNAt-infected but not in the control scrambled shRNA (shRNAs)-infected neurons. We employed measures of cell death and cell viability to test if AIF knock down was neuroprotective. Abrogation of AIF expression both significantly reduced LDH release and increased neuronal viability over controls (Figures 2B and C, respectively). Although the protective effect of AIF knock down was not complete, these data confirm that AIF plays a role in NMDA-induced neurotoxicity.

AIF cleavage and translocation is calpain-dependent

We and others have demonstrated that AIF truncation and release is dependent upon calpain activation in isolated mitochondria (Polster et al., 2005), primary neurons and in rat brain (Cao et al., 2007). We next sought to demonstrate involvement of calpain in AIF truncation and translocation to the nucleus following NMDA exposure. Calpastatin, the only known endogenous inhibitor of calpain (Murachi et al., 1980), was transfected into neurons using AAV expressing calpastatin-HA (Figure 3A). Calpastatin overexpression significantly reduced AIF truncation (Figure 3B) and nuclear translocation (Figure 3C) following NMDA exposure. In addition, calpain inhibition also resulted in a significant increase in cell viability. These experiments demonstrate the requirement of calpain activation for AIF truncation, translocation and neuronal death following NMDA exposure.

PARP-1 inhibition abrogates AIF translocation and calpain activity

We next confirmed the dependence of AIF release on PARP-1 activation. Using PAR polymer as a marker of PARP-1 activity, we found that exposure of neurons to NMDA resulted in the progressive accumulation of PAR polymer, an effect that was blocked using the PARP-1 specific inhibitor 3-ABA (Figure 4A). Inhibition of PARP-1 (and PAR polymer formation) also attenuated AIF truncation, nuclear translocation, and neuronal death (Figures 4B, C and E, respectively). Thus, PARP-1 is also critical for AIF release and neuronal death due to NMDA exposure.

Activation of PARP-1 has also been associated with increased calpain activity in nonneuronal cells (Moubarak et al., 2007). We therefore tested the hypothesis that calpain activity is dependent upon PARP-1. Exposure of neurons to NMDA resulted in elevated calpain activity in mitochondria, which was prevented by PARP-1 inhibition (Figure 4D). This indicates that calpain-mediated truncation of AIF is contingent upon PARP-1 activity.

Calpain and PARP-1 inhibition attenuate AIF translocation

To confirm that AIF translocation was dependent upon both calpain and PARP-1, we performed immunocytochemistry to visualize AIF. In control neurons, AIF colocalized with the mitochondrial marker cytochrome *c* (Figure 5Aa-c). At 6h following NMDA exposure, approximately 50% of the neurons had AIF present in the nucleus (Figure A, d-f and m-p). In contrast, treatment with either calpastatin and 3-ABA treatment attenuated AIF translocation to the nucleus (Figure 5A, g-l). Quantification demonstrated a significant reduction in the number of neurons containing nuclear AIF with calpain and PARP-1 inhibition (Figure 5B and C, respectively). These results demonstrate that both PARP-1 and calpain activation are involved in AIF translocation to the nucleus.

3-ABA inhibits NMDA-induced Ca^{2+} accumulation in mitochondria

Lastly, we sought to determine the mechanisms governing PARP-1-dependent calpain activation. Since calcium dysregulation is a common phenomenon underlying neuronal demise, and calpain is a calcium-dependent protease, we hypothesized that PARP-1 activation contributed to mitochondrial calcium dysregulation and ultimately to calpain activation.

NMDA-induced changes in mitochondrial Ca^{2+} were investigated using Rhod-2. Under control conditions, punctate mitochondrial Ca^{2+} dye Rhod-2 staining colocalized with mitochondrial probe MitoTracker green (Figure 6A). Exposure to 100 μM NMDA resulted in a gradual increase in the mitochondrial Ca^{2+} signal that reached a plateau by 25 min and ~2.5 fold of control (Figure 6B). Increased mitochondrial Ca^{2+} levels were sustained at 120 min in the presence of NMDA (Figure 6A and B). Loss of Rhod-2 fluorescence signal from mitochondria

(>90%) was induced by adding the mitochondrial depolarizing agent FCCP (1 μ M) to verify that the increased Ca^{2+} localized specifically to the mitochondria (Figure 6A and B).

To investigate the role PARP-1 plays in mitochondrial dysfunction following NMDA-mediated neurotoxicity, the effects of PARP1 inhibition using 3-ABA on changes in mitochondrial Ca^{2+} was studied. Inhibition of PARP-1 completely abolished the NMDA-induced increases in Rhod-2 fluorescence over the entire 120 min exposure period (Figure 6A-C). In addition, no mitochondrial swelling was observed with the MitoTracker green signal in the presence of 3-ABA and 100 μ M NMDA. Together, these data show that PARP-1 activation mediates NMDA-induced elevation of mitochondrial Ca^{2+} .

DISCUSSION

There are two main contemporary hypotheses regarding the mechanism underlying AIF translocation from the mitochondria to induce caspase-independent neuronal death: PARP-1-induced PAR polymer formation (Yu et al., 2006) and calpain activation (Cao et al., 2007). Here we report the novel finding that PARP-1 induced mitochondrial Ca^{2+} dysregulation mediates calpain activation and subsequent AIF truncation and translocation to the nucleus. This is the first time PARP-1 and calpain activation have been examined simultaneously in the context of neuronal NMDA toxicity. Moreover, it reconciles the two seemingly independent hypotheses.

This study confirmed that ischemia-related injury induces neuronal death via AIF translocation as shown previously (Cregan et al., 2002; Yu et al., 2002; Zhu et al., 2003; Wang et al., 2004; Culmsee et al., 2005). We also demonstrate that both PARP-1 and calpain trigger truncation and nuclear localization of AIF, and that calpain activity is dependent upon PARP-1. This finding is supported by a previous study showing that calpain acts downstream of PARP-1 following MNNG-induced necrosis in mouse embryonic fibroblasts (Moubarak et al., 2007). An important addition to understanding the sequential nature of PARP-1 and calpain activation is that PARP-1 activates calpain through PAR polymer-induced mitochondrial Ca^{2+} dysregulation. How PAR polymer induces alterations in mitochondrial Ca^{2+} levels remains unknown. One potential mechanism is that PAR polymer induces mitochondrial permeability pore formation, resulting in Ca^{2+} overload. This is supported by the finding that the mitochondrial permeability pore inhibitor cyclosporine A blocked calpain mediated calcium-induced AIF release in isolated mitochondria (Polster et al., 2005).

The validity of the hypothesis that PARP-1 induces mitochondrial Ca^{2+} dysregulation to induce calpain activation is dependent on mitochondrial calpain. Indeed, the most calcium-sensitive form of calpain, μ -calpain (or calpain I), possesses a mitochondrial localization sequence and is found in the mitochondrial intermembrane space (IMS) (Garcia et al., 2005; Badugu et al., 2008). We have also found μ -calpain to be localized to the IMS under control conditions. However, calpain was also present in both the outer and inner mitochondrial (IM) membrane fractions following ischemia-related injury (Cao et al., 2007). Notably, AIF is normally located in the IM. Ischemic injury therefore results in the colocalization of calpain and AIF.

Mitochondria start accumulating Ca^{2+} when intracellular Ca^{2+} increases to ~ 400 nM (Szabadkai and Duchon, 2008). Free mitochondrial Ca^{2+} will only increase when the robust Ca^{2+} buffering capacity of mitochondrial matrix becomes nearly saturated. Therefore, the NMDA-induced increase in free mitochondrial Ca^{2+} levels shown in this study indicates intracellular Ca^{2+} levels increased prior to mitochondrial Ca^{2+} changes and were unremitting, although we did not monitor them concurrently. We believe that sustained increases in free mitochondrial Ca^{2+} leads to opening of the permeability transition pore and release of AIF.

Ablation of mitochondrial Ca^{2+} increases by 3-ABA will then reduce AIF release and cell death following excitotoxicity.

Neuronal stress induced by NMDA toxicity produced an early (<5 min) increase in mitochondrial Ca^{2+} that was responsible for mitochondria-induced ROS production and PARP-1 activation (Duan et al., 2007). This study complements our findings, and we propose the following two-phase model as shown in Figure 7. First, excessive NMDA receptor activation leads to massive Ca^{2+} influx. The mitochondria serve as a sink to absorb a portion of this excess Ca^{2+} . However, excessive Ca^{2+} levels overload the oxygen reducing capacity of the mitochondria, and results in release of superoxide radicals into the cytosol. Cytosolic superoxide can then react with the nitric oxide that is concomitantly produced during NMDA receptor activation (Dawson et al., 1991; Dawson et al., 1996) to form peroxynitrite (Stout et al., 1998; Urushitani et al., 1998), a molecule known to produce severe DNA damage (Hara and Snyder, 2007).

Next, DNA damage activates PARP-1 producing PAR-polymer and severe NAD depletion in the second phase of our model (>10 minutes following excitotoxicity). Levels of PAR polymer that exceed the processing capacity of the PAR polymer catabolic enzyme, PAR glycohydrolase (PARG) (Andrabi et al., 2006; Cozzi et al., 2006), result in secondary mitochondrial Ca^{2+} dysregulation. Alternatively, NAD depletion could create a severe energy crisis (Eliasson et al., 1997; Endres et al., 1997), contributing to mitochondrial Ca^{2+} dysregulation. Abnormal Ca^{2+} homeostasis results in mitochondrial calpain activation, which cleaves AIF located in the inner mitochondrial space. Truncated AIF is then released from the mitochondria and translocates to the nucleus. The final stage is AIF-induced DNA processing and eventual neuronal death.

In summary, we provide novel evidence that PARP-1 activation following NMDA exposure induced mitochondrial Ca^{2+} dysregulation, subsequent calpain activation and AIF release from the mitochondria. This study further reconciles two seemingly disparate hypotheses regarding the mechanisms of AIF-induced neuronal death. Furthermore, it provides the groundwork for future endeavors to investigate how PARP-1 contributes to mitochondrial Ca^{2+} dysregulation. Additional characterization of these mechanisms will lead to greater understanding of neuronal death and potentially to viable therapeutics to combat neurodegenerative diseases.

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Fig. 1. Translocation of truncated AIF in neurons after NMDA neurotoxicity

A, Western blots based on total-cell extracts show that AIF was truncated to yield the 57 kDa fragment in neurons at 2, 6, and 24 h after NMDA exposure (100 μ M). Recombinant proteins AIF62 (representing the endogenous AIF) and AIF57 (representing the calpain-truncated AIF) served as size controls. **B**, Western blots based on nuclear protein extracts show that a truncated AIF at the size of 57 kDa was increased in the nucleus at 2, 6, and 24 h after NMDA exposure (100 μ M). The graphs at the bottom of blots illustrate the time-dependent increases in the truncated AIF in both whole-cell extracts (**A**) and nuclear extracts (**B**) after NMDA exposure. Data are based on 3 independent experiments. * $p < 0.05$ versus control neurons without NMDA exposure.

Fig. 2. Neuroprotective effect of AIF knockdown against NMDA toxicity in primary cortical neurons

A, Western blots show that AIF expression was decreased in neurons infected with AAV expressing the AIF-targeting shRNA sequence (shRNAt), but not in neurons expressing the scramble shRNA sequence (shRNAs). **B-C**, Cell death and cell viability were measured based on LDH release (**B**) and Alamar blue fluorescence (**C**), respectively, at 24 h after NMDA exposure (100 μ M). * $p < 0.05$ versus nontransfected neurons. Data are mean $\pm$ SE; $n = 12$ per experimental condition from three independent transfection experiments.

Fig. 3. Calpain inhibition prevents NMDA-induced AIF truncation and nuclear translocation in neurons

A, Neurons were infected for 3 d with empty AAV or AAV carrying the calpastatin (cps) cDNA (AAVcps), and the expression of cps (tagged with HA) was confirmed by Western blot using the anti-HA antibody. **B**, Western blots for AIF using whole-cell extracts from control neurons (Con) or 0.5, 2 and 6 h after NMDA exposure (100 μ M) in neurons infected with empty AAV or AAVcps. **C**, Western blots for AIF using nuclear extracts from control neurons (Con) or 2 and 6 h after NMDA exposure in neurons infected with empty AAV or AAVcps. The graphs at the bottom of blots illustrate the effect of cps transfection on NMDA-induced truncation (B) and nuclear translocation (C) of AIF, respectively. **D**, Cps transfection increases cell viability 24 h after NMDA exposure, based on the Alamar blue assay. All data are mean $\pm$ SE, ** p <0.01; *** p <0.001 versus neurons infected with empty AAV, based on 3 independent experiments.

Fig. 4. PARP-1 inhibition attenuates NMDA-induced AIF truncation and nuclear translocation in neurons

A, Neurons were treated with 3-ABA (1 mM) or vehicle for 30 min and then incubated with NMDA (100 μ M). Western blot was performed at 0.5, 2, 6 and 24 h after NMDA exposure to detect the formation of poly(ADP-ribose)polymers (PARS). The blots are representatives of two independent experiments with similar results. **B**, Western blots for AIF using nuclear extracts from control neurons (C) or 2, 6 and 24 h after NMDA exposure in neurons pre-treated with 3-ABA (1 mM) or vehicle. The graph at the bottom illustrates the effect of 3-ABA on NMDA-induced nuclear translocation of AIF, based on 3 independent experiments. * $p < 0.05$ versus vehicle-treated neurons. **C**, Western blots for AIF using whole-cell extracts from control neurons (C) or 2, 6 and 24 h after NMDA exposure in neurons pre-treated with 3-ABA (1 mM) or vehicle. The blots are representatives of two independent experiments with similar results. **D**, Calpain activity measured in isolated mitochondria (30 μ g protein/reaction) before or 2 and 6 h after NMDA exposure from neurons neurons pre-treated with 3-ABA (1 mM) or vehicle. **E**, PARP-1 inhibition by 3-ABA decreases cell death 24 h after NMDA exposure, as determined by measuring LDH release. All data are mean $\pm$ SE, * $p < 0.05$; ** $p < 0.01$ versus vehicle-treated neurons, from three experiments.

Fig. 5. Attenuation of NMDA-induced AIF nuclear translocation in neurons by inhibiting calpain or PARP-1

A, Immunostaining for AIF (green) and cytochrome *c* (red). In control neurons (a-c), AIF and cytochrome *c* fluorescence exhibit a coincided cytoplasmic pattern (combined image in panel c). At 6 h after NMDA exposure (100 μ M), many neurons show AIF nuclear localization (arrows in panel d and in combined panel f) in which cytochrome *c* fluorescence remains cytosolic or is lost (e). AIF nuclear translocation is less frequently seen in cultures that are either infected with AAVcps (g-i) or pre-treated with 3-ABA (j-l). The higher power images in panels m-p show the triple-label of AIF (green), cytochrome *c* (red) and DAPI (blue) in NMDA-challenged neurons. Note that AIF and DAPI fluorescence is co-localized in NMDA-challenged neurons (arrows). **B-C**, Percentages of neurons showing nuclear translocation of AIF at 2, 6, and 24 h NMDA exposure (100 μ M). The effects of AAVcps transfection or 3-ABA on AIF translocation are illustrated in B and C, respectively. * $p < 0.05$, ** $p < 0.01$ versus control treatment (empty AAV or vehicle), from 4 independent experiments.

Fig. 6. PARP-1 activation contributes to mitochondrial Ca^{2+} dysregulation after NMDA neurotoxicity

Neuronal cultures grown on coverslips were loaded with MitoTracker green and the mitochondrial Ca^{2+} dye Rhod-2. Changes in MitoTracker and Rhod-2 fluorescence were monitored on a confocal microscope. In some studies, cells were incubated with 1.0 mM 3-ABA for 30 min prior to and during the subsequent exposure to NMDA (100 μM). **A**, Representative images showing single neurons loaded with MitoTracker green and Rhod-2. **B**, Time course of changes of Rhod-2 fluorescence signals. **C**, Summary of relative change in Rhod-2 fluorescence in response to 100 μM NMDA with and without 3-ABA treatment. Mean $\pm$ S.E.; $n = 3-4$, $*p < 0.05$.

Fig 7. Model of PARP-1 and calpain-mediated AIF release

Excitotoxic activation of NMDA receptors result in **1**) massive influx of Ca^{2+} and increased NO production. **2**) Uptake of Ca^{2+} by mitochondria leads to increased O_2^- production that **3**) reacts with NO to form ONOO $^-$. **4**) Production of ONOO $^-$ causes DNA damage, **5**) activating PARP-1. **6**) PARP-1 activation, either by increased PAR polymer formation or decreased NAD cause further mitochondrial Ca^{2+} dysregulation, potentially *via* the MPT. **7**) Further Ca^{2+} dysregulation activates mitochondrial calpain, which **8**) truncates AIF. **9**) Truncated AIF translocates to the nucleus where it causes **10**) DNA fragmentation and neuronal death. AIF, apoptosis-inducing factor; MPT, mitochondrial permeability transition pore; NAD, nicotinamide adenine dinucleotide; O_2^- , superoxide radical; PARP-1, poly(ADP-ribose) polymerase-1; ONOO $^-$; peroxynitrite.