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μ-Calpain regulates caspase-dependent and apoptosis inducing factor-mediated caspase-independent apoptotic pathways in cisplatin-induced apoptosis

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Cisplatin, an effective anticancer agent, can induce tumor cell apoptosis via caspase-dependent and-independent pathways. However, the precise mechanism that regulates the pathways remains unclear. In this study, we showed that μ-calpain mediated both caspase-dependent and-independent pathways during cisplatin-induced apoptosis in human lung adenocarcinoma cells. After cisplatin treatment, calpain activation, as measured by a fluorescent substrate, was an early event, taking place well before apoptosis inducing factor (AIF) release and caspase-9/-3 activation. Confocal imaging of cells transfected with AIF-GFP demonstrated that AIF release occurred about 9 hr after cisplatin treatment. The increase of μ-calpain activity proved to be a crucial event in the apoptotic machinery, as demonstrated by the significant protection of cell death in samples suppressed the endogenous μ-calpain expression level, as well as cotreated with the calpain inhibitors, calpeptin and PD150606. Inhibition of μ-calpain not only significantly reduced caspase-9/-3 activities but also completely blocked AIF redistribution. Our study also showed that endogenous mitochondrial μ-calpain could directly induce the truncation and release of AIF, while caspases and cathepsins were not necessary for this process. In conclusion, the study demonstrated that activation of μ-calpain played an essential role in regulating both caspase-dependent and AIF-mediated caspase-independent apoptotic pathways in cisplatin-induced apoptosis.

Key words: cisplatin-induced apoptosis; μ-calpain; caspase-dependent; caspase-independent; AIF

Cisplatin [*cis*-diammine dichloroplatinum; *cis*-Platinum(II)] is an important chemotherapeutic drug for the treatment of various malignancies.[1,2] Cisplatin treatment causes DNA damage resulting in apoptosis and cell death.[1,2] The most widely studied mediator of cisplatin-induced apoptosis in cells is caspase cascade of enzymes. Many studies have reported that caspase-8, −9, −7 and −3 are involved in response to cisplatin treatment in various experimental settings.[1–4] Although caspases are important regulators of apoptosis, there is accumulating evidence indicating the existence of caspase-independent mechanisms in cisplatin-induced cell apoptosis.[5–7] It has been reported that AIF may regulate a complementary, cooperative or redundant pathway, along with caspase cascades in cisplatin-induced apoptosis.[8,9] Despite these discoveries, however, a key question remains to be addressed: How does the caspase-dependent and-independent signaling pathways are regulated in cisplatin-induced apoptosis.

AIF is a 62-kDa mitochondrial redoxactive enzyme capable of oxidizing NAD(P)H *in vitro* and exhibiting proapoptotic properties.[10,11] Many studies demonstrated that AIF is anchored to the outer face of the mitochondrial inner membrane in healthy cells.[11–13] Upon apoptosis induction, processing of 62-kDa AIF to a 57-kDa form occurred caspase-independently in the intermembrane space. Then the processed form is released to the cytosol, and it translocates to the nucleus where it mediates chromatin condensation and large-scale DNA fragmentation.[14,15] This apoptogenic function of AIF is essential in some relevant experimental models of cell death.[16–23] The AIF release from mitochondria is likely to involve 2 steps, namely, detachment from the inner membrane (IM) and translocation into the cytosol after mitochondrial outer membrane permeabilization (MOMP).[11,20] During the whole process, AIF needs to be cleaved into 57 kDa tAIF by calpains or cathepsins.[11,24–32] Other intracellular signaling events also have been mechanistically linked to AIF release. These include poly(ADP–ribose) polymerase-1 (PARP-1) activation,[22,27,33–35] activation of p53 and Bax,[17,36] mitochondrial translocation of Bid.[37–39] Despite these discoveries, however, the precise mechanism responsible for AIF release in cisplatin-induced apoptosis remains elusive. Several key questions remain to be addressed: Which specific factor is responsible for mitochondrial AIF release? Are calpains, caspases and/or cathepsins implicated in such release?

Calpains are a family of calcium-dependent cysteine proteases found in all eukaryotes. There are 2 ubiquitous isoforms, μ-calpain (calpain-I) and m-calpain (calpain-II), that are activated by micromolar and millimolar concentrations of Ca^{2+} *in vitro*, respectively.[40] It has been implicated that calpains perform an important role in various cellular processes in mammals, such as signal transduction, cell proliferation and differentiation, apoptosis and necrosis.[40,41] The potential role of calpains in apoptosis is indicated by a growing list of calpains substrates, including p53, PARP, Bax, Bid, AIF and several cytoskeletal proteins.[3,24,25,27,28,30,31,40–45] Though the contribution of calpains to apoptosis is generally accepted, further studies are still needed to precisely elucidate the role of calpains in apoptosis.

Cisplatin has been introduced into clinical trials for almost 30 years. Studies still continue in an effort to understand exactly how cisplatin works. Understanding the molecular basis of cisplatin-mediated apoptosis could lead to strategies resulting in improved therapeutic benefits. Recently, we attempted to characterize some biochemical mechanisms of the cisplatin-induced apoptosis, and we showed that calpain-mediated pathway dominated cisplatin-induced apoptosis in human lung adenocarcinoma cells.[44] There is accumulating evidence indicating that calpain

Additional Supporting Information may be found in the online version of this article.

Abbreviations: AIF, apoptosis inducing factor; IMS, inter membrane space; IM, inner membrane; MOMP, mitochondrial outer membrane permeabilization; CCK-8, Cell Counting Kit-8; GFP, green fluorescent protein; Calpain, calcium-activated proteinase; Cyt-c, cytochrome c.

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regulates both caspase-dependent and-independent pathways in apoptosis induced by different apoptotic stimuli in various cell system.[40,41,44,46] However, how calpain plays its role in cisplatin-induced apoptotic cell death so far is unclear. The aim of the present study was to further investigate the cisplatin-induced apoptotic machinery. To determine the molecular involvement of calpain, this study focuses on (*i*) the contribution of μ-calpain and m-calpain in the experimental system, (*ii*) the crosstalk between calpains and caspases, 2 cytosolic proteolytic systems, and (*iii*) the role of calpain in regulating both caspase-dependent and-independent apoptotic pathways.

Material and methods

Cell culture, transfection and treatment

The human lung adenocarcinoma cell lines ASTC-a-1 and A549 were obtained from the Department of Medicine, Jinan University (Guangzhou, China) and cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 15% fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 mg/ml) in 5% CO_2 at 37°C in a humidified incubator. The plasmid AIF-GFP was kindly provided by Dr. Douglas R. Green (Department of Immunology, St Jude Children's Research Hospital, Memphis, TN),[47] and the plasmid DsRed-Mit was kindly provided by Dr. Y. Gotoh (University of Tokyo, Yayoi, Tokyo, Japan).[48] Transfections were performed with Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

The concentration of cisplatin (Sigma-Aldrich, St Louis, MO) used in our experiments was 40 μM. In the experiments using calpeptin (10 μM) (Alexis Biochemicals, Coger, Paris, France), PD150606 (20 μM) (Alexis Biochemicals, Coger, Paris, France), Z-LEHD-fmk (25 μM), Z-DEVD-fmk (25 μM) (BioVision, Mountain View, CA) or Z-VAD-fmk (25 μM) (Sigma-Aldrich, St Louis, MO), all the drugs were added to the cells 30 min before cisplatin.

Time-lapse confocal fluorescence microscopy

GFP and DsRed emissions were monitored confocally using a laser scanning microscope (LSM 510/ConfoCor 2) combination system (Zeiss, Jena, Germany). GFP was excited at 488 nm with an argon ion laser and its fluorescence emission was recorded through a 500–530 nm IR band-pass filter. DsRed was excited at 543 nm with a helium–neon laser and its emitted light was recorded through a 560-nm long-pass filter.

For detection of AIF release, the samples were cotransfected with DsRed-Mit and AIF-GFP, and imaged by confocal microscope. The images of AIF-GFP and DsRed-Mit were obtained separately and then merged. The AIF-GFP released from mitochondria was determined based on the overlap of AIF-GFP and DsRed-Mit fluorescence images.

Calpain, caspase-3 and caspase-9 activity assays

The activities of calpain, caspase-9 and caspase-3 were measured using the following fluorogenic enzyme substrates: Ac-LLY-AFC (BioVision, Mountain View, CA), LEHD-AFC and DEVD-AFC (Alexis Biochemicals, Coger, Paris, France), respectively. After the desired duration of different treatments, the cells were harvested at 1,200*g* and lysed with extraction buffer provided by the manufacturer. Cell lysates were centrifuged at 10,000*g* at 4°C for 10 min, and the supernatants were collected. After incubation at 37°C for 1 hr, the samples were read in a fluorometer equipped with a 400-nm excitation filter and a 505-nm emission filter. The enzyme activity was expressed as relative fluorescence units per milligram of protein. The arbitrary values were presented as the mean ± SD of 3 experiments.

Cell viability assays

Cells were cultured in a 96-well microplate at a density of 5×10^3 cells/well. The cells were treated with different agents, and their viability was assessed with CCK-8 (Cell Counting Kit) (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. OD_{450}, the absorbance value at 450 nm, was read with a 96-well plate reader (DG5032, Huadong, Nanjing, China) to determine the viability and proliferation of the cells.

Flow cytometry

We used annexin V-fluorescein isothiocyanate (FITC; 0.1 μg/ml) for the assessment of phosphatidylserine (PS) exposure, propidium iodide (PI; 0.5 μg/ml) for cell viability analysis. Cell death was measured in a FACSCanto™ II cytofluorimeter (Becton Dickinson, Mountain View, CA).

Subcellular fractionation

Nuclear, cytosolic and mitochondria-enriched fractions were prepared using the Subcellular Proteome Extraction Kit (ProteoExtract™, Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions.

Preparation of mitochondria

Adult mouse liver mitochondria were isolated according to previously described method.[25,49] In brief, liver tissues were homogenized in mitochondria isolation buffer (MIB) containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, pH 7.4, 1 mM EGTA and 1 mg/ml bovine serum albumin, and centrifuged at 1,200*g* for 10 min. The mitochondria were collected by centrifugation at 12,000*g* for 10 min, resuspended in 10 ml of MIB containing 0.02% digitonin and centrifuged at 12,000*g* for 10 min. The mitochondrial pellets were then resuspended in MIB and further purified by a sucrose step gradient consisting of 2 ml each of 1.2 and 1.6 M sucrose by centrifugation at 40,000*g* for 1 hr at 4°C. The brownish-colored mitochondria-containing band, which was located at the interface of 1.2 and 1.6 M sucrose, was recovered, washed once with MIB and resuspended in MIB without BSA and EGTA.

To generate mitochondrial subfractions, a protocol was used as described previously.[29] In brief, the obtained mitochondrial fraction was resuspended in 2 volumes of 20 mM potassium phosphate buffer containing 0.2 mg/ml bovine serum albumin at pH 7.4 and allowed to stand at 4°C for 1 hr. The resuspended sample was centrifuged at 3,000*g* for 10 min. The supernatant was centrifuged at 105,000*g* for 30 min, and the pellet was used as the OM fraction and the supernatant was used as the IMS fraction. The remaining pellet was sonicated (15 sec × 4) and centrifuged at 77,000*g* for 60 min, and this pellet was used as the IM fraction and the supernatant was used as the matrix fraction. All of the procedures were carried out at 4°C. After protein concentration determinations, the purity of these fractions was analyzed by immunoblot with antibodies against mitochondrial outer membrane [voltage-dependent anion channel (VDAC)], inner membrane (COX IV) and intermembrane space [adenylate kinase 2 (AK2)] markers. High purities of the mitochondrial compartments were observed (data not shown).

Western blotting analysis

Western blotting was performed as described previously.[44] Briefly, 20–50 μg of proteins were loaded on SDS/PAGE, transferred to nitro-cellulose membranes and blotted with primary antibodies reactive to the detected proteins (anti-AIF, 1:500; anti-μ-calpain, 1:500; anti-m-calpain, 1:500; anti-cyto *c*, 1:500; antiactin, 1:1,000; anti-Cox IV, 1:1,000; antihistone, 1:1,000), followed by secondary antibodies, goat antirabbit conjugated to IRDye™800 (Rockland Immunochemicals, Gilbertsville, PA) or goat anti-mouse conjugated to Alexa Fluor 680 (Invitrogen, Carlsbad, CA). Detection was performed using the LI-COR Odyssey Scanning Infrared Fluorescence Imaging System (LI-COR, Lincoln, NE).

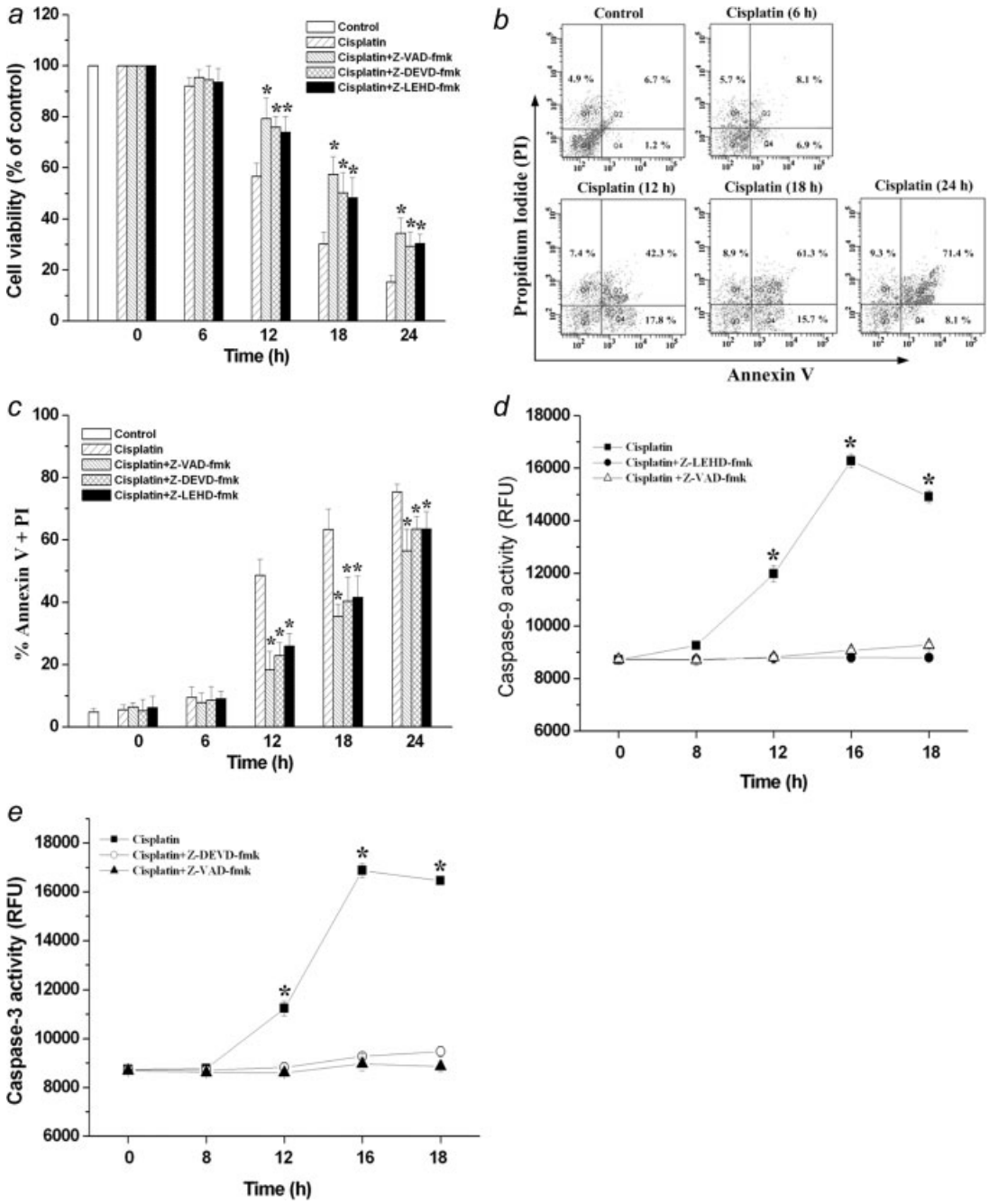

Figure 1 – Cisplatin induces caspases activation. (*a*) Effects of cisplatin on cell viability. Viability of ASTC-a-1 cells, after different treatments as indicated, was assessed by the CCK-8 assays at different time points. Data represent the mean ± SD of 4 independent experiments, $*p < 0.05$ *versus* cisplain-only treatment. (*b*) After the indicated time post-cisplatin treatment, the cells were stained with annexin V-FITC and PI; the percentages refer to the levels of positive staining. (*c*) After different treatments, the percentage of apoptotic cells was assessed by flow cytometry at indicated time points. Data represent the mean ± SD of 4 independent experiments, $*p < 0.05$ *versus* cisplain-only treatment. (*d*) An increase in caspase-9 activities induced by cisplatin. After different treatments as indicated, the activity of caspase-9 was assayed by using its substrate LEHD-AFC (relative fluorescence units/mg of protein and expressed as arbitrary units). Data represent the mean ± SD of 4 independent experiments, $*p < 0.05$ *versus* cisplain + Z-LEHD-fmk treatment. (*e*) After different treatments as indicated, the induction of caspase-3 activity was measured by cleavage of the substrate DEVD-AFC (relative fluorescence units/mg of protein and expressed as arbitrary units). Data represent the mean ± SD of 4 independent experiments, $*p < 0.05$ *versus* cisplain + Z-DEVD-fmk treatment.

RNA interference

The suppression of AIF, μ-calpain and m-calpain was accomplished using siRNA sequences. The sequences of the siRNAs specific for human large subunits of m-calpain and μ-calpain were 5′-CCAGGACUACGAGGCGCUGdTdT-3′ and 5′-GCUAGUGUUCGUGCACUCUdTdT-3′, respectively. The sequence of the siRNA specific for human AIF was 5′-CCGGUCCCAGGCAACUUGdTdT-3′. A scrambled siRNA (5′-CCUAGACCGAACGAACUGGdTdT-3′) was used as a negative control. The siRNA sequences were transfected into cells using Lipofectamine reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. In the experiments using siRNAs, the cells were

treated by specific siRNA for 48 hr firstly, and then the treated cells were used to perform different experiments as desired.

Image processing and statistical analysis

To quantify cells showing cytosolic AIF-GFP, the cells cotransfected with AIF-GFP and DsRed-Mit were treated with different agents. At selected time points, the percentage of cells showing AIF-GFP released from mitochondria was assessed by counting the number of cells exhibiting cytosolic AIF-GFP. Data were collected from $n = 150–200$ cells per treatment in 10–15 randomly selected image frames from different experiments.

Data are represented as the mean ± standard deviation (SD). Statistical analysis was performed with Student's paired *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

Cisplatin induces caspase activation in ASTC-a-1 cells

Treatment of ASTC-a-1 cells with cisplatin (40 μM) for 24 hr significantly decreased cell viability compared to untreated cells (Fig. 1*a*). Correspondingly, the number of apoptotic cells increased with time following treatment with cisplatin compared to control (Fig. 1*b*). Treatment of ASTC-a-1 cells with cisplatin also significantly increased caspase-9 and −3 activities (Figs. 1*d* and 1*e*). Pretreatment with Z-LEHD-fmk (inhibitor of caspase-9) and Z-DEVD-fmk (inhibitor of caspase-3) increased cell viability (Fig. 1*a*) and decreased apoptosis (Fig. 1*c*). These observations implied that caspase-mediated apoptotic pathway was activated following cisplatin treatment in ASTC-a-1 cells.

Because cisplatin treatment increased apoptosis and caspases activities in ASTC-a-1 cells, we explored the effect of Z-VAD-fmk, a pan-caspase inhibitor, on cisplatin-induced effects on cell viability and apoptosis in cells. Compared with cisplatin-only treatment, pretreatment with Z-VAD-fmk increased cell viability (Fig. 1*a*) and decreased apoptosis (Fig. 1*c*). However, even though Z-VAD-fmk obviously reduced cisplatin-induced apoptosis, it failed to completely prevent cell death, suggesting that a caspase-independent mechanism was involved in this experimental model.

Protective effect of AIF knockdown against cisplatin-induced cell death

Previous studies demonstrated that AIF mediated caspase-independent apoptotic pathway. To identify whether AIF played a role in our experimental model, we detected cell viability and cell death after knocking down AIF using RNA interference approach. The data demonstrated that the designed sequence was highly effective in knocking down AIF (see Supporting Information Figs. 1*a* and 1*b*). Silencing AIF obviously increased cell viability (Fig. 2*a*) and decreased apoptosis (Fig. 2*b*). Interestingly, compared to the results by the treatment of Z-VAD-fmk or siRNA-AIF alone, cotreatment with Z-VAD-fmk and siRNA-AIF had significant effects on cell viability and cell death (Figs. 2*a* and 2*b*). These results implied that AIF complemented caspase-mediated apoptosis in cisplatin-treated ASTC-a-1 cells.

Cisplatin treatment induces nuclear translocation of mitochondrial AIF

To observe the dynamic behavior of AIF during cisplatin-induced apoptosis, the fusion protein AIF-GFP was utilized to follow AIF migration and DsRed-Mit was utilized to label the mitochondria.

As shown in typical images, before AIF release, the distribution patterns of both AIF and DsRed-Mit were the same as those of mitochondria, appearing as filamentous structures as shown in the control cell (Fig. 3*a*). However, about 9 hr post-treatment with cisplatin, AIF released from mitochondria and then translocated to nuclei (Figs. 3*b* and 3*c*). These results were also confirmed by western blotting analysis (Figs. 3*d* and 3*e*).

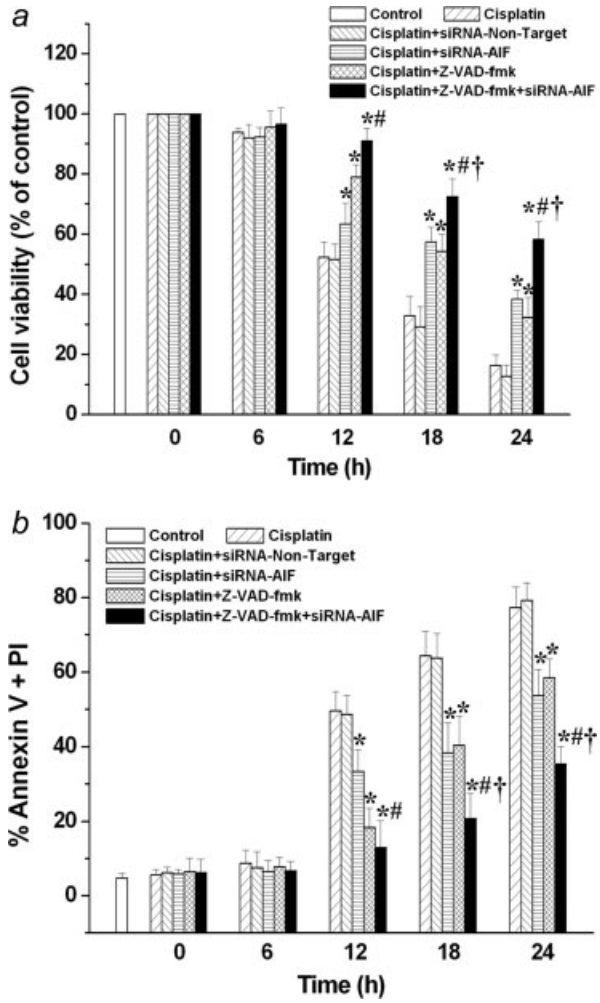

Figure 2 – Protective effect of AIF knockdown against cisplatin-induced cell death. In the experiments using siRNA-AIF, the cells were treated by siRNA-AIF for 48 hr firstly, and then the treated cells were used to perform different experiments as desired. (*a*) The viability of cells after different treatments as indicated was assessed by the CCK-8 assays at different time points. Data represent the mean ± SEM of 4 independent experiments, $^{*}p < 0.05$ *versus* cisplain-only treatment; $^{\#}p < 0.05$ *versus* cisplatin + siRNA-AIF treatment; $^{\dagger}p < 0.05$ *versus* cisplatin + Z-VAD-fmk treatment. (*b*) After different treatments, the percentage of apoptotic cells was assessed by flow cytometry at indicated time points. Data represent the mean ± SD of 4 independent experiments, $^{*}p < 0.05$ *versus* cisplain-only treatment; $^{\#}p < 0.05$ *versus* cisplatin + siRNA-AIF treatment; $^{\dagger}p < 0.05$ *versus* cisplatin + Z-VAD-fmk treatment.

Mitochondrial μ-calpain induces truncation and release of AIF in cisplatin-induced apoptosis

Our previous studies showed that calpain played an important role in cisplatin-induced apoptosis.[44] We further identify the role of μ-calpain and m-calpain using siRNA. Therefore, siRNA sequences specifically targeting μ-calpain and m-calpain were constructed, respectively, together with the scramble-sequence control. As the results shown (see Supporting Information Fig. 2), transfection of siRNA sequences specifically suppressed the expression of m-calpain and μ-calpain, respectively, whereas the negative control had no effect. Then we determined the role of m-calpain and μ-calpain in cisplatin-induced calpain activation. As shown in Figure 4*a*, calpain activation occurred early following cisplatin treatment and was prevented by inhibitors, calpeptin and

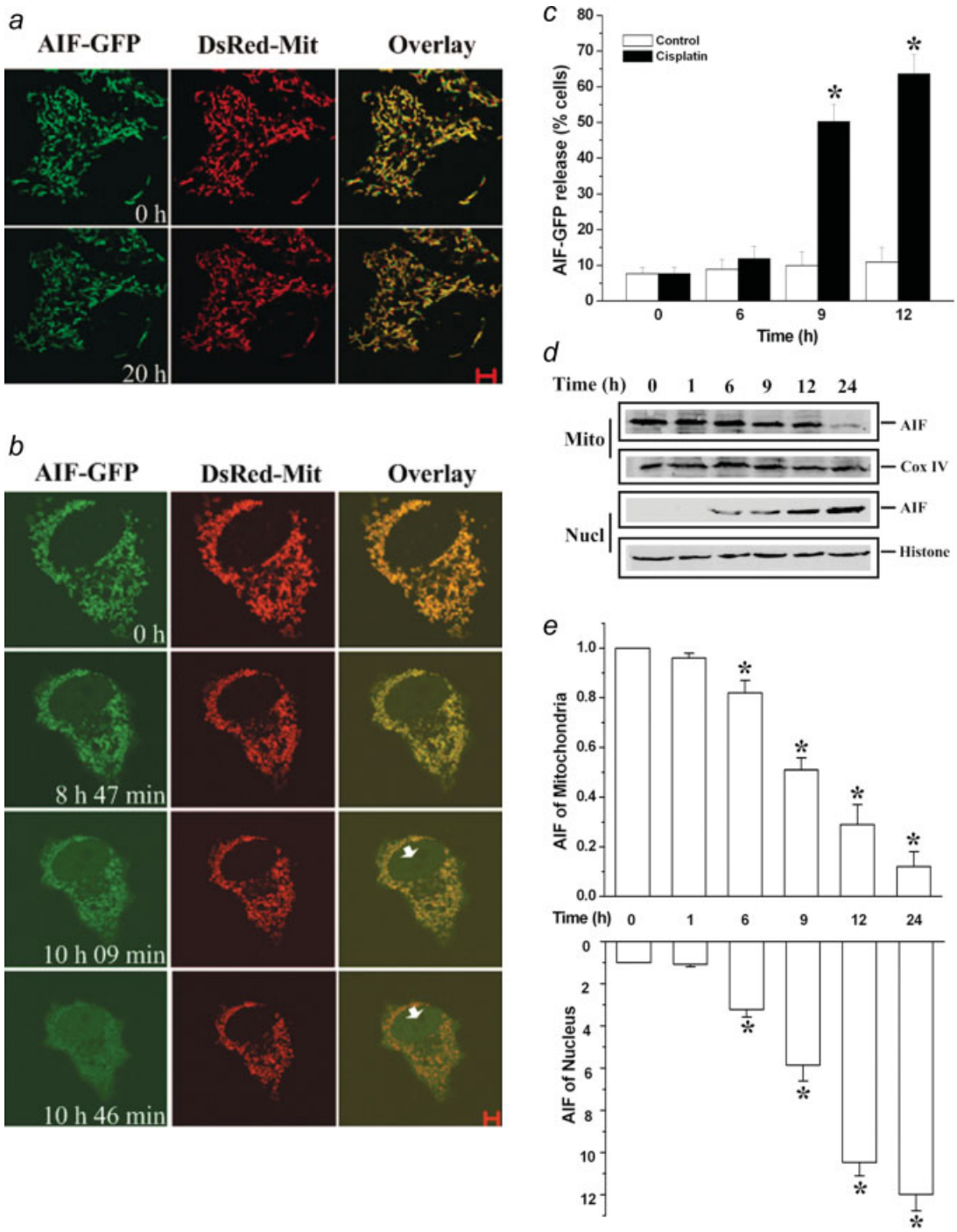

FIGURE 3 – Spatial and temporal changes in AIF subcellular localization during cisplatin-induced apoptosis. (*a*) Control cells without AIF translocation over time. (*b*) Time-lapse images of AIF-GFP redistribution after cisplatin treatment. The arrows in the images show the nuclear translocation of AIF. Similar results were obtained from 3 independent experiments. Bar, 5 μm. (*c*) Quantification of cells showing AIF translocation. At indicated time points, the percentage of cells showing AIF translocation to nucleus was assessed by counting the number of cells exhibiting nuclear AIF. Data represent the mean ± SD of 3 independent experiments, $*p < 0.05$ *versus* control group. (*d*) Time course of AIF relocation from the mitochondrial fraction (Mito) to nuclear fraction (Nucl) in cells treated with cisplatin. CoxIV is a marker for mitochondria, Histone for nuclear proteins. Similar results were obtained from 3 independent experiments. (*e*) Quantitative data of (*d*); densitometric results of 3 separate blots were used for quantitative analysis. For densitometry, values were normalized according to the control sample lanes that were arbitrarily set as 1; data represent the mean ± SD, $*p < 0.05$ *versus* control sample. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

PD150606; suppression of μ-calpain blocked calpain activities, while siRNA targeting m-calpain had no major effect on calpain activation, suggesting that only μ-calpain was activated in our experimental model.

Then we hypothesize that μ-calpain may mediate the truncation and release of AIF from mitochondria following cisplatin treatment. We tested this hypothesis using western blotting technique. Suppression of μ-calpain (Figs. 4*b* and 5), as well as pretreatment

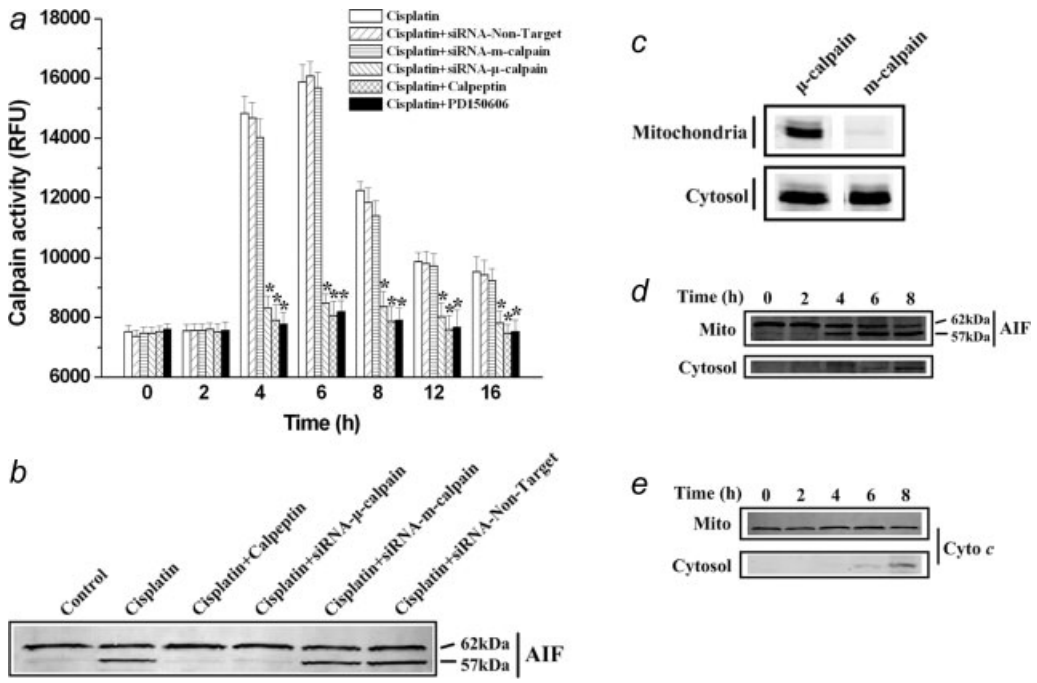

FIGURE 4 – Mitochondrial μ-calpain induces the truncation of AIF in cisplatin-induced apoptosis. (*a*) Knockdown of μ-calpain blocks calpain activities. After different treatments as indicated, calpain activities, as assayed by the use of calpain substrate Ac-LLY-AFC, are indicated as relative fluorescence unit (RFU)/mg of protein and is expressed as arbitrary units. $*p < 0.05$ *versus* cisplain-only treatment. (*b*) Western blotting shows that suppression of μ-calpain blocks AIF truncation. (*c*) Western blotting shows the mitochondrial localization of μ-calpain, but not m-calpain. (*d*) Detection of the truncation and release of AIF in response to cisplatin exposure at different time points as indicated. (*e*) Detection of the release of cytochrome *c* in response to cisplatin exposure at different time points as indicated. From (*a*) to (*e*), similar results were obtained from at least 3 independent experiments. In the experiments using siRNAs, the cells were treated by specific siRNA for 48 hr firstly, and then the treated cells were used to perform the desired experiments.

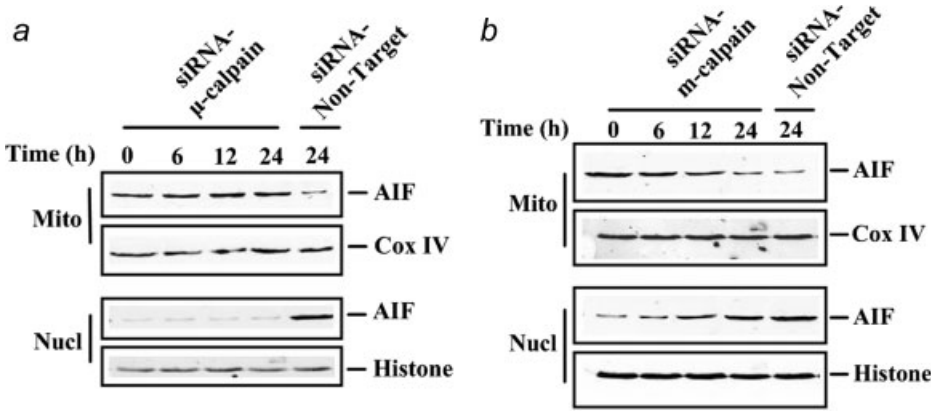

FIGURE 5 – Knockdown of μ-calpain blocks AIF translocation in cisplatin-induced apoptosis. (*a*) Western blotting shows that suppression of μ-calpain blocks AIF relocation from the mitochondrial fraction (Mito) to nuclear fraction (Nucl) in cisplatin-induced apoptosis. (*b*) Suppression of m-calpain cannot block AIF relocation in cisplatin-induced apoptosis. Similar results were obtained from 3 independent experiments.

with calpeptin or PD150606 (Figs. 7*a*, 7*c* and 7*d*), blocked the truncation and translocation of AIF, while downregulation of m-calpain had no such effect. Further study demonstrated that μ-calpain, but not m-calpain, localizes in mitochondria (Fig. 4*c*). Then we hypothesize that mitochondrial μ-calpain could cleave AIF directly. The results demonstrated that the truncation of AIF in cisplatin-treated cells occurred at about 4 hr and increased progressively (Fig. 4*d*), while AIF and cytochrome *c* release (Figs. 4*d* and 4*e*) from mitochondria started at 8 hr post-treatment, indicating that AIF was subject to truncation by μ-calpain before MOMP. These observations implied that original mitochondrial μ-calpain caused truncation and release of AIF in cisplatin-induced apoptosis.

To further confirm that the activated mitochondrial μ-calpain could cause the proteolytic processing of intact AIF, we further investigated the effect of μ-calpain on AIF truncation in isolated mitochondria. In IMS, the amount of 57-kDa truncated AIF (tAIF) increased in time-dependent manner (see Supporting Information Fig. 3*a*), while calpeptin could block the cleavage of AIF and release into IMS. The mixture of inner membrane (IM) and IMS incubated with Ca^{2+} for 90 min (see Supporting Information Fig. 3*b*). Sixty-two-kiloDalton intact AIF in IM and 57-kDa truncated

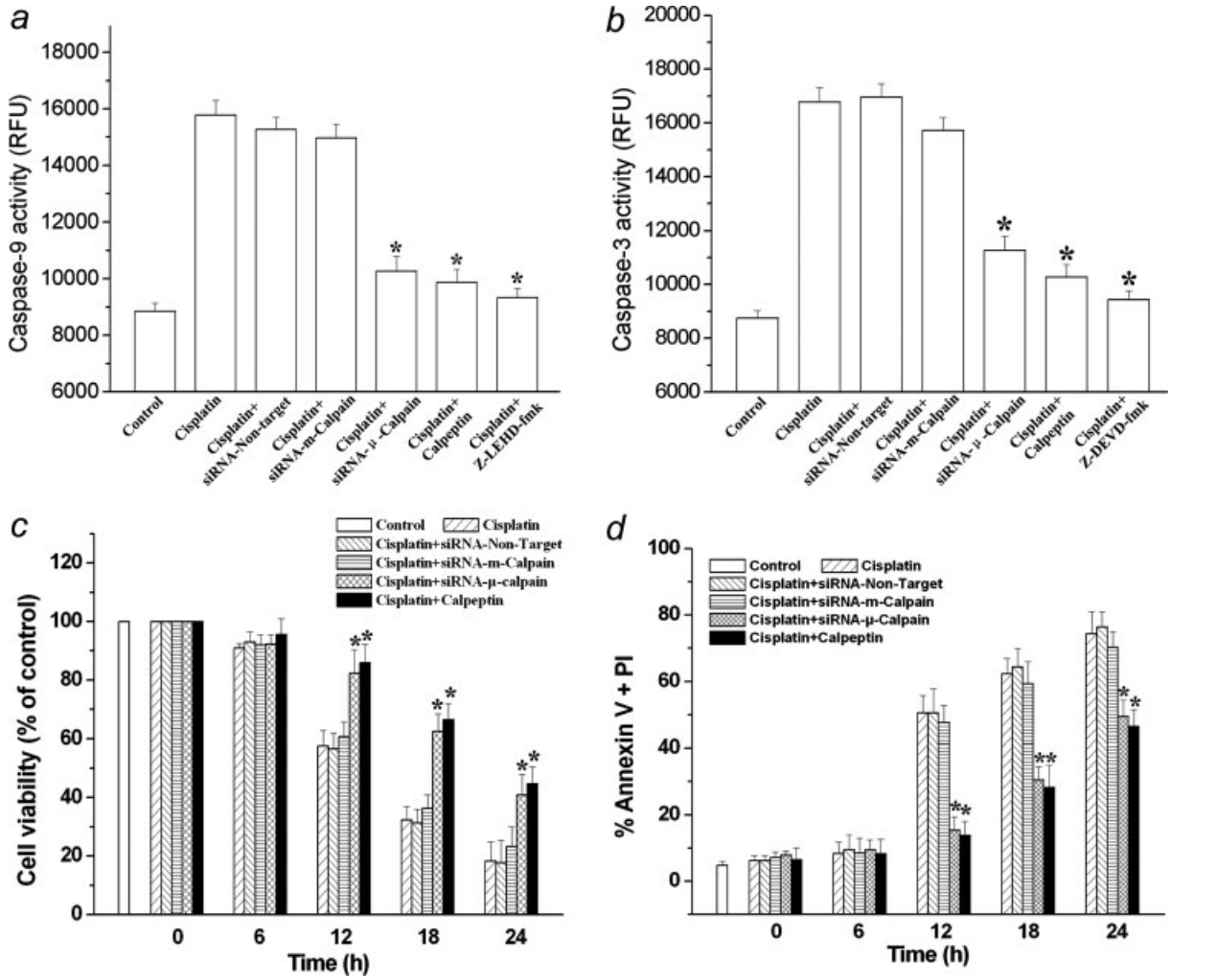

FIGURE 6 – Knockdown of μ-calpain reduces cisplatin-induced caspases activation. (*a*) Downregulation of μ-calpain reduces caspase-9 activities. After different treatments as indicated for 16 hr, caspase-9 activities were assayed (relative fluorescence units/mg of protein and expressed as arbitrary units). Data represent the mean ± SD of 4 independent experiments, $*p < 0.05$ *versus* cisplain-only treatment. (*b*) Downregulation of μ-calpain reduces caspase-3 activities. After different treatments as indicated for 16 hr, the induction of caspase-3 activity was measured (relative fluorescence units/mg of protein and expressed as arbitrary units). Data represent the mean ± SD of 3 independent experiments, $*p < 0.05$ *versus* cisplain-only treatment. (*c*, *d*) Knockdown of μ-calpain reduces cisplatin-induced cell death. (*c*) The viability of cells after different treatments as indicated was assessed by the CCK-8 assays at different time points. Data represent the mean ± SD of 4 independent experiments, $*p < 0.05$ *versus* cisplain-only treatment. (*d*) After different treatments, the percentage of apoptotic cells was assessed by flow cytometry at indicated time points. Data represent the mean ± SD of 4 independent experiments, $*p < 0.05$ *versus* cisplain-only treatment.

form (tAIF) in IMS could be detected clearly. These results implied that activated endogenous mitochondrial calpain in IMS mainly plays a role in direct cleavage of 62-kDa intact AIF to 57-kDa tAIF.

Knockdown of μ-calpain reduces caspases activation in cisplatin-induced apoptosis

To determine whether the alterations of caspases activities are associated with suppression of μ-calpain, we measured caspase-9/-3 activities after downregulation of μ-calpain. Compared with cisplatin treatment alone, suppression of μ-calpain, as well as pretreatment with calpeptin, obviously reduced the activities of caspase-9 and -3 (Figs. 6*a* and 6*b*). Accordingly, the cell viability was increased (Fig. 6*c*) and the induced-apoptosis was decreased (Fig. 6*d*) after knockdown of μ-calpain.

Cathepsins and caspases are not necessary for cisplatin-induced AIF release

A number of cysteine proteases, including caspases, cathepsins and calpains, may be responsible for AIF cleavage. Our study results demonstrated that μ-calpain played an essential role in AIF release; therefore, we tested whether cathepsins and caspases were implicated in cisplatin-induced AIF release. To address this issue, we used a panel of inhibitors for cathepsins and caspases. As shown in Figures 7*b* and 7*e*, inhibition of cathepsins has no effect on AIF release. The pan-caspase inhibitor, Z-VAD-fmk, has no effect on AIF release either (Figs. 7*b* and 7*c*). These results implied that cathepsins and caspases are not necessary for cisplatin-induced AIF release.

Discussion

Cisplatin is one of the most potent anticancer agents displaying significant clinical activity against a variety of solid tumors, par-

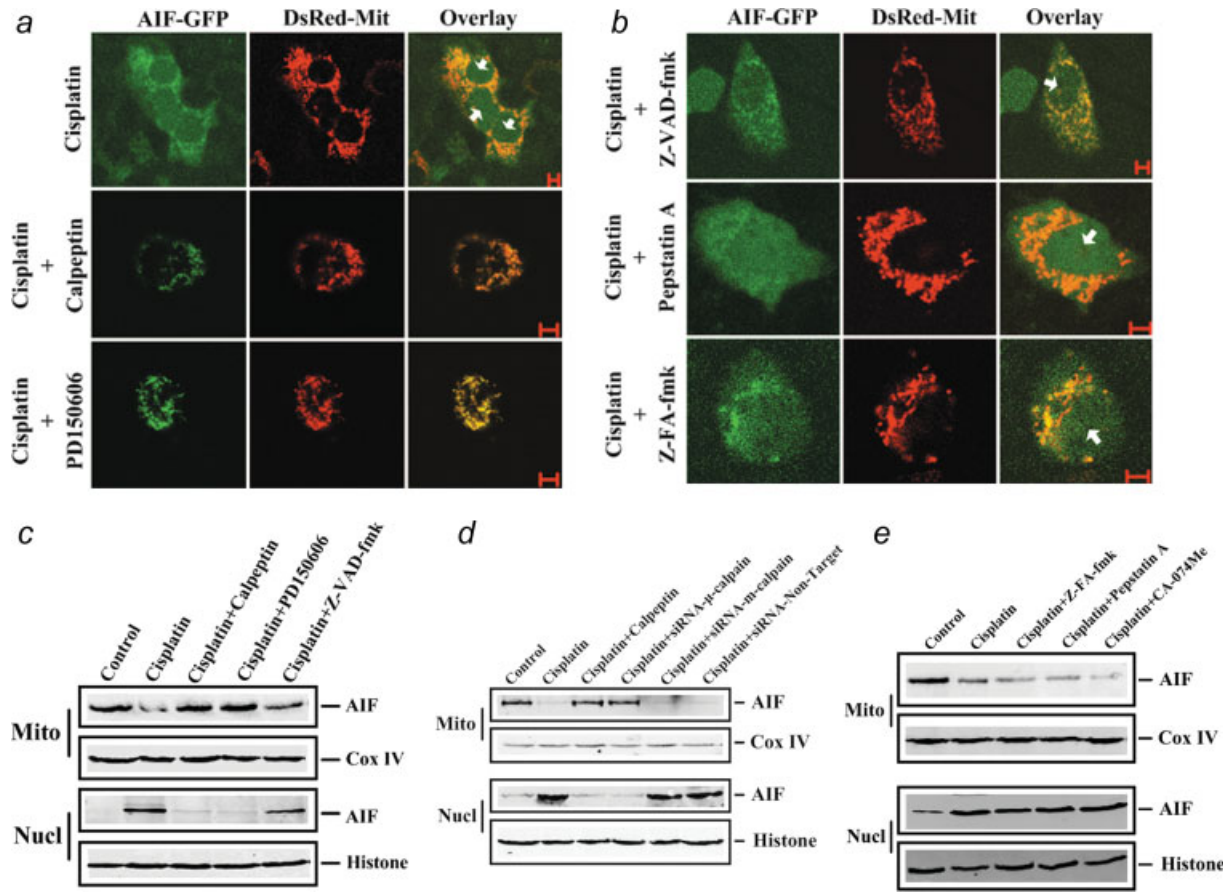

FIGURE 7 – Inhibition of calpain, but not cathepsin, blocks AIF translocation in cisplatin-induced apoptosis. (*a*) Representative confocal images show inhibitory effect of calpain on cisplatin-induced AIF release. (*b*) Representative confocal images show that the inhibitors of cathepsins and caspase family had no effect on AIF release and nuclear translocation in cisplatin-induced apoptosis. From (*a*) to (*b*), the cells were stained with AIF-GFP and DsRed-Mit. The experiments were performed at 12 hr after different treatments as indicated. The arrows in the images show the nuclear translocation of AIF. Similar results were obtained from 3 independent experiments. Bar, 5 μm. (*c*, *d*) Western blotting shows that inhibition of calpain blocks AIF relocation from the mitochondrial fraction (Mito) to nuclear fraction (Nucl) in cisplatin-induced apoptosis in (*c*) ASTC-a-1 cells and (*d*) A549 cells. (*e*) Western blotting shows that inhibition of cathepsins had no effect on AIF relocation from the mitochondrial fraction (Mito) to nuclear fraction (Nucl) in cisplatin-induced apoptosis. For all western blotting experiments, CoxIV is a marker for mitochondria and Histone is for nuclear proteins. Similar results were obtained from 3 independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ticularly for lung cancers. A better understanding of the molecular biological mechanisms of cisplatin could further improve its therapeutic outcome. Our previous studies showed that calpain played an important role in cisplatin-induced apoptosis.[44] It is also important to understand how calpain regulates cisplatin-induced apoptosis. In the present study, we showed for the first time that μ-calpain regulated both the caspase-dependent and AIF-mediated caspase-independent apoptotic pathways in cisplatin-treated human lung adenocarcinoma cells.

The mechanisms involved in cisplatin-induced apoptosis are complex. Previous investigations demonstrated that caspases cascade was activated in response to cisplatin exposure and such activation led to an irreversible commitment to apoptotic cell death.[1–4] In our study, the activities of caspase 9/-3 sharply increased after cisplatin treatment (Fig. 1), suggesting that caspase-mediated apoptotic pathway was activated following cisplatin treatment. We also found that calpain mediated the caspases cascade, because suppression of μ-calpain, as well as pretreatment with calpeptin, obviously reduced the activities of caspase-9 and -3 (Fig. 6). However, the pathways involved in the crosstalk between the calpain and caspase proteolytic system is controversial. Calpain activation may be upstream or downstream of caspases.[46] In our study, we found that calpain activation was an early event following cisplatin treatment, taking place well before caspase-9/-3 activation (Figs. 1*d*, 1*e* and 4*a*); pretreatment with calpeptin and PD150606, the calpain inhibitors, could greatly reduce caspase-9/-3 activities (Fig. 6). These results implied that calpain activation was the upstream of caspases in our experimental model. Some previous studies reported that apoptosis induced by cisplatin also occurred through the Fas/FasL-activated caspase-8/caspase-3 pathway.[4] However, as reported earlier, caspase-8 was not activated, ruling out the apoptotic pathway in our experimental model.[44]

Although caspases are important regulators of apoptosis, many recent reports indicated that caspase-independent mechanism was involved in cisplatin-induced apoptosis.[5–9] We found that apoptosis following cisplatin treatment was significantly reduced by suppressing AIF expression level (Fig. 2), suggesting that AIF-mediated caspase-independent apoptotic pathway was involved. This AIF-mediated apoptosis was associated with nuclear translocation of AIF (Fig. 3).

It is also known that various factors can regulate AIF activation, such as PARP, p53, Bax, Bid, calpains and cathepsins.[11,17,24–38] However, the nature of the involvement of these regulating factors is not clearly established. In this study, we demonstrated that suppression of μ-calpain, either by siRNA or calpeptin, could

completely block the AIF release and nuclear translocation (Figs. 4*b*, 5, 7*a*, 7*c* and 7*d*) in cisplatin-induced apoptosis. We also tested the role of cathepsins and caspases in AIF release; the results implied that both cytosolic proteolytic systems are not necessary for cisplatin-induced AIF release.

A critical issue is how μ-calpain gains access to the inner membrane-bound AIF. The results reported by Polster et al. implied that cytosolic μ-calpain could gain access to AIF in isolated liver mitochondria after MOMP mediated by Bax and Bak.[31] Cao et al. found that μ-calpain normally localized in the IMS in isolated brain mitochondria moved to the inner membrane of mitochondria to cleave AIF during oxygen-glucose deprivation. These previous studies suggested that, in ischemic neurons, μ-calpain (from cytosolic and/or mitochondrial origin) could translocate to the mitochondrial inner membrane, leading to the truncation and release of AIF.[25] Norberg et al. reported that mitochondrial calpain may cleave AIF to release it.[28,29]

In our study, we detected the mitochondrial localization of μ-calpain (Fig. 4*c*) in ASTC-a-1 cells; the truncation of AIF in cisplatin-treated cells occurred before MOMP (Figs. 4*d* and 4*e*); further study revealed that activated endogenous mitochondrial could induce AIF truncation (Supporting Information Fig. 3). These results indicated that μ-calpain of mitochondrial origin initiated the truncation of AIF; the cytosolic μ-calpain may translocate to mitochondria to accelerate the process after MOMP. Therefore, based on the previous studies and our findings, we speculate a working model for proteolytic processing-dependent release of AIF from mitochondria in response to cisplatin exposure: apoptotic information from the cytoplasm is transmitted by proapoptotic Bcl-2 family proteins to alter mitochondrial environments (Step 1), thus triggering mitochondrial μ-calpain activation (Step 2). The IMS portion of AIF is proteolytically cleaved from the inner membrane (Step 3). The mature AIF fragment is released from the mitochondria after MOMP, possibly through a specific channel regulated by proapoptotic Bcl-2 family proteins, such as Bid, Bax and Bak (Step 4).

In conclusion, the present study demonstrated that cisplatin treatment induced not only caspase-dependent but also AIF-mediated caspase-independent apoptotic pathways in human lung adenocarcinoma cells. In response to cisplatin treatment, μ-calpain activation was an early event, and such activation played an important role in regulating both caspase-dependent and AIF-mediated caspase-independent apoptotic pathways.

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