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Mobilization of Ca^{2+} from endoplasmic reticulum to mitochondria plays a positive role in the early stage of UV- or TNFα-induced apoptosis

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ABSTRACT

Previously it was known that cytosolic Ca^{2+} elevation was involved in regulating UV- or TNFα-induced apoptosis. Here, we reported new evidence that mitochondrial Ca^{2+} signal is also involved in the apoptotic process. First, using living cell imaging techniques, we observed multiple mitochondrial Ca^{2+} spikes during the early stage of UV- or TNFα-induced apoptosis. Second, the mitochondrial Ca^{2+} spikes were synchronous with cytosolic Ca^{2+} spikes observed in apoptosis, which preceded cytochrome *c* (cyt-c) release. Third, blocking the mitochondrial Ca^{2+} elevation by applying a mitochondrial uniporter inhibitor could suppress UV-induced apoptosis in HeLa cells. Finally, overexpressing an anti-apoptotic protein, Bcl-2, could suppress the mitochondrial Ca^{2+} elevation. Furthermore, it appeared that the elevation of mitochondrial Ca^{2+} during apoptosis was caused by a direct coupling between endoplasmic reticulum (ER) and mitochondria through IP_3 receptors. Taken together, these findings suggest that Ca^{2+} mobilization from ER to mitochondria can play a significant role in the apoptotic signaling pathway.

Apoptosis is a very important cellular process, in which Ca^{2+} signaling is known to be involved. Most of the studies so far focused on the effects of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) elevation on apoptotic cells [1–9]. For example, it was reported that a sustained increase in $[Ca^{2+}]_c$ concentration was observed at the execution stage of apoptosis, which appeared to be required for endonuclease activation [4]. Later, we showed that $[Ca^{2+}]_c$ elevation also occurred in the commitment stage of programmed cell death and the Ca^{2+} signal was upstream of cyt-*c* release during UV- or TNFα-induced apoptosis [6,7]. Besides Ca^{2+} elevation in cytosol, it was suggested that Ca^{2+} depletion from the ER could also play an important role in the initiation of apoptosis [8,9]. The remaining question is whether mitochondrial Ca^{2+} ($[Ca^{2+}]_{mito}$) uploading could also play an important role on regulating the apoptotic process (see Fig. 1A). It is well known that, in response to a variety of apoptotic stimuli, mitochondria can release many apoptotic factors (including cyt-*c*, Smac) to the cytosol to activate the caspase cascades [10]. It was previously reported that mitochondrial Ca^{2+} homeostasis is a key regulator to modulate the mitochondrial function [11]. For example, Ca^{2+} loading into mitochondria can stimulate more ATP generation from mitochondria [12]. On the other hand, under apoptotic stimuli, Ca^{2+} overloading might cause opening of the permeability transition pore (PTP) or generation of reactive oxygen species (ROS), which could lead to mitochondrial dysfunction [13,14]. At present, however, it is still not clear about the functional role of mitochondrial Ca^{2+} signal in apoptosis. Particularly, there is very little information about the characteristics of mitochondrial Ca^{2+} signals during the apoptotic process.

In this study, we conducted a series of experiments to examine the following issues:

(1) Are there dynamic changes in the $[Ca^{2+}]_{mito}$ pool during apoptosis? If the answer is yes, what are the characteristics of the $[Ca^{2+}]_{mito}$ changes in apoptotic cells?
(2) What is the relationship between the $[Ca^{2+}]_{mito}$ and $[Ca^{2+}]_c$ elevations during apoptosis?
(3) Will the $[Ca^{2+}]_{mito}$ increase have any functional significance in the apoptotic process?

Here, we used a highly sensitive $[Ca^{2+}]_{mito}$ indicator, Rhod-2-AM, and a confocal microscope to monitor the free $[Ca^{2+}]_{mito}$ during UV or TNFα-induced apoptosis in living HeLa cells. At the same time the $[Ca^{2+}]_c$ changes were monitored using another Ca^{2+} indicator, Fluo-4-AM. We also investigated whether the mitochondrial Ca^{2+} uploading during apoptosis was caused by Ca^{2+} release from ER through IP_3Rs. Furthermore, we examined if inhibition of $[Ca^{2+}]_{mito}$ elevation by applying Ruthenium Red (RuRed, a uniporter inhibitor) can suppress the apoptotic process. Finally, we tested if overexpressing the anti-apoptotic protein, Bcl-2, can inhibit the $[Ca^{2+}]_{mito}$ increase in our cell model.

Materials and methods

Cell culture and chemical loading. HeLa cells grown on glass coverslips were cultured in humidified CO_2 (5%) incubator at 37 °C in

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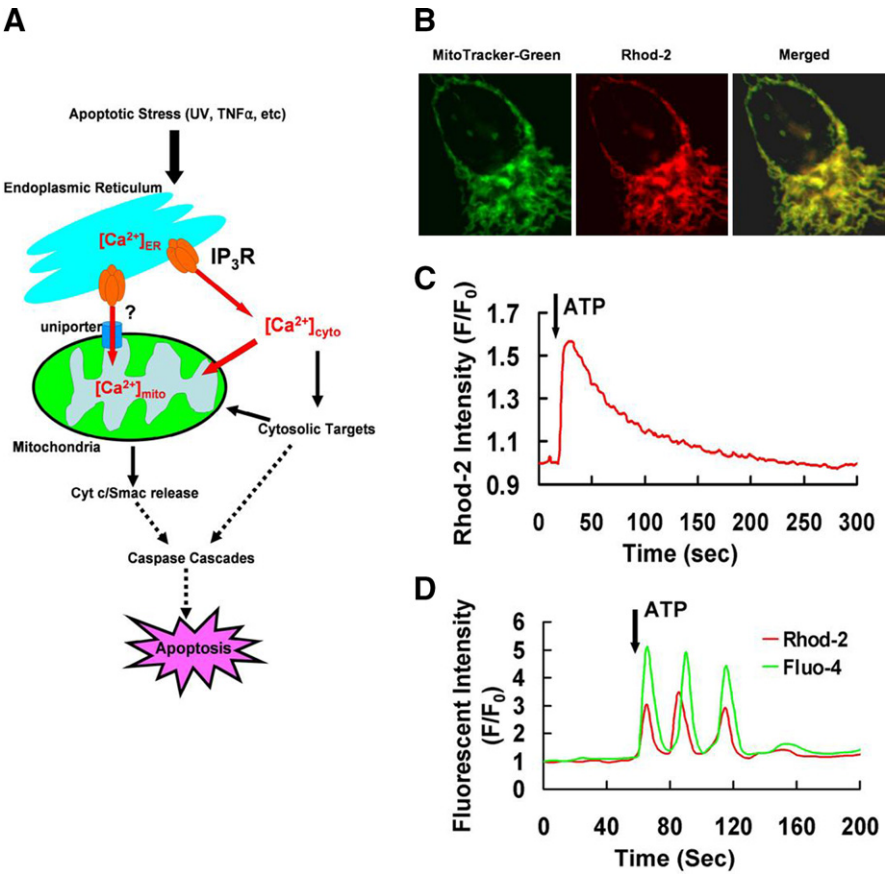

Fig. 1. (A) Schematic diagram showing the possible roles of Ca^{2+} signaling in the apoptotic pathway. (B) Fluorescent image of Mitotracker Green (green) and Rhod-2-AM (red) in a HeLa cell. (C) Increase of $[Ca^{2+}]_{mito}$ triggered 10 μM ATP (average of 20 cells). (D) Simultaneous measurements of $[Ca^{2+}]_c$ (green, using Fluo-4-AM) and $[Ca^{2+}]_{mito}$ (red, using Rhod-2-AM) induced by 1 μM ATP in a single HeLa cell.

MEM, supplemented with 10% fetal bovine serum (FBS). To load Ruthenium Red (RuRed, Sigma), cells were pre-incubated with RuRed for 2 h at 37 °C before imaging experiments. To load Mitotracker Green (Invitrogen), cells were incubated with 1 μM Mitotracker Green for 5 min before imaging experiments.

Gene transfection by electroporation. The electroporation protocol is as following: Cells grown in the mid-log phase were trypsinized and then suspended in the poration medium (PM) in a concentration of 4×10^5 cells per ml [6]. One hundred microliters of the cell suspension was mixed with 1 μg of plasmid DNA and put into the cuvette (Bio-Rad Labs). Two trains of electric pulses were applied to the cell and plasmid DNA mixture suspension with an interval of 10 s. After electroporation, cells were incubated in the recovery medium (PM supplemented with 10% FBS and 2 mM $MgCl_2$) for 20 min, and then returned to CO_2 incubator for culturing. Cells were usually cultured for 24 h for overexpressing the introduced gene. Human Bcl-2 gene is fused in the YFP-C1 or mRFP vector between the EcoRI/KpnI restriction sites.

In situ electroporation. The membrane-impermeant BAPTA potassium salt (Molecular Probes Inc., Eugene, OR) was loaded into HeLa cells grown on the coverslip using a specially designed *in situ* electroporation method [6].

Ca^{2+} imaging in living cells. HeLa cells were seeded on a 25 mm circular glass coverslip and cultured in CO_2 incubator at 37 °C 1 day before the Ca^{2+} measurement. For $[Ca^{2+}]_c$ measurement, 1.5 μM Fluo-4-AM (Molecular Probes) was loaded into HeLa cell in room temperature for 30 min. Before loading into cells, Fluo-4-AM was mixed with Pluronic F127 (Molecular Probes) with 1:1 ratio. For $[Ca^{2+}]_{mito}$ measurement, 5 μM dihydroRhod-2-AM (Molecular Probes) was loaded into HeLa cell in 37 °C for 30 min. DihydroRhod-2-AM was prepared by adding 10% 26 μM sodium borohybride ($NaBH_4$) in methanol solution. Fluorescent images were acquired by Leica DM IRE2 confocal microscope (Leica Microsystems, German). Laser lines of 488 and 543 nm were used to excite Fluo-4-AM and Rhod-2-AM, and the emission wavelength is 495–540 nm, 550–600 nm respectively. Hepes-buffered Hanks balanced salt solution (HHBSS) was used as imaging buffer [15]. To avoid the stored operated Ca^{2+} entry, all the experiments using ATP or thapsigargin to trigger ER Ca^{2+} release were performed in Ca^{2+} free buffer [16]. UV- or TNFα-induced apoptotic cells are incubated in Ca^{2+} containing buffer.

Induction of apoptosis. Apoptosis was induced by exposing cells to UV-irradiation (300 mW/cm^2) for 3 min or incubated with TNFα (10 ng/ml) plus cycloheximide (CHX) (10 μg/ml) for several hours. The number of apoptotic cells was scored by counting the round-up cells. At least 500 cells from five random fields were counted in each control or drug-treated sample. The percentage of apoptotic cells was presented as means ± SD in the following studies and analyzed by *t* test. Each result was from at least four independent experiments.

Results

Mitochondrial Ca^{2+} spikes were observed during UV-induced apoptosis in HeLa cells

In this study, we used single living cell imaging technique to measure $[Ca^{2+}]_{mito}$ in individual apoptotic cells. We used Rhod-2-AM to monitor the $[Ca^{2+}]_{mito}$ change [17]. It is evident from Fig.

1B that Rhod-2 distributed only in mitochondria since it was colocalized with Mitotracker Green. To test the sensitivity of Rhod-2-AM, we applied ATP to trigger Ca^{2+} release from the ER [15]. Upon ATP treatment in HeLa cells, the IP_3 generation can trigger the activation of IP_3R to release Ca^{2+} from ER, causing a $[Ca^{2+}]_c$ elevation. We found that treatment of 10 μM ATP to HeLa cells could clearly trigger an elevation of $[Ca^{2+}]_{mito}$ (Fig. 1C), indicating that part of the Ca^{2+} flux released from the ER can be taken up by mitochondria.

To verify that the $[Ca^{2+}]_{mito}$ elevation is indeed correlated with the release of Ca^{2+} from ER to cytosol, we applied both Rhod-2-AM and a $[Ca^{2+}]_c$ indicator (Fluo-4-AM) to the same group of cells. Because the excitation and emission wavelengths of Fluo-4-AM are different from that of Rhod-2-AM, we were able to measure the $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mito}$ changes simultaneously within an individual cell. Then, we applied ATP to induce Ca^{2+} release from ER. It is known that, when ATP was applied at a lower concentration (1 μM), it could trigger multiple Ca^{2+} spikes [18]. Results of our measurement indicated that, under this condition, the cytosolic and mitochondrial Ca^{2+} spikes were triggered together at the same time (Fig. 1D). In fact, results of our statistical analysis indicated that there was no significant delay between the $[Ca^{2+}]_c$ spikes and $[Ca^{2+}]_{mito}$ spikes, suggesting that the Ca^{2+} mobilization from ER to mitochondria may be through direct coupling rather than through $[Ca^{2+}]_c$ increase (see Supplementary material).

Next, using this Ca^{2+} imaging system, we measured the changes of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mito}$ in HeLa cells undergoing UV-induced apoptosis. A typical time-dependent record is shown in Fig. 2A. Here, a series of transient increases of the intracellular free Ca^{2+} concentration, as indicated by the fluorescence intensity of Fluo-4-AM, can be observed in the first hour following the UV-irradiation. Using the $[Ca^{2+}]_{mito}$ indicator, Rhod-2-AM, we also observed many $[Ca^{2+}]_{mito}$ spikes in the first hour after UV-irradiation (Fig. 2B). These two records, although obtained from different apoptotic cells, showed a certain degree of similarity between their temporal patterns. In order to more accurately compare the characteristics of the cytosolic and mitochondrial Ca^{2+} within an individual cell, we co-stained HeLa cells with both Rhod-2-AM and Fluo-4-AM and measured their fluorescent intensities in the same cell following the UV treatment. Our results indicated that the cytosolic and mitochondrial Ca^{2+} spikes were synchronous within the same cell during UV-induced apoptosis (Fig. 2C).

Inhibition of mitochondrial Ca^{2+} uptake could partially suppress apoptosis

In order to determine whether $[Ca^{2+}]_{mito}$ increase has a functional role in the apoptotic process or not, we investigated if inhibiting $[Ca^{2+}]_{mito}$ elevation will affect UV-induced apoptosis in HeLa cells. RuRed is a potent inhibitor of $[Ca^{2+}]_{mito}$ elevation by blocking the putative uniporter in mitochondria [19]. We pretreated HeLa cells with 1 μM RuRed for 2 h, followed by $[Ca^{2+}]_{mito}$ measurement. We observed that RuRed can effectively prevent $[Ca^{2+}]_{mito}$ elevation under the 10 μM ATP treatment (Fig. 3A). The $[Ca^{2+}]_{mito}$ peak as indicated by Rhod-2-AM was significantly decreased. More importantly, RuRed was found to suppress UV-induced apoptosis in HeLa cells (Fig. 3B). Here, HeLa cells were pretreated with RuRed for 2 h before UV-irradiation. After UV-irradiation, the cells were continuously incubated in RuRed. The apoptotic cells were assayed based on their morphological change. Our results show that the inhibitory effect of RuRed was dosage-dependent (Fig. 3B). We also showed that RuRed could suppress the caspase-3 cleavage during UV-induced apoptosis (Fig. 3C).

Mitochondrial Ca^{2+} elevation was mainly through direct ER-mitochondria coupling

After recognizing the important role of $[Ca^{2+}]_{mito}$ during apoptosis, we wanted to determine whether the $[Ca^{2+}]_{mito}$ elevation is due to direct transport of Ca^{2+} between ER and mitochondria or through $[Ca^{2+}]_c$ elevation. Thus, we injected membrane-impermeable BAPTA into HeLa cells by electroporation to block the $[Ca^{2+}]_c$ elevation during ER Ca^{2+} release. Since the membrane-impermeable BAPTA was only loaded into cytosol but not the internal organelles, it did not affect the ER Ca^{2+} pool [6]. We measured the changes in $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mito}$ stimulated by ATP in the same cell under this BAPTA treatment. Our results showed that application of 1 mM BAPTA could almost completely suppress $[Ca^{2+}]_c$ elevation as indicated by measurement using Fluo-4-AM (Fig. 3D). At

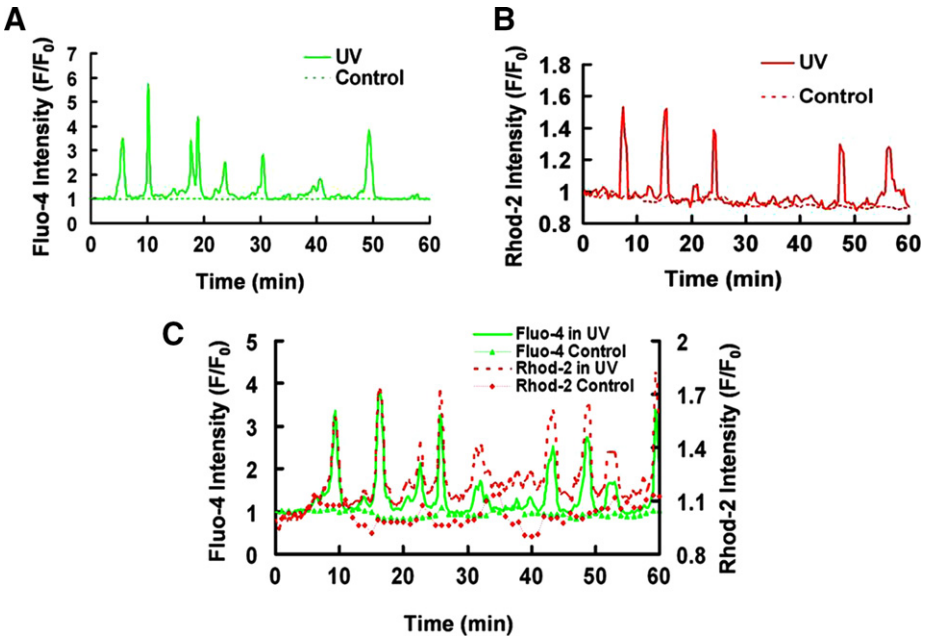

Fig. 2. (A) Cytosolic Ca^{2+} elevation (Fluo-4-AM) with or without UV treatment. (B) Mitochondrial Ca^{2+} elevation (Rhod-2-AM) with or without UV treatment. (C) Simultaneous measurements of $[Ca^{2+}]_c$ (Fluo-4-AM) and $[Ca^{2+}]_{mito}$ (Rhod-2-AM) with or without UV treatment.

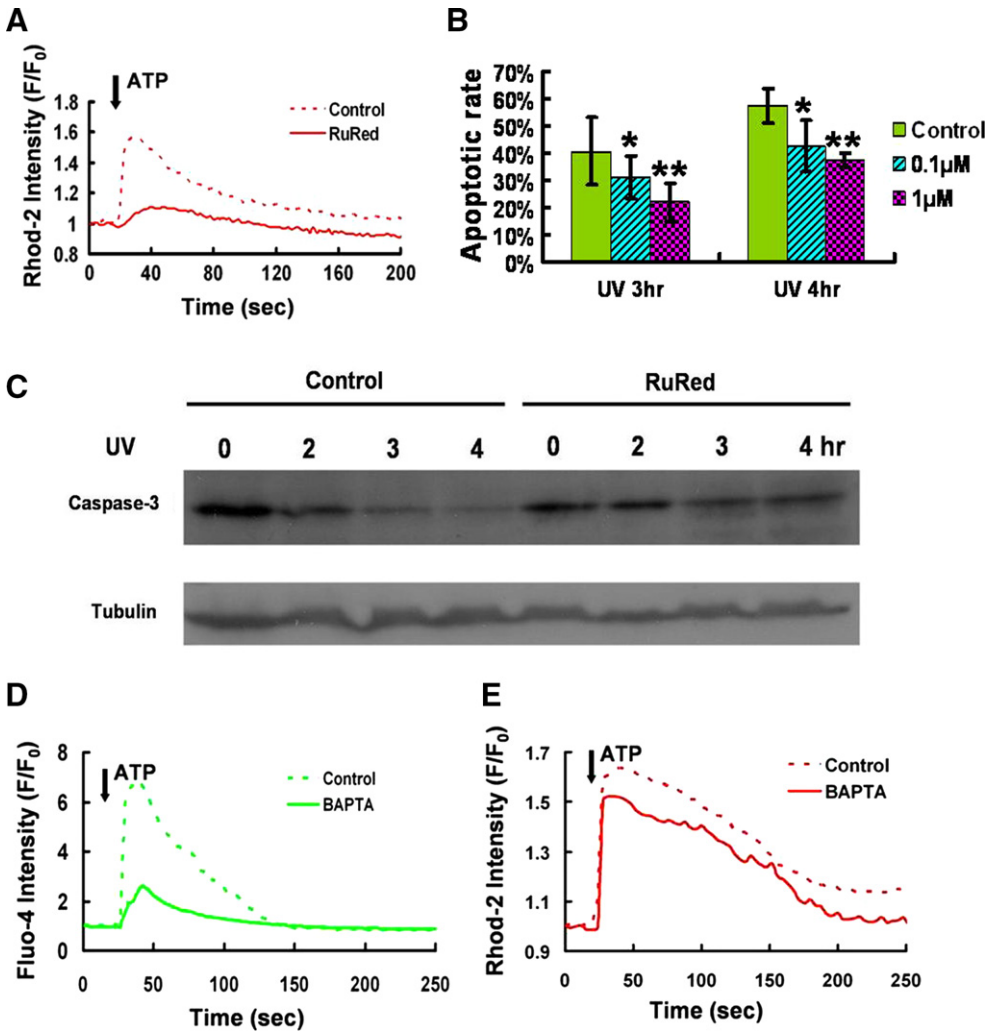

Fig. 3. (A) $[Ca^{2+}]_{mito}$ elevation (using Rhod-2-AM) in HeLa cells triggered by ATP treatment with or without RuRed. Cells were treated with 1 μM RuRed for 2 h before applying 10 μM ATP. Data were averaged from 20 cells. (B) Effects of RuRed on UV-induced apoptosis in HeLa cells. Cells were treated with 0.1 μM or 1 μM RuRed for 2 h before UV-irradiation. Apoptotic cells were counted at 3 and 4 h after UV-irradiation ($^{*}P < 0.2$, $^{**}P < 0.02$, *t* test). (C) Caspase-3 cleavage during UV-induced apoptosis with or without 1 μM RuRed pre-treatment. (D)$[Ca^{2+}]_c$ elevation (Fluo-4-AM) in HeLa cells triggered by 10 μM ATP treatment with (solid line) or without (dash line) preloading of membrane-impermeable BAPTA. (E) $[Ca^{2+}]_{mito}$ elevation (Rhod-2-AM) triggered by 10 μM ATP with (solid line) or without (dash line) preloading of membrane-impermeable BAPTA. Data were averaged from the same 40 HeLa cells as in (D).

the same time, the $[Ca^{2+}]_{mito}$ increase was only slightly suppressed in the same cells (i.e., the Rhod-2 intensity was reduced from 1.62 ± 0.1 to 1.52 ± 0.1) (Fig. 3E). These results suggested that the $[Ca^{2+}]_{mito}$ elevation in HeLa cells is mainly due to direct coupling between ER and mitochondria instead of through the $[Ca^{2+}]_c$ elevation.

The intensity of Ca^{2+} spikes observed under TNFα-induced apoptosis was correlated with the ATP-triggered Ca^{2+} oscillation in the same cell

One interesting observation in the study of Ca^{2+} signaling in apoptosis is that not all apoptotic cells showed Ca^{2+} spikes. In our previous study, we found that only about 50–70% of cells showed clear $[Ca^{2+}]_c$ elevation during UV or TNFα-induced apoptosis [7]. Thus, there was an unanswered question on why some cells were less capable in generating Ca^{2+} spikes than others during the apoptotic process. We speculated that this could be due to the varying degree of efficiency in the IP_3-mediated Ca^{2+} releasing system in different cells. To test this hypothesis, we treated cells with low concentration ATP (1 μM) and recorded the $[Ca^{2+}]_c$ with Fluo-4-AM. We found that some cells showed strong Ca^{2+} oscillation but others showed no response (Fig. 4A and B). After that, we washed out the ATP and waited for the ER Ca^{2+} refilling for 1 h. Then, we applied TNFα to the same cell sample and recorded the $[Ca^{2+}]_c$ changes within the first hour (Fig. 4A and C). Fig. 4A showed a comparison of the typical Ca^{2+} response in the same group of cells under these two drug treatments. The upper panels show the results of a time-dependent Ca^{2+} measurement in four HeLa cells treated by 1 μM ATP. The results of Ca^{2+} measurement in response to TNFα treatment are shown in the lower panels. There was a strong correlation between the ATP-induced Ca^{2+} spikes and TNFα-triggered Ca^{2+} spikes. For example, the upper two cells showed in Fig. 4A gave no Ca^{2+} spikes, while the lower two cells showed strong Ca^{2+} spikes under both conditions. To further verify the correlation between the Ca^{2+} signaling response to the treatments of ATP and TNFα in the same cells, we conduct a statistical analysis on the pattern of Ca^{2+} spikes in 39 individual cells. We counted the number of Ca^{2+} spikes happened within the first 10 min under the 1 μM ATP treatment, and compared it with the number of Ca^{2+} spikes within the first hour under the TNFα treatment. The results are shown Fig. 4D. It was apparent that those cells showing little response to the ATP treatment had very few Ca^{2+} spikes during TNFα-induced

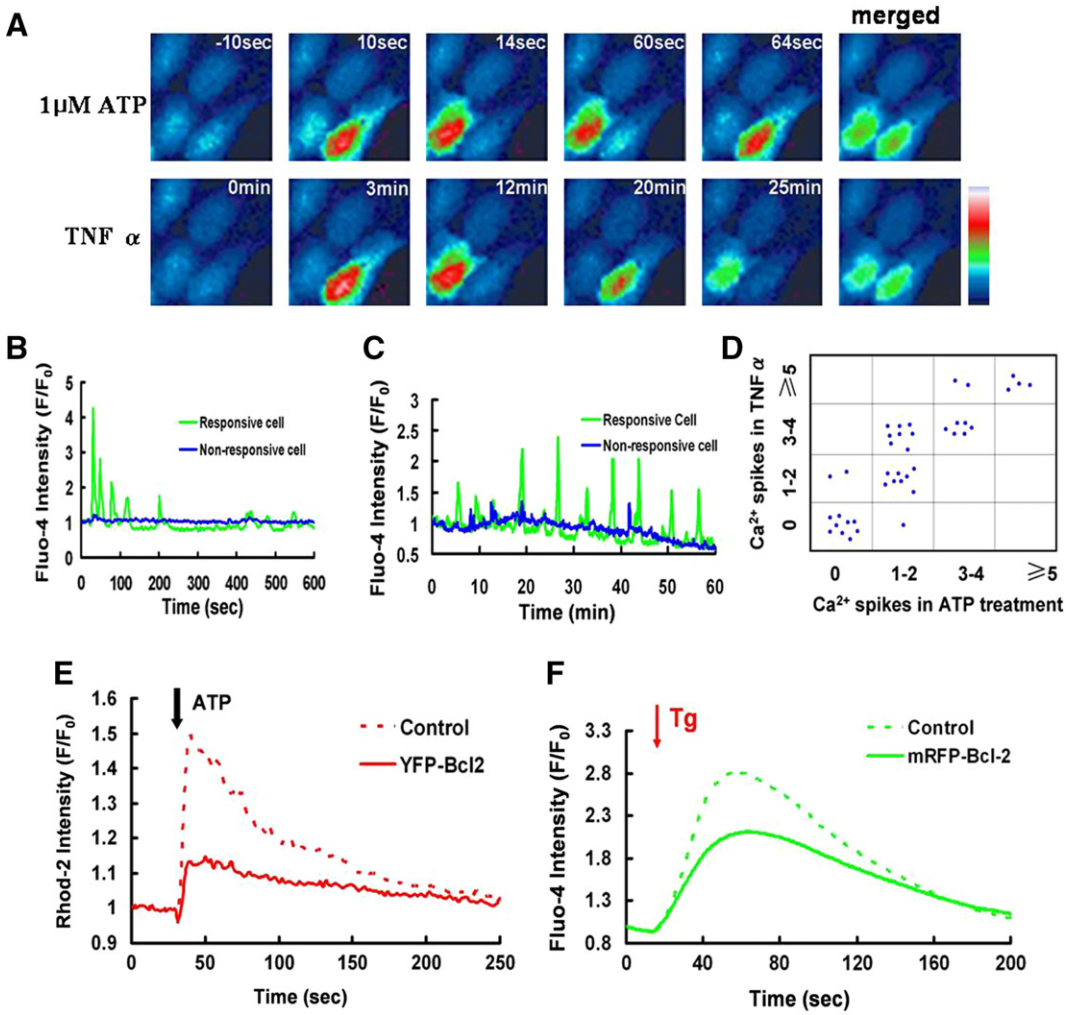

Fig. 4. (A) Comparison of $[Ca^{2+}]_c$ oscillation (using Fluo-4-AM and displayed in pseudo-color) in the same HeLa cells triggered by either treatment of 1 μM ATP (upper panel) or TNFα (lower panel). (B) Sample record the $[Ca^{2+}]_c$ variation in two different HeLa cells under 1 μM ATP treatment. (C) Time dependent variation of $[Ca^{2+}]_c$ in the same two cells shown in (B) under TNFα treatment. (D) Correlation between the number of ATP-triggered Ca^{2+} spikes (*x* axis) and the number of TNFα-induced Ca^{2+} spikes (*y* axis) observed in the same cell. Each dot represents the result from one cell. Data were from four independent experiments. (E) $[Ca^{2+}]_{mito}$ elevation (Rhod-2-AM) triggered by 10 μM ATP treatment with (solid line) or without (dash line) overexpressing the YFP-Bcl-2. (F) $[Ca^{2+}]_c$ elevation (Fluo-4-AM) triggered by 5 μM thapsigargin treatment with (solid line) or without (dash line) overexpressing mRFP-Bcl-2. Results were averaged from 20 cells.

apoptosis, and *vise versa*. The correlation coefficient of these two groups is 0.90 based on Pearson Correlation Coefficient analysis. These results suggest that the reason for some apoptotic cells failing to show Ca^{2+} spikes is because they have very low sensitivity in their IP_3-mediated Ca^{2+} releasing signaling mechanism.

Anti-apoptotic Bcl-2 can inhibit the mitochondrial Ca^{2+} uploading

From the above results, it is evident that Ca^{2+} mobilization from ER to mitochondria through IP_3R appears to play a significant role in regulating UV and TNFα-induced apoptotic progression. To further demonstrate that the Ca^{2+} uploading in mitochondria is relevant to the apoptotic signaling, we investigated whether $[Ca^{2+}]_{mito}$ is affected by the anti-apoptotic protein, Bcl-2. Recently, it was suggested that Bcl-2 can reduce the Ca^{2+} efflux from ER [20]. If this suggestion is true, we would expect that overexpressing Bcl-2 should suppress the $[Ca^{2+}]_{mito}$ elevation during apoptosis. The result of our experiment confirmed that this expectation is correct. When we transfected the YFP-Bcl-2 fusion gene into HeLa cells by electroporation, we found that overexpression of YFP-Bcl-2 could partially inhibit the $[Ca^{2+}]_{mito}$ elevation triggered by an ATP treatment (Fig. 4E). Based on average data from 20 cells, we estimated that overexpression of YFP-Bcl-2 can reduce the $[Ca^{2+}]_{mito}$ peak by about 70% in comparison to non-transfected cells. This reduction is at least partially due to the reduction of ER Ca^{2+} level under the overexpression of Bcl-2. Indeed, when we expressed mRFP-Bcl-2 in HeLa cells, thapsigargin induced ER Ca^{2+} release was observed to reduce by about 40% (Fig. 4F).

Discussion

The involvement of Ca^{2+} in cell death has been clearly documented from many previous studies [1–9]. Up to now, however, it is not clear whether mitochondrial Ca^{2+} signal could also play a significant role in the apoptotic pathway. One early study on this topic was focused on metabolism requirement for apoptotic cells [14]. It was reported that apoptotic stimuli could induce a switch in mitochondrial calcium signaling at the beginning of the apoptotic process by facilitating Ca^{2+}-induced opening of the mitochondrial PTP. After the decay of Ca^{2+} spikes, resealing of PTP occurred to allow mitochondrial metabolism to recover [14]. More

recently, several other studies had suggested that some PTP components such as voltage-dependent anion channel (VDAC) and cyclophilin D could play roles on the tolerance of mitochondrial Ca^{2+} uploading [21,22]. So far, there is still a lack of detailed study focusing on the functional role of mitochondrial Ca^{2+} signal on the apoptotic process.

In this study, we measured the changes of $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mito}$ in HeLa cells undergoing UV- or TNFα-induced apoptosis. We observed many $[Ca^{2+}]_{mito}$ spikes in the first hour (Fig. 2B). Furthermore, our results indicated that the $[Ca^{2+}]_c$ and $[Ca^{2+}]_{mito}$ spikes were synchronous within the same cell during apoptosis (Fig. 2C). This synchrony was due to the fact that both $[Ca^{2+}]_c$ spikes and $[Ca^{2+}]_{mito}$ spikes were caused by the release of Ca^{2+} from ER through the IP_3R. In fact, our data suggest that the $[Ca^{2+}]_{mito}$ spikes were generated by a direct coupling between the ER and mitochondria, since the rise of the $[Ca^{2+}]_{mito}$ spikes in some cases could be slightly ahead of that of $[Ca^{2+}]_c$ spikes. Also, injecting BAPTA into the cytosol could not significantly suppress the $[Ca^{2+}]_{mito}$ spikes during ER Ca^{2+} release.

Results of our studies suggest that mitochondrial Ca^{2+} signal could play a significant role in the apoptotic pathway. First, $[Ca^{2+}]_{mito}$ elevation appears to be an upstream signal; it was observed in the early stage of apoptosis, before the release of cyt-*c*. Second, inhibition of $[Ca^{2+}]_{mito}$ elevation by RuRed can suppress the apoptotic progression in a significant fraction of cells. At this point, it is not yet clear what the downstream target of mitochondrial Ca^{2+} signal is. It had been suggested that mitochondrial calcium increase could facilitate opening of the mitochondrial PTP which, in turn, results in cyt-*c* release from mitochondria. More recent studies, however, indicated that the release of cyt-*c* from mitochondria is mainly through the oligomers of Bax and Bak formed at the mitochondrial outer membrane instead of PTP opening [23]. One of the possible mechanisms is that the Ca^{2+} overloading could trigger the ROS generation under apoptotic stimuli [13]. Thus, inhibition of $[Ca^{2+}]_{mito}$ elevation by applying RuRed, may suppress the ROS production and result in delaying the apoptotic progression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.172.

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