Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2007;20:569-578

Accepted: April 18, 2007

Spatio-Temporal Dynamic Analysis of Bid Activation and Apoptosis Induced by Alkaline Condition in Human Lung Adenocarcinoma Cell

Tongsheng Chen*[,1], Jinjun Wang *[,1], Da Xing[1] and Wei R. Chen [1,2]

[1]MOE Key Libratory of Laser Life Science & Institute of Laser Life Science, South China Normal University; [2]Biomedical Engineering Program, Department of Physics and Engineering, University of Central Oklahoma, Edmond, *These authors contributed equally to this work

Key Words

Alkaline conditions • Apoptosis • Bid/tBid • Caspase-8 • Caspase-3 • FRET • GFPs

Abstract

Activation of initiator and effector caspases and Bid cleavage are apoptotic characteristic features. They are associated with cell alkalization or acidification in some models of apoptosis. The alteration of culture conditions such as extracellular pH value and the overexpression of Bid plasmids may induce cell apoptosis. In present report, we used fluorescence confocal imaging and fluorescence resonance energy transfer (FRET) techniques based on green fluorescent proteins (GFPs) to monitor the spatio-temporal dynamics of Bid translocation and caspase-3 activation in real time in living human lung adenocarcinoma (ASTC-a-1) cells under neutral (pH 7.4) and alkaline (pH 8.0) conditions. The cells transfected with Bid-CFP plasmid did not show apoptotic characteristics for 96 hours under an atmosphere of 95% air, 5% CO_2 at pH 7.4 and 37°C, implying that the overexpression of Bid-CFP plasmid does not induce cell apoptosis. However, all the cells underwent apoptosis after being placed in the alkaline culture (pH 8.0). The dynamic results in single living cell showed that the alkaline condition at pH of 8.0 induced Bid cleavage and tBid translocation to mitochondria at about 1.5 hour, and then induced the caspase-3 activation and cell apoptosis. These results show that the alkaline sondition (pH=8.0) induces cell apoptosis by activating caspase-8, which cleaves Bid to tBid, tBid translocation to mitochondria, and then activating the caspase-3 in the ASTC-a-1 cells.

Introduction

Apoptosis plays an important role in cell development and homeostasis, and caspase family is crucial in apoptosis [1]. Bid, a proapoptotic member of the Bcl-2 family, contains only the BH3 domain. It was first noted for its ability to bind to Bcl-2 and Bax [2]. In proliferating cells, inactive Bid (a protein of 22 kDa; p22) is located in the cytoplasm. Upon being exposed to tumor necrosis factor-α or Fas, caspase-8 in the cell is activated by death-inducing signaling complex, and cleavage of Bid by caspase-8 yields two fragments (p15 (tBid) and p7) [3].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/07/0205-0569$23.50/0

Accessible online at:
www.karger.com/cpb

Prof. Da Xing
Institute of Laser Life Science
South China Normal University; Guangzhou 510631 (China)
Tel. +86-20-85210089, Fax +86-20-85216052
E-Mail xingda@scnu.edu.cn; chentsh@scnu.edu.cn

An exposed glycine residue at the C terminus of Bid (p15) undergoes N-myristoylation [4], and the resulting C-terminal fragment of Bid (p15) is translocated to the mitochondrial outer membrane, where it binds to Bax or Bak [5, 6] and induces the release of cytochrome c from the mitochondria as well as subsequent caspase-3 activation [7]. Caspase-8-mediated Bid processing therefore bridges the extrinsic death receptor-mediated pathway of apoptosis to the intrinsic mitochondrial pathway [3, 8, 9]. This provides a mechanism to amplify the execution signal and exacerbate the pace of cell demise.

The effect of pH on cell fate is dependent on the cell type and stimulation factors. Recent work suggests that changes in pH and mitochondrial function play an important role in regulating several components of apoptotic signal transduction pathways, including caspase activation, poly (ADP-ribose) polymerase (PARP) cleavage and DNA fragmentation in some cell types [10-14]. Previous studies showed that extracellular acidosis exerts a protective effect on the onset of cell death in different cell lines, including neuronal, cardiac, renal, hepatic, and endothelial cells [13, 15]. Collins et al. found that intracellular acidification induces apoptosis by stimulating ICE-like protease activity [11]. Schwerdt et al. found that inhibition of mitochondria and extracellular acidification enhanced ochratoxin A-induced apoptosis in renal collecting duct-derived MDCK-C7 cells [14]. Cutaia et al. investigated the effect of altered extracellular pH on the apoptosis in human pulmonary artery endothelial cells after treatment with staurosporine (STS) [15, 16]. They demonstrated that pH, mitochondrial function, and ATP supply are important variables that regulate STS-induced apoptosis. STS produced a concentration- and time-dependent increase in caspase-3 activity in neutral (pH 7.4) medium that reached a peak at 6 hours. Fluorescent imaging of treated monolayers in pH 7.4 medium with Hoechst-33342-propidium iodide demonstrated a large percentage of apoptotic cells (40%) with no evidence of necrosis. However, caspase activity, DNA fragmentation, and percentage of apoptotic cells were reduced after STS treatment in acidic media (pH 7.0 and 6.6) [16]. Segal et al. reported that although both cations and alkalinity inhibited caspase activation by different mechanisms [17], a pH value above 7.4 severely suppressed the activation of procaspase-3 but not the activity of caspase-3. Segal et al. also demonstrated that cytochrome c-mediated activation of caspase-3 was inhibited by alkaline pH and that the inhibition by high pH could be overcome with an excess of cytochrome c [17]. By comparing the ability of a variety of salts to inhibit this activity, they demonstrated that cations appeared to exert a stronger influence than anions on the activation of caspase-3 [17]. Walters et al. found that alkaline conditions could accelerate polymorphonuclear leukocyte apoptosis [18].

Farber et al. found that a change in intracellular pH occurred upon induction of apoptosis in HeLa cells by staurosporine or TNF [19]. The change in intracellular pH (pH_i) is followed by Bax translocation to the mitochondria, cytochrome c release, and cell death. The chloride channel inhibitor furosemide prevented all these changes. No necessary role for Bid translocation could be established. In fact, the translocation of Bax, the release of cytochrome c, and the death of the cells were observed in the absence of mitochondrial translocation of either full-length Bid or tBid. Alkalization itself induced Bax translocation, as demonstrated by two experiments with D1 cells maintained in IL-7 [20]. First, Bax translocation occurred upon incubation of homogenates in buffers of pH 7.8 or higher [20]. In the second experiment, nigericin was used to equilibrate intact cells with the extracellular pH. Again, Bax translocation was observed only upon incubation in alkaline buffers [20].

Confocal fluorescence imaging and FRET technology have been widely used to study protein-protein interactions in living cells [21-26]. Traditional biophysical and/or biochemical methods can only measure the average behavior of cell populations and the static spatial information from fixed cells and thus cannot provide direct access to the interactions of these protein partners in their natural environment [23]. SCAT3 is a FRET indicator of caspase-3 activation [25], which is composed of enhanced cyan fluorescence protein (ECFP) as the FRET donor and Venus, a variant of enhanced yellow fluorescence protein (EYFP), as the FRET acceptor, linked by peptides containing the caspase-3 cleavage sequence, DEVD [21, 22]. This sequence is found in many cytosolic and nuclear caspase substrates and is cleaved by several effector caspases including caspase-3 and -7 [22, 25]. Caspase-3 is believed to play a central role in the execution of apoptosis, because this enzyme is required for oligonucleosomal DNA fragmentation and promotes the activation of other effector caspases [22, 27].

The present report for the first time used confocal fluorescence imaging and FRET technology based on GFPs to study the mechanism of alkalization-induced cell apoptosis in single living human lung adenocarcinoma (ASTC-a-1) cells. A human lung cancer (ASTC-a-1) cell line stably expressing SCAT3 [28, 29] was used in this

report to study caspase-3 activation and cell apoptosis under alkaline conditions (pH 8.0). The Bid cleavage, means caspase-8 activation, and tBid translocation have been monitored in real-time in the single living cells transfected with Bid-CFP plasmid under alkaline conditions (pH 8.0).

Materials and Methods

Material

Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO (Grand Island, NY). The DMEMs with different pH value were obtained by adding H_2O_2, and the pH value was measured using waterproof pH/Temperature Tester (pHTestr 30, Oakton Instruments, USA). Lipofectamine reagent was purchased from Invitrogen (Carlsbad, CA). DNA Extraction kit was purchased from Qiagen (Valencia, CA). SCAT3 was provided by Professor Masayuki Miura [25], and pBid-CFP was provided by Professor Taira [7].

Cell culture

ASTC-a-1 cells were grown in DMEM supplemented with 15% fetal calf serum (FCS), and the cells were maintained at 37°C in a humidified atmosphere (95% air and 5% CO_2) at pH 7.4. The constant temperature can be controlled by the temperaturecontrol 37-2 (Carl Zeiss MicroImaging, Inc., Germany).

Cell viability assay

Cell viability assays were performed by using Cell Counting Kit-8(CCK-8) (WST-8, Dojindo, Kumamoto, Japan), according to the supplier recommendations. Cells were plated in 96-well plates at $5x10^3$ cells per well and cultured in the medium with different pH value. At the indicated time points, the cell numbers in five wells were measured as the absorbance (450 nm) of reduced WST-8. The WST-8 reagent solution (10 µl) was added to each well of a 96 well microplate containing 100 µl of cells in the culture medium at various densities, and the plate incubated for 2 hours at 37°C. Absorbance was measured at 450 nm using auto microplate reader (infinite M200, Tecan, Austria). Cell viability was expressed as the percentage of viable cells relative to untreated cells using the absorbance at 450 nm. All experiments were performed in five wells on three separate occasions.

Minitype CO_2 culture chamber

In order to keep cells in the normal culture condition during the imaging in the laser confocal scanning fluorescence microscope, a minitype CO_2 culture chamber with a temperaturecontrol 37-2 (Carl Zeiss MicroImaging, Inc., Germany) was used in our experiment. The temperature, the concentration of CO_2 and the humidity in the minitype culture chamber can be controlled very well by this minitype CO_2 culture chamber.

Cell transfection and screening

ASTC-a-1 cells were cultured in DMEM supplemented with 1% serum at a density of $4x10^3$ cells/well in 35-mm glass dish. After 24 hours, when the cells reached 30-50% confluence in DMEM containing 10% FBS at 37°C in 5% CO_2, plasmid DNA of Bid-CFP was transfected into the cells by using Lipofectamine reagent. Serum-free medium of 0.8 ml was added to the tube containing the Lipofectin Reagent-DNA complexes. After 5-24 hours, the medium containing DNA was replaced with 2-ml DMEM medium containing FBS. After 24-48 hours, the cells can be used for experiments.

Plasmid DNA of SCAT3 was transfected into ASTC-a-1 cells by using Lipofectin reagent (Carlsbad, CA). The cells stably expressing SCAT3 reporter were screened with 0.8 mg/ml G418, and positive clones were picked up with micropipettes [28, 29].

Confocal microscopy and image analysis

Fluorescence confocal imaging and FRET were performed on a commercial Laser Scanning Microscopes (LSM510/ConfoCor2) combination system with C-Apochromat 40x NA 1.3 and 100x1.4 NA oil objective(Carl Zeiss MicroImaging, Inc., Germany). The excitation wavelengths were 458 nm for SCAT3 and CFP-Bid, 514 nm for Venus, 633nm for Mitotracker Red The emission fluorescence channels were 470-500 nm bandpass for CFP, 530 nm longpass for Venus, 650 nm longpass for Mitotracker Red. To quantify the results, the images of CFP and Venus emission intensities were processed with Zeiss Rel3.2 image processing software (Carl Zeiss MicroImaging, Inc., Germany).

To monitor the fluorescence of Hochest33342 (EX352/EM461), the mercury lamp was used to excite Hochest33342, reflected by a filter set (BP 365/12, FT 395, LP397). The BP 390-465 nm bandpass filter was used to record the emission fluorescence for Hochest33342 channel.

FRET and acceptor photobleaching

Acceptor photobleaching experiments were carried out to assess the correct expression and the cleavage of the SCAT3 plasmid in single living cell. During the experiments, the acceptor (Venus) in the region of interesting cell was selectively bleached with the highest 514nm laser line. A quantitative analysis of acceptor bleaching showed the absolute fluorescence values for CFP and Venus for a single cell. Upon photobleaching there was a marked decrease of the acceptor fluorescence (Venus), which coincided with an increase of the donor fluorescence (CFP) because of an inability of the acceptor to accept energy from the donor after bleaching. Therefore, the increase of CFP fluorescence upon Venus bleaching confirmed that FRET exists between the two fluorescent proteins in the SCAT3 probe in vivo. The same method was also used to verify the cleavage of SCAT3 by the activated caspase-3 4 hours after alkalization treatment in single living cell.

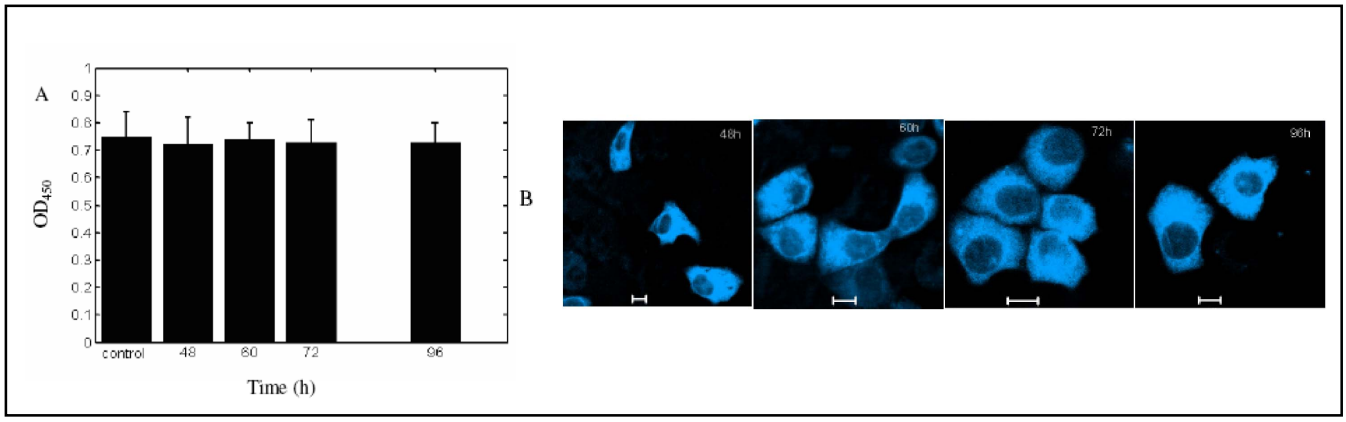

Fig.1. Effect of the overexpression of Bid-CFP on the ASTC-a-1 cells. (A) The effect of the overexpression of Bid-CFP on the survival rate of ASTC-a-1 cells. The cells were cultured at pH 7.4 medium for 48, 60, 72 and 96 hours, and the control was the cells without transfection of Bid-CFP; (B) Confocal fluorescence images of Bid-CFP distribution in ASTC-a-1 cells under neutral culture condition (pH 7.4) for 48, 60, 72 and 96 hours. The cells transfected with Bid-CFP were cultured in the minitype CO_2 chamber on LSM510 microscope, and the temperature in the mintype CO_2 chamber is 37°C. Bid-CFP was distributed evenly in the cytoplasm throughout the experiments. Bar: 10μm.

Statistical Analyses

Experiments are means of five plicates, and each experiment was performed five times. Data are expressed as mean ± SD. Statistical analyses were performed with SPSS12 (SPSS, Chicago) by using the two-sample t-test. Differences were considered statistically significant when $P \leq 0.005$.

Results

Overexpression of Bid-CFP plasmid does not induce Bid activation and apoptosis

The effect of the overexpression of Bid-CFP on the survival rate of ASTC-a-1 cells was investigated by a Cell Counting Kit-8 (CCK-8) assay. The cells transfected with Bid-CFP were cultured at neutral medium (pH 7.4) for 48, 60, 72 and 96 hours, and the control was the cells without transfection of Bid-CFP. The results revealed that the overexpression of Bid-CFP plasmid did not decrease the survival of ASTC-a-1 cells within 96 hour (Fig.1 A).

To monitor whether the overexpression of Bid-CFP plasmid induce Bid cleavage, tBid translocation and cell apoptosis under neutral condition (pH 7.4), ASTC-a-1 cells transfected with Bid-CFP plasmid were cultured in the minitype CO_2 culture chamber on the LSM510 confocal microscope at pH 7.4 for 96 hours. Bid-CFP was evenly distributed in the cytoplasm during the entire time period, and the cells showed no apoptotic characteristics, such as Bid cleavage, tBid translocation, and cells shrinkage in all our independent experiments (Fig. 1 B). Fig. 1 B shows some typical confocal fluorescence images of Bid-CFP distribution in living cells 48, 60, 72 and 96 hours after the cells were maintained under the neutral condition (pH 7.4).

Bid activation and cell apoptosis induced by alkalization

The effect of culture condition with different pH value on the survival rate of ASTC-a-1 cells was investigated by a CCK-8 assay. The cells were cultured in different pH medium for 48 hours. The results revealed that the alkaline condition (pH=8.0) decreased the survival rate of ASTC-a-1 cells (Fig.2 A), and the P is 0.053 for pH7.8, 0.002 for pH 8.0 and pH8.5.

Fig.2 B shows the fluorescence images of cells stained by Hoechst 33242 in pH7.4 and pH8.0 conditions for 8h, respectively. The alkaline condition (pH=8.0) induced the nuclei condensation and fragmentation (fig. 2 B), which are the apoptotic character.

To investigate whether the alkaline culture condition (pH 8.0) induces caspase-8 activation, Bid cleavage, tBid translocation and cell apoptosis, the cells transfected with Bid-CFP plasmid were cultured under alkaline condition (pH 8.0) for 48 hours. Most of the cells incubated for 48 hours under alkaline conditions (pH 8.0) underwent apoptotic changes such as Bid cleavage and tBid translocation (Fig. 2 C). The fluorescence clusters of tBid-CFP distribution are due to the tBid translocation to mitochondria (Fig. 2 D). Not only Bid cleavage and tBid

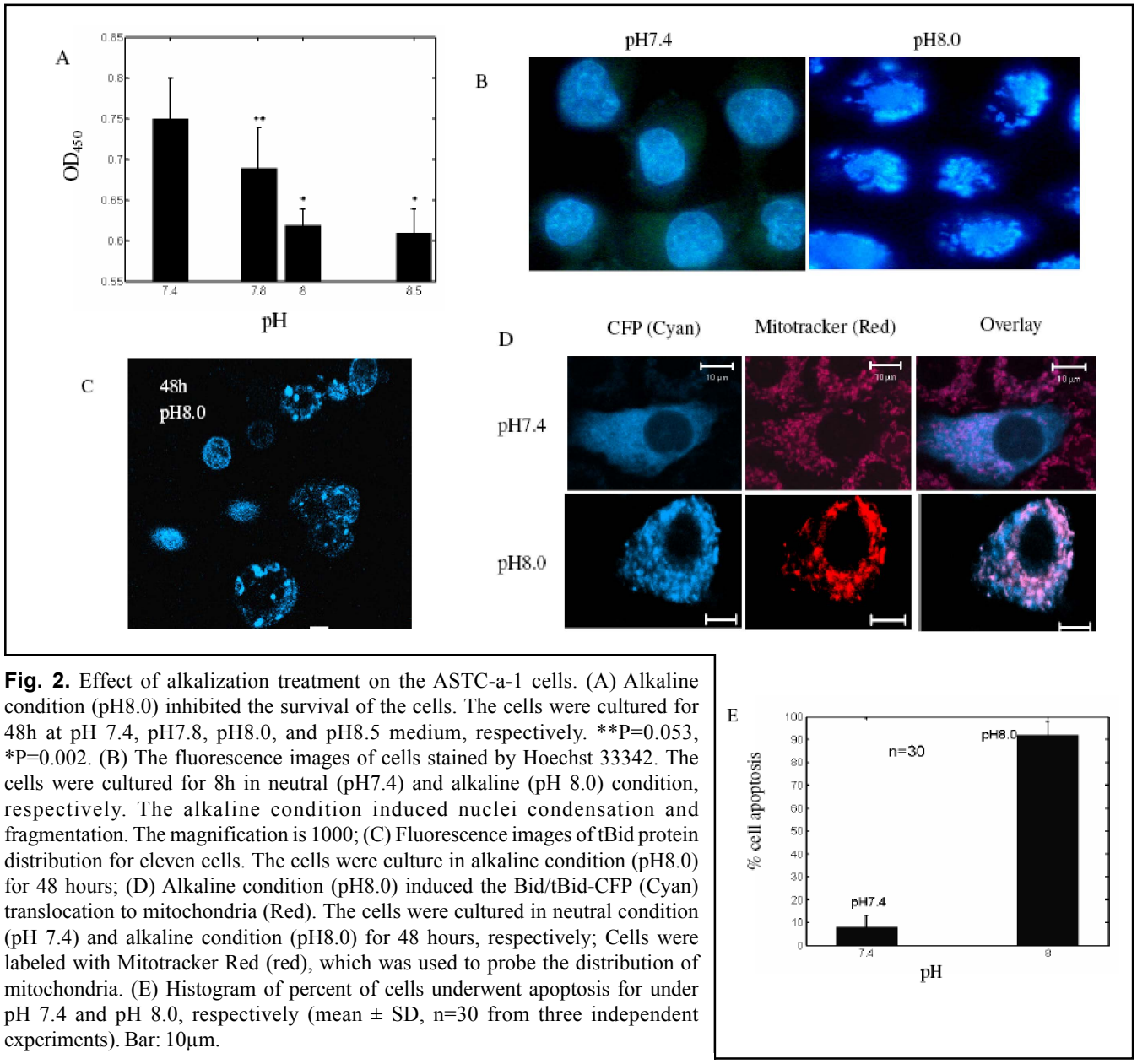

Fig. 2. Effect of alkalization treatment on the ASTC-a-1 cells. (A) Alkaline condition (pH8.0) inhibited the survival of the cells. The cells were cultured for 48h at pH 7.4, pH7.8, pH8.0, and pH8.5 medium, respectively. **P=0.053, *P=0.002. (B) The fluorescence images of cells stained by Hoechst 33342. The cells were cultured for 8h in neutral (pH7.4) and alkaline (pH 8.0) condition, respectively. The alkaline condition induced nuclei condensation and fragmentation. The magnification is 1000; (C) Fluorescence images of tBid protein distribution for eleven cells. The cells were culture in alkaline condition (pH8.0) for 48 hours; (D) Alkaline condition (pH8.0) induced the Bid/tBid-CFP (Cyan) translocation to mitochondria (Red). The cells were cultured in neutral condition (pH 7.4) and alkaline condition (pH8.0) for 48 hours, respectively; Cells were labeled with Mitotracker Red (red), which was used to probe the distribution of mitochondria. (E) Histogram of percent of cells underwent apoptosis for under pH 7.4 and pH 8.0, respectively (mean ± SD, n=30 from three independent experiments). Bar: 10μm.

translocation were formed, but also cell shrinkage occurred in all of the cells under alkaline condition (pH 8.0), implying that the alkaline conditions activated the caspase-8, which in turn cleaved the Bid into tBid, tBid translocation to mitochondria, and then induced cell apoptosis. Fig. 2 E shows the percent of cell apoptosis under pH 7.4 and pH 8.0 conditions (n=30 from three independent experiments). All of the cells underwent apoptosis after 48 hours of alkalization treatment (pH 8.0).

Spatio-temporal dynamics of Bid cleavage and tBid translocation during alkalization-induced apoptosis

In order to monitor the dynamic process of Bid cleavage and tBid translocation when cells were cultured under the alkaline atmosphere (pH 8.0), spatio-temporal fluorescence imaging of Bid/tBid-CFP distribution was recorded by time-lapse confocal microcopy during the alkalization treatment-induced apoptosis (Fig.3 A). For

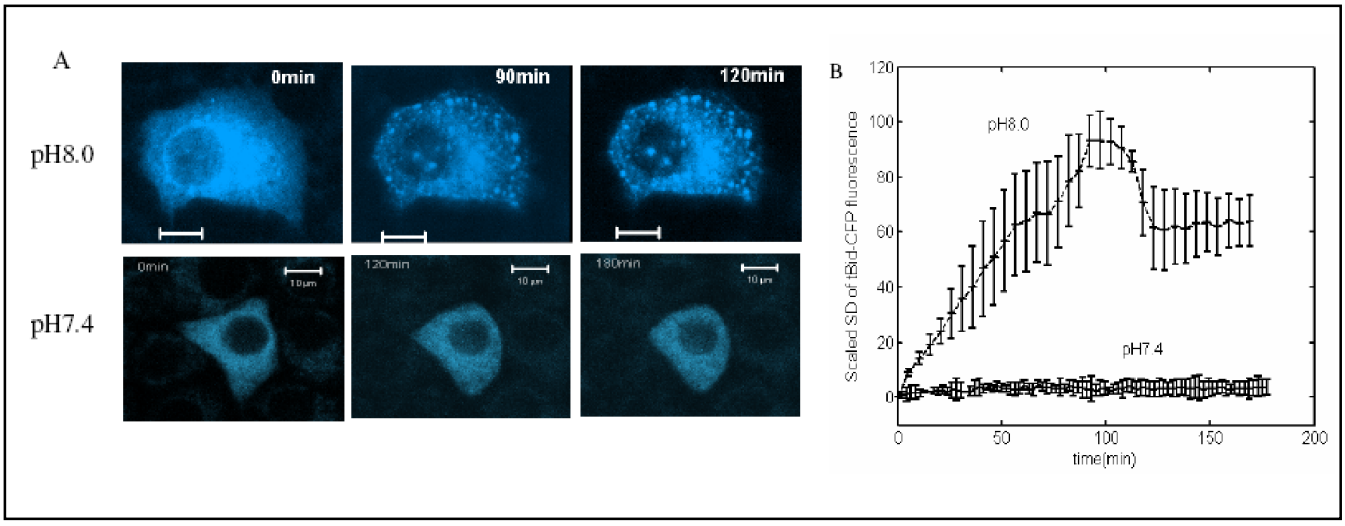

Fig. 3. Spatio-temporal dynamics of the Bid cleavage and tBid translocation by alkalization treatment (pH 8.0). The image series was recorded every 5 minutes for 3 hours. (A) Confocal images of a typical cells transfected with Bid-CFP. The cell shows a redistribution of fluorescence signal according to the Bid cleavage and tBid translocation to mitochondria within 1.5 ± 0.3 hours (mean ± SD, n=15 from four independent expriments). (B) The individual traces of the SD, standard deviation, of the pixel intensities for three cells. The translocation of tBid-CFP was detected as an increase in the SD of the CFP pixel intensity. For direct comparison of tBid translocation, the traces were scaled from 0 (initial SD) to 100 (the maximal SD). Bar: 10μm.

the cells cultured in neutral condition (pH 7.4), the Bid-CFP distributed evenly in the cytoplasm for 3 hours (Low panel in Fig.3 A), implying that the laser scanning did not induce the Bid cleavage and tBid translocation. However, the alkaline condition (pH 8.0) induced the Bid cleavage and the tBid translocation from cytoplasm to mitochondria (the top panel in Fig. 3 A). The tBid-CFP translocation to mitochondria was indicated by an increase in the standard deviation of the average pixel intensity. Confocal fluorescence imaging of tBid-CFP redistribution revealed a comparable type of translocation from cytoplasm to mitochondria for the alkaline condition (Fig. 3 A, B). Fig. 3B shows a quantitative analysis of the Bid cleavage and tBid translocation to mitochondria. The standard deviation (SD) of the pixel intensity in response to tBid-CFP fluorescence increases rapidly to the maximal level after 1.5 ± 0.3 hour (mean ± SD, n=15 from four independent expriments) of alkalization treatment. Subsequent decrease of SD may be due to the evolution of mitochondria during cell apoptosis. This suggests that the tBid-CFP translocates to mitochondria distinctly after 1.5 hour of alkalization treatment. For direct comparison of tBid translocation, the traces were scaled from 0 (the initial SD) to 100 (the maximal SD). These results imply that the alkaline culture condition may activate caspase-8, and the activated caspase-8 cleaved Bid into tBid within 1.5 hour, and then the tBid translocated into mitochondria.

Dynamics of caspase-3 activation inside living cells under alkaline condition (pH 8.0)

Fig.4 A gives the fluorescence images of an ASTC-a-1 cells stably expressing SCAT-3 [28, 29] for the CFP (BP470-500nm) and Venus (LP 530nm) channels in neutral condition (pH 7.4). The SCAT-3 distributed evenly in both nuclei and cytoplasm (Fig.4 A). We used the acceptor (Venus) photobleaching for the circle area (in Fig.4 A) to verify the natural expression of SCAT-3 in single living cell, and found that the fluorescence intensity of CFP channel increased during the Venus photobleaching (Fig.4 B), implying that there were FRET between CFP and Venus probes in the SCAT-3 FRET reporter. The Venus photobleaching in the nuclei gave the similar results (unpublished data).

The real-time FRET imaging of the ASTC-a-1 cells stably expressing SCAT3 [28, 29] were performed in single living cells under both the neutral (pH7.4) and the alkaline (pH 8.0) conditions by time-lapse confocal fluorescence microscope. For the neutral condition (pH7.4), the fluorescence intensities of both the CFP and Venus channels were almost constant for 200min (Right panel in Fig.4 C), implying that the laser scanning did not activate caspase-3 and photobleach obviously the probes. However, for the alkaline condition (pH 8.0), the fluorescence intensity of Venus channel decreased, and that of CFP channel increased gradually for 120min (Left

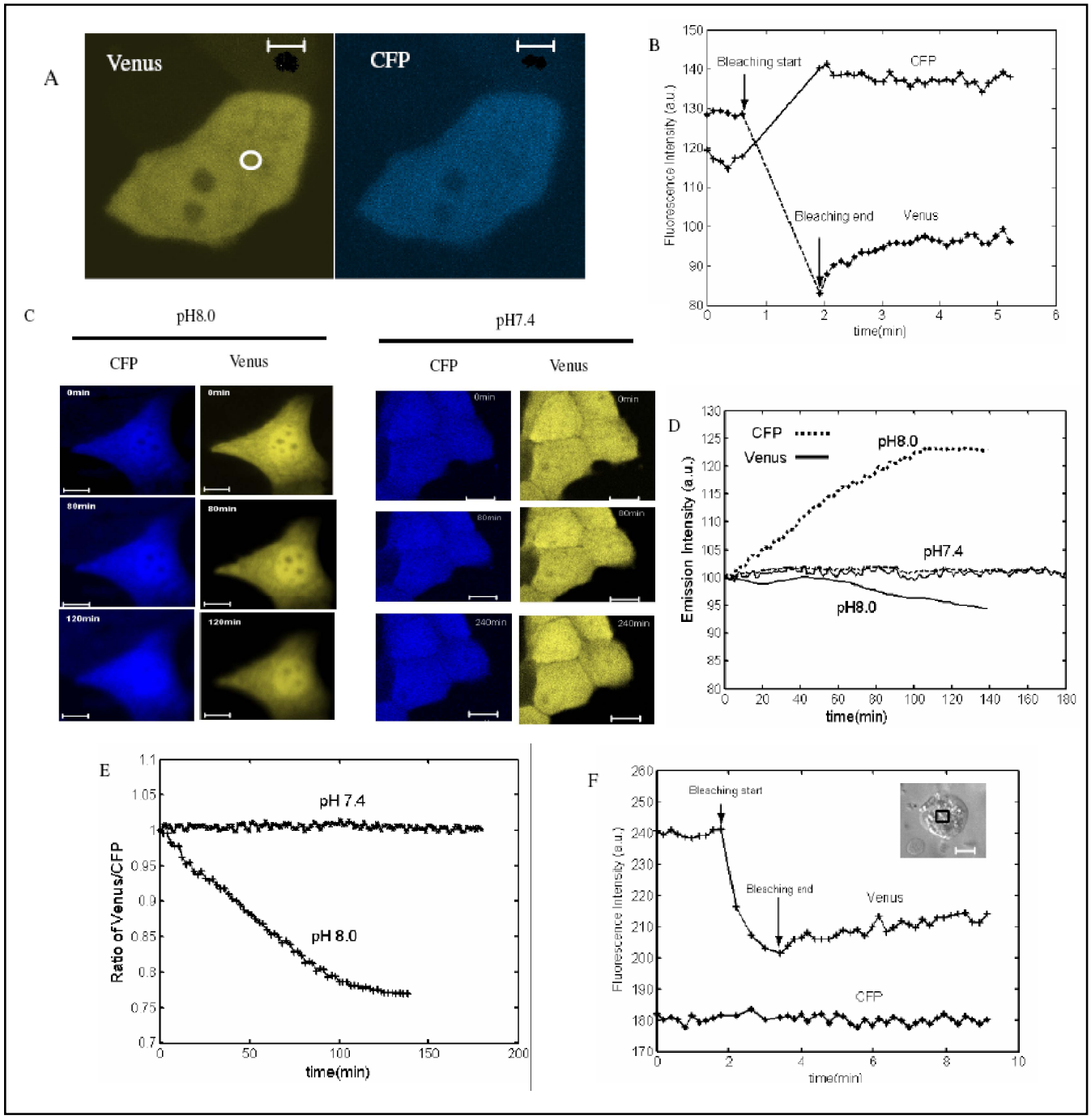

Fig. 4. Dynamics of caspase-3 activation under alkaline condition (pH 8.0). (A) Confocal fluorescence images of CFP and Venus channels under neutral condition (pH7.4). (B) Acceptor (Venus) photobleaching for the cell under neutral condition (pH7.4) corresponding to the circle area in Fig. 4 A. The Venus was photobleached by the highest 514nm laser. The fluorescence intensity of CFP channel increases during Venus photobleaching; (C) Imaging series of CFP and Venus channels under neutral (pH7.4) and alkaline (pH8.0) conditions, respectively. The cells transfected with SCAT3 plasmid were cultured in the medium of pH7.4 and pH 8.0, respectively. The images were recorded every 5 minutes for 2.5 hours; (D) Dynamics of the fluorescence intensities of CFP and Venus emission channels corresponding to Fig.4C. For direct comparison of changes of fluorescence, the initial intensities were modified to 100; (E) Dynamics of the ratio of Venus/CFP channel corresponding to Fig.4 D; (F) Acceptor (Venus) photobleaching for the apoptotic cell induced by alkaline condition (pH8.0). The cells were cultured for 4 hous under alkaline condition (pH8.0). The Venus was photobleached by the highest 514nm laser, and the fluorescence intensity of CFP channel was constant during the Venus photobleaching. Bar: 10μm.

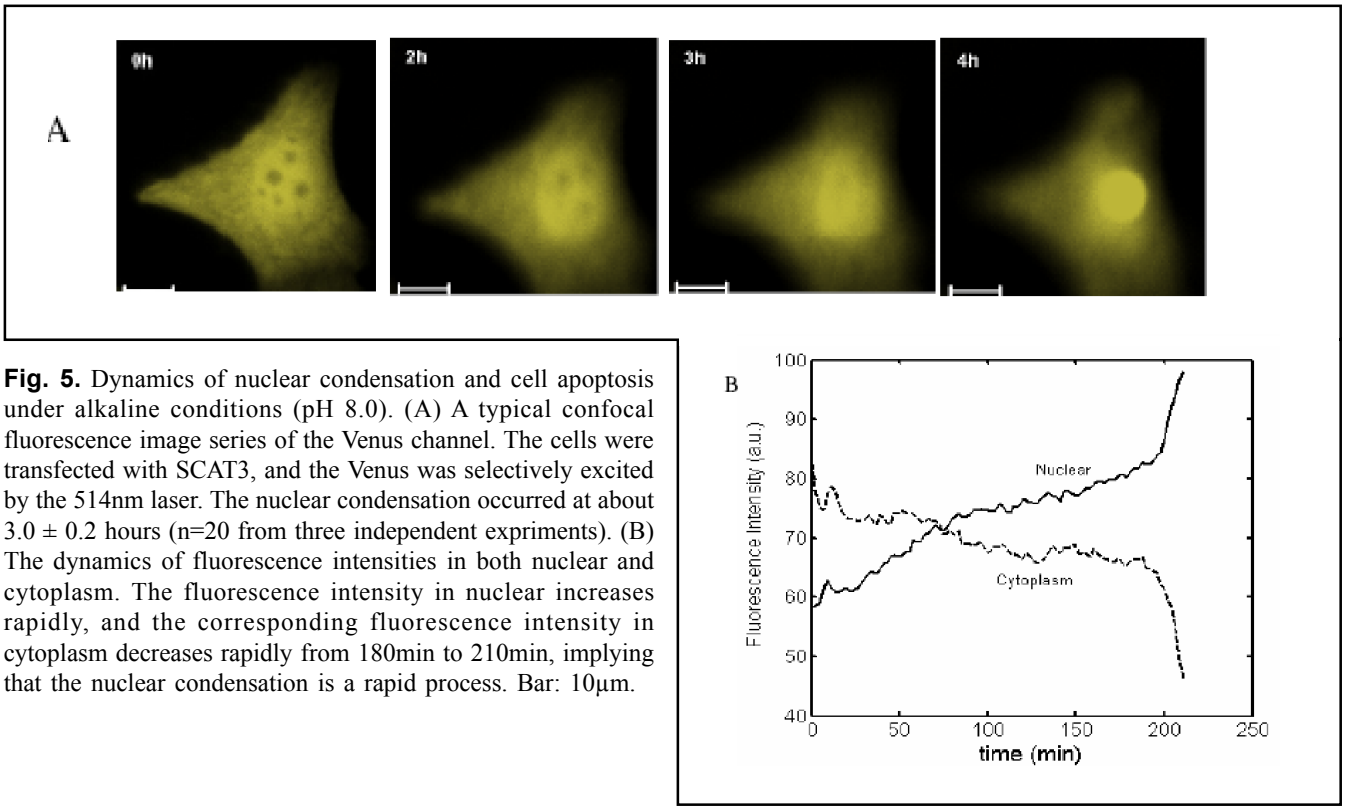

Fig. 5. Dynamics of nuclear condensation and cell apoptosis under alkaline conditions (pH 8.0). (A) A typical confocal fluorescence image series of the Venus channel. The cells were transfected with SCAT3, and the Venus was selectively excited by the 514nm laser. The nuclear condensation occurred at about 3.0 ± 0.2 hours (n=20 from three independent expriments). (B) The dynamics of fluorescence intensities in both nuclear and cytoplasm. The fluorescence intensity in nuclear increases rapidly, and the corresponding fluorescence intensity in cytoplasm decreases rapidly from 180min to 210min, implying that the nuclear condensation is a rapid process. Bar: 10μm.

panel in Fig. 4 C), implying that the alkaline condition (pH8.0) activated the caspase-3, which cleaved the DEVD sequence between the CFP and Venus, thus the FRET of SCAT3 disappeared. Fig. 4D shows the dynamics of CFP and Venus channels corresponding to the Fig.4C, and Fig. 4E shows the corresponding dynamics of the ratio of Venus/CFP. For direct comparisons, the initial intensities of all channels were modified to 100.

After 4 hours of alkalization treatment, we also used the acceptor (Venus) photobleaching to verify the cleavage of SCAT-3 by the activated caspase-3 (Fig.4 F). The fluorescence intensity of CFP channel was constant during the Venus photobleaching (Fig.4 F), implying that the SCAT-3 had been cleaved by the activated caspase-3 in alkaline condition (pH8.0). The insert image in Fig.4 F is the phase contrast image of an ASTC-a-1 cell stably expressing SCAT3 after 4 hours of alkalization treatment.

These above results revealed that the alkaline condition (pH8.0) activated the caspase-3.

We also found that most of the cells under alkaline culture condition (pH 8.0) underwent shrinkage and nuclear condensation at about 3 hour (Fig. 5). Fig. 5A shows a typical fluorescence image series of cell shrinkage and nuclear condensation in a single cell, Fig. 5B gives the corresponding dynamics of fluorescence intensity in both nuclear and cytoplasm. Nuclear condensation is a rapid process. The cell death occurred about 4.0 ± 0.5 hours (n=20 from three independent experiments) after alkalization treatment, implying that the alkaline conditions (pH 8.0) can induce cell shrinkage, nuclear condensation, and cell apoptosis within 4 hours.

Discussion

In this report we used the fluorescence imaging and FRET techniques to study the molecular mechanism of alkalization-induced cell apoptosis in single ASTC-a-1 cells. The cells were transfected with Bid-CFP plasmid and SCAT3 plasmid, respectively, for determination of Bid cleavage and translations, as well as the caspase-3 activation in single living cell.

It was reported in some previous studies that full-length Bid could also be a potent inducer of apoptosis, and the cleavage of Bid was not essential for triggering apoptotic activity [30, 31]. In order to determine if the overexpression of Bid-CFP plasmid could induce Bid translocation and cell death in our experimental system, the cells transfected with Bid-CFP plasmid were cultured in the minitype CO_2 culture chamber (pH 7.4, 37°C, 95%

air and 5% CO_2) on our LSM 510 confocal microscope (Carl Zeiss MicroImaging, Inc., Germany). Due to the movement of living cells, we obtained randomly the fluorescence images of cells. Our experimental results showed that the overexpression of Bid-CFP plasmid did not induce any apoptotic characteristic features under the neutral culture conditions for a time period of up to 96 hours, as shown in Fig. 1B.

Bid, a unique BH3-only pro-apoptotic protein [32], plays an important role in the cell apoptosis induced under alkaline condition. Bid activation depends on the proteolytic processing of intact Bid into truncated forms of tBid. tBid, thus generated, translocates to mitochondria and leads to disruption of the organelles and the release of apoptogenic molecules such as cytochrome c [32]. Caspase-8 has been shown to be the major protease responsible for Bid cleavage during death receptor-mediated apoptosis [32]. Our results showed that, at pH 8.0, Bid cleavage and tBid translocation to mitochondria occurred within 1.5 hours of the alkaline treatment, as shown in Fig. 3, and cell shrinkage and nuclear condensation occurred within 4 hours, and 100% of the cells underwent apoptosis within 48 hours, as shown in Fig. 2. These results imply that the alkaline conditions induce cell apoptosis through caspase-8/Bid cleavage-mediated apoptosis pathway.

SCAT3, which can report the caspase-3 activation, is highly resistant to the changes in intracellular pH, Cl^- and H^+, and it is especially useful for monitoring the caspase-3 activation in living cells [25]. There are two pathways of caspase-8 and capase-3 activation in TNF-α-induced apoptosis: direct activation of caspase-3 by caspase-8, and indirectly activation of caspase-3 through Bid /tBid/mitochondria/caspase-9 pathways [21]. Our experimental data, shown in Fig. 4, demonstrated that alkaline conditions (pH=8.0) may activate caspase-8, which cleaved Bid to tBid, followed by tBid translocation to mitochondria and cytochrome c release from mitochondria, and then activated caspase-3 through caspase-9 activation.

However, Farber et al. demonstrated that the alkaline conditions (pH 7.8 or higher) could induce Bax translocation to mitochondria, cytochrome c release, and cell death in HeLa cell apoptosis with staurosporine [18]. Therefore, it is necessary to further study if Bax is involved in the alkalization-induced apoptosis in human lung adenocarcinoma cells.

In conclusion, we use the fluorescence techniques based on GFPs to monitor the dynamics of Bid cleavage, tBid translocation, caspase activation, cell shrinkage, and nuclear condensation in single living cell under neutral and alkaline cell physiological conditions. Our results show that alkaline condition (pH 8.0) induces apoptosis by the pathway of activating caspase-8, Bid cleavage into tBid, tBid translocation into mitochondria, and caspase-3 activation. Further studies are required to understand the mechanisms of apoptosis induced by alkaline conditions.

Acknowledgements

We wish to thank Professor K. Taira for providing us with Bid-CFP plasmid, Professor M. Miura for providing us with SCAT3 plasmid, and Fang Wang for experimental helps. This study was supported by National Natural Science Foundation of China (Grant No. 30470494, 30670507, and 30627003), and Natural Science Foundation of Guangdong Province (Grant No. 015012).

References

1 Li H, Yuan J: Deciphering the pathways of life and death. Curr Opin Cell Biol 1999;11:261–266.

2 Wang K, Yin X M, Chao DT, Milliman CL, Korsmeyer S J: BID: a novel BH3 domain-only death agonist. Genes Dev 1996;10:2859–2869.

3 Li H, Zhu H, Xu CJ, Yuan J: Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94:491–501.

4 Zha J, Weiler S, Oh KJ, Wei MC, Korsmeyer SJ: Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science 2000;290:1761–1765.

5 Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson C B, Korsmeyer S J: tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev 2000;14:2060–2071.

6 Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, Maundrell K, Antonsson B, Martinou JC: Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol 1999;144:891–901.
7 Onuki R, Nagasaki A, Kawasaki H, Baba T, Uyeda TQ, Taira K: Confirmation by FRET in individual living cells of the absence of significant amyloid??-mediated caspase 8 activation. Proc Natl Acad Sci USA 2002;99:14716-14721.
8 Luo X, Budihardjo I, Zou H, Slaughter C, Wang X: Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 1998;94:481–490.
9 Li S, Zhao Y, He X, Kim T H, Kuharsky D K, Rabinowich H, Chen J, Du C, Yin X M: Relief of Extrinsic Pathway Inhibition by the Bid-dependent Mitochondrial Release of Smac in Fas-mediated Hepatocyte Apoptosis. J Biol Chem 2002;277:26912–26920.
10 D'Arcangelo D, Facchiano F, Barlucchi LM, Melillo G, Testolin L, Gaetano C, Capogrossi MC: Acidosis inhibits endothelial cell apoptosis and function and induces basic fibroblast growth factor and vascular endothelial growth factor expression. Circ Res 2000;86:312–318.
11 Furlong IL, Ascaso R, Rivas AL, Collings MKL: Intracellular acidification induces apoptosis by stimulating ICE-like protease activity. J Cell Sci 1997;110:653–661.
12 Green DR, Reed J C: Mitochondria and apoptosis. Science 1998;281:1309–1312.
13 Xu L, Glassford AJ, Giacca AJ, Giffard RG: Acidosis reduces neuronal cell apoptosis. Neuroreport 1998;9:875–879.
14 Schwerdt G, Freudinger R, Schuster C, Silbernagl S, Gekle M: Inhibition of mitochondria and extracellular acidification enhance ochratoxin A-induced apoptosis in renal collecting duct-derived MDCK-C7 cells. Cell Physiol Biochem 2004;14:47-56.
15 Cutaia M, Tollefson K, Kroczynski J, Parks N, Rounds S: Role of the Na^+/H^+ antiport in pH-dependent cell death in pulmonary artery endothelial cells. Am J Physiol Lung Cell Mol Physiol 2000;278:L536–L544.
16 Terminella C, Tollefson K, Kroczynski J, Pelli J, Cutaia M: Inhibition of apoptosis in pulmonary endothelial cells by altered pH, mitochondrial function, and ATP supply. Am J Physiol Lung Cell Mol Physiol 2003;283:L1291–L1302.
17 Segal MS And Beem E: Effect of pH, ionic charge, and osmolality on cytochrome c-mediated caspase-3 activity. Am J Physiol Cell Physiol. 2001;281:C1196–C1204.
18 Leblebicioglu B, Walters J: Alkaline conditions accelerate polymorphonuclear leukocyte apoptosis in vitro. Infect Immun 1999;67:2019-21.
19 Tafani M, Joshua AC, Natalie OK, Ronald JR, Russo MA, Farber JL: Regulation of Intracellular pH Mediates Bax Activation in HeLa Cells Treated with Staurosporine or Tumor Necrosis Factor-α???J Biol Chem 2002;277(51):49569–49576.
20 Khaled AR, Kim K, Hofmeister R, Muegge K, and Durum SK: Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. Proc Natl Acad Sci 1999;96:14476–14481.
21 Luo KQ, Yu VC, Pu Y, Chang DC: Measuring dynamics of caspase-8 activation in a single living HeLa cell during TNF-alpha-induced apoptosis. Biochem Bioph Res Comm 2003;304:217–222.
22 Rehm M, Dussmann H, Janicke RU, Tavare JM, Kogel D, Prehn JH: Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3. J Biol Chem 2002;277:24506–24514.
23 Sekar RB, Periasamy A: Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. J Cell Biol 2003;160:629-633.
24 Lin JQ, Zhang ZH, Zeng SQ, Zhou SX, Liu BF, Liu Q, Yang J, Luo QM: TRAIL-induced apoptosis proceeding from caspase-3-dependentand -independent pathways in distinct HeLa cells. Biochem Biophys Res Commun 2006;346:1136–1141.
25 Takemoto K, Nagai T, Miyawaki A, Miura M: Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects. J Cell Biol 2003;160:235-43.
26 Gao XJ, Chen TS, Xing D, Wei XB: Single Cell Analysis of PKC Activation During Proliferation and Apoptosis Induced by Laser Irradiation., J Cell Physiol 2006;206:441-448.
27 Hoeppner DJ, Hengartner MO, Schnabel R.: Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans. Nature 2001;412:202–206.
28 Wang F, Chen TS, Xing D, Wang J., Wu YX: Measuring dynamics of caspase-3 activity in living cells using fret technique during apoptosis induced by high fluence low power laser irradiation. Lasers Surg Med 2005;36:2-7.
29 Wu YX, Xing D, Luo SM, Tang YH, Chen Q: Detection of caspase-3 activation in single cells by fluorescence resonance energy transfer during photodynamic therapy induced apoptosis. Cancer Lett 2006;235:239–247.
30 Sarig R, Zaltsman Y, Marcellus RC, Flavell R, Mak TW, Gross A: BID-D59A is a potent inducer of apoptosis in primary embryonic fibroblasts. J Biol Chem 2003;278:10707-10715
31 Madesh M, Antonsson B, Srinivasula SM, Alnemri ES, Hajnóczky G: Rapid Kinetics of tBid-induced Cytochrome c and Smac/DIABLO Release and Mitochondrial Depolarization. J Biol Chem 2001;277:5651-5659.
32 Yi XL, Yin XM, Dong Z: Inhibition of Bid-induced Apoptosis by Bcl-2. J Biol Chem 2003;278:16992–16999.