High Q1 Fluence Low-Power Laser Irradiation Induces Mitochondrial Permeability Transition Mediated by Reactive Oxygen Species

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High fluence low-power laser irradiation (HF-LPLI) can induce cell apoptosis via the mitochondria/caspase-3 pathway. Here, we further investigated the mechanism involved in the apoptotic process in human lung adenocarcinoma cells (ASTC-a-1) at a laser irradiation fluence of 120 J/cm² (633 nm). Cytochrome c release was ascribed to mitochondrial permeability transition (MPT) because the release was prevented by cyclosporine (CsA), a specific inhibitor of MPT. Furthermore, mitochondrial permeability for calcium (~620 Da) was another evidence for the MPT induction under HF-LPLI treatment. A high-level intracellular reactive oxygen species (ROS) generation was observed after irradiation. The photodynamically produced ROS caused onset of MPT, as the ROS scavenger docosahexaenoic acid (DHA) prevented the MPT. However, CsA failed to prevent cell death induced by HF-LPLI, indicating the existence of other signaling pathways. Following laser irradiation, Bax activation occurred after mitochondrial depolarization and cytochrome c release, indicating Bax activation was a downstream event. In the presence of CsA, Bax was still activated at the end-stage of apoptotic process caused by HF-LPLI, suggesting that Bax was involved in an alternative-signaling pathway, which was independent of MPT. Under HF-LPLI treatment, cell viabilities due to pre-treatment with DHA, CsA, or Bax small interfering RNA (siRNA) demonstrated that the MPT signaling pathway was dominant, while Bax signaling pathway was secondary, and more importantly ROS mediated both pathways. Taken together, these results showed that HF-LPLI induced cell apoptosis via the CsA-sensitive MPT, which was ROS-dependent. Furthermore, there existed a secondary signaling pathway through Bax activation. The observed link between MPT and triggering ROS could be a fundamental phenomenon in HF-LPLI-induced cell apoptosis.


Low-power laser irradiation (LPLI) has been shown to modulate various biological processes (Karu, 1989), such as cell proliferation and differentiation (Nadav et al., 1999), cell viability (Lubart et al., 2005), and cell apoptosis (Wang et al., 2005). It can induce changes in reactive oxygen species (ROS) production, Ca²⁺ concentration, mitochondrial transmembrane potential (ΔΨm), intracellular pH (Alexandrou et al., 2002), and ATP quantity (Passarella et al., 1984). The biological effects of LPLI rely upon some proteins, such as protein kinase Cs (PKC) (Gao et al., 2006), extracellular signal-regulated kinase (ERK), protein kinase B (Akt/PI3K), CyclinD1 (Shefer et al., 2001), platelet-derived growth factor (PDGF), interleukin-8/1α (IL-8/1α) (Yu et al., 1994), vascular endothelial growth factor (VEGF) (Kipshidze et al., 2001), and nerve growth factor (NGF) (Fidi et al., 2002).

When cells were treated with LPLI at 0.5–0.8 J/cm², a typical low fluence dosage, proliferation of human lung adenocarcinoma (ASTC-a-1) cells was observed, while at 60 J/cm², a typical high fluence dosage, cell apoptosis was observed (Ga o et al., 2006). Early results obtained by Zhang et al. (2008) showed an increased viability in HeLa cells treated with LPLI at 3–15 J/cm²; irradiation at 25–50 J/cm² evidently decreased the cell viability and caused cell morphologic damage. LPLI at high fluence interfered with cell cycling and inhibited cell proliferation, thus could be used to control certain types of hyperplasia (Gross and Jelkmann, 1990; O’Kane et al., 1994; Ocana-Quero et al., 1998). This phenomenon is consistent with our early results of induced ASTC-a-1 cell apoptosis when irradiated with a fluence between 60 and 120 J/cm², and its dependence on the dosage of laser irradiation (Wang et al., 2005). For the mechanism studies, recent works by Wu et al. (2007) showed that high fluence low-power laser irradiation (HF-LPLI) (80, 120 J/cm²) induced cell apoptosis via the mitochondrial signaling pathway (mitochondria/caspase-3), accompanied by a large amount of ROS generation in both ASTC-a-1 cells and transformed African green monkey kidney fibroblast (COX-7) cells. The definition for high or low fluence for LPLI is not clear. We refer to a high fluence irradiation when an obvious inhibition of cell viability is observed (Wang et al., 2005; Wu et al., 2007) in order to distinguish the proliferation effect of low fluence LPLI, which has been reported previously (Karu, 1989).

Abbreviations: ASTC-a-1, human lung adenocarcinoma cells; CsA, cyclosporine; CCK-8, cell counting kit-8; DHA, docosahexaenoic acid; GSK3, glycogen synthase kinase; H2DCFDA, dichlorodihydrofluorescein diacetate; HF-LPLI, high fluence low-power laser irradiation; MPT, mitochondrial permeability transition; OMMP, outer mitochondrial membrane permeabilization; PDT, photodynamic treatment; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl esters; ΔΨm, mitochondrial transmembrane potential.

Contract grant sponsor: National Natural Science Foundation of China and Natural Science Foundation of Guangdong Province; Contract grant numbers: 30627003, 30870676, 7117865.

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Received 1 June 2008; Accepted 8 October 2008

Published online in Wiley InterScience (www.interscience.wiley.com.), 00 Month 2008. DOI: 10.1002/jcp.21636
The mechanism of apoptosis induced by HF-LPLI is still not well understood. Such mechanism is necessary for the clinical applications of low-power laser therapies. It can provide a new stimulator to trigger cell apoptosis, since it is well-known that apoptosis plays an important role in physiological and pathological conditions (Steven et al., 2000; Ferreira et al., 2002). Furthermore, it can be used to treat some kind of hyperplasia (Gross and Jelkmann, 1990; O’Kane et al., 1994; Ocana-Quero et al., 1998) and to treat cancers, like photodynamic treatment (PDT) (Sharman et al., 1999) in the absence of photosensitizers.

Mitochondrial injury is central to apoptosis (Green and Reed, 1998). The permeabilization of outer mitochondrial membrane leads to the release of apoptogenic factors (Kroemer et al., 2006). In general, two non-exclusive models have been proposed for the mechanism of outer mitochondrial membrane permeabilization (OMMP) (Kroemer et al., 2006). In one, pro-apoptotic members of the Bcl-2 family act to create discontinuities (perhaps pores) in the outer mitochondrial membrane, without affecting the functions of the inner membrane or matrix (Wills et al., 2003). In another, a variety of signals, such as Ca\(^{2+}\) (Jacobson and Duchen, 2002) and ROS (Zorott et al., 2000; Jacobson and Duchen, 2002), trigger the opening of small channels on the inner mitochondrial membrane, allowing water to enter and swell the matrix, effectively bursting the outer membrane, an effect referred to as the mitochondrial permeability transition (MPT) (Kim et al., 2003; Zorott et al., 2005).

Zorott et al. (2000) reported that mitochondrial triggered ROS generation via photoactivation of tetramethylrhodamine derivatives could induce MPT, since they observed increased ROS production at sites of spontaneously de-energized mitochondria. Jacobson and Michael developed a model in which an intramitochondrial photosensitizing agent was used to explore the consequences of mitochondrial ROS generation for mitochondrial function and cell fate in primary cells (Jacobson and Duchen, 2002). They found that in astrocytes the interplay between mitochondrial ROS and endoplasmic reticulum (ER) sequestered Ca\(^{2+}\) increased the frequency of transient mitochondrial depolarizations and caused mitochondrial Ca\(^{2+}\) loading from ER stores. The depolarizations were attributed to the opening of the mitochondrial permeability transition pore (MPTP) (Jacobson and Duchen, 2002). Agents, such as cyclosporin (CsA) and bongkrekic acid (BA), that blocked the MPT could also block apoptosis (Kroemer et al., 2006). Both models have been widely used to explain OMMP in apoptosis via the mitochondrial pathway (Kroemer et al., 2006).

Mitochondria are both a major source of ROS and a target for their damaging effects due to ROS. Oxidants stimulate, while antioxidants inhibit, apoptosis, suggesting a role for ROS as initiators or downstream mediators of apoptosis (Jacobson, 1996). Recent studies showed that HF-LPLI induced a high-level intracellular ROS generation (Wu et al., 2007). Understanding the roles of ROS in both normal and pathological conditions has led to renewed interest in mitochondrial functions.

Combining the two facts that intracellular ROS generation induces MPT and HF-LPLI triggers a large amount of ROS generation, we speculate that HF-LPLI induces cell apoptosis mediated by MPT induction. Using fluorescent image techniques, we further investigate the mitochondrial mechanism of cell apoptosis induced by HF-LPLI.

**Materials and Methods**

**Cell culture**

ASTC-a-1 cells were grown on 22 mm culture glasses, in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies Co. Ltd.) supplemented with 15% fetal bovine serum (FBS) (GIBCO Co. Ltd.), 50 units/ml penicillin, and 50 µg/ml streptomycin, 5% CO\(_2\), 95% air at 37°C in a humidified incubator. In all experiments, 70–85% confluent cultures were used.

**HF-LPLI treatment**

For irradiation of cells, a 633 nm He–Ne laser inside a confocal laser scanning microscope (LSM510-Confocor2) (Zeiss, Jena, Germany) was used. Laser irradiation was performed through the objective lens (100x/NA1.45) of the inverted microscope in laser scanning mode. In this setup, only the cells under observation were irradiated by the laser. The output laser power through the objective lens was 4.3 µW (633 nm), which was measured by a power meter when acousto-optical tunable filter (AOTF) was set as 100%. The cells in selected area were irradiated for 10 min with the fluence of 120 J/cm\(^2\). The power intensity was kept as 0.2 W/cm\(^2\) by changing the AOTF (%) and irradiation area. For fluorescent imaging, the excitation laser powers were 0.2875 µW for 488 nm, 0.0479 µW for 458 nm, and 0.0534 µW for 543 nm. A mini-type culture chamber with CO\(_2\) supply (Tempcontrol 37-2 digital, Zeiss, Germany) was used to keep cells under normal culture conditions (37°C, 5% CO\(_2\)) during irradiation.

**Chemicals**

The following fluorescent probes were used: dichlorodihydrofluorescein diacetate (H\(_2\)DCFDA, 10 µM) to detect the generation of ROS, calcein AM (1 µM) to monitor MPT, rhodamine 123 (5 µM), and tetramethylrhodamine methyl esters (TMRM) (100 nM) to monitor ΔΨm. All the probes were purchased from Molecular Probes (MP). The optimal incubation time for each probe was determined experimentally.

The following reagents were used: CsA (5 µM) to inhibit MPT, docosahexaenoic acid (DHA) (100 µM) to scavenge ROS, CoCl\(_2\) (1 mM) to quench the fluorescence of calcein, CaCl\(_2\) (200 mM) to induce MPT. These reagents were purchased from Sigma–Aldrich (St. Louis, MO). We used LipofectamineTM 2000 reagent (Invitrogen Life Technologies, Inc. Grand Island, NY) to transfect plasmid DNA and small interfering RNA (siRNA) into cells. Cells were examined 36–48 h after transfection.

**Bax gene silencing by siRNA**

RNA interference of Bax was performed using 24-bp siRNA duplexes purchased from Gene Pharma (Shanghai, China). The sense strand nucleotide sequence for Bax siRNA was AACATGGAGCTGAGAGGATGAdTdT. A control siRNA specific to the GFP DNA sequence CCACCTCTGAGCAGGAGGCTGAdTdT. A control siRNA duplexes purchased from Gene Pharma (Shanghai, China) was used as a negative control. For transfection, ASTC-a-1 cells were seeded in six-well plates at 30% confluency, and siRNA duplexes (200 nmol/L) were introduced into the cells using LipofectamineTM 2000 according to the manufacturer’s recommendations. Assays were performed 48 h after transfection. The protein levels of Bax were detected in the cell lysate by Western Blot.

**MPT monitoring**

MPT was monitored using a calcein–Co\(^{2+}\) technique (Kroemer et al., 2006). This method relies on the loading of cells with the fluorescent probe calcein (~620 Da) and its quencher, Co\(^{2+}\). Co\(^{2+}\) was added to cells 1 h before experiment and calcein AM was loaded to cells 30 min before experiment. After attainment of quenching, cells were washed free of calcein AM and Co\(^{2+}\) in order to remove the sections in the medium. When loaded into cells in its acetoxymethyl ester form, calcein was trapped in all subcellular compartments, including mitochondria, whereas Co\(^{2+}\) was excluded from mitochondrial matrix due to the inner membrane impermeability to this ion. As a consequence, when the barrier provided by inner membrane was functional, a distinct punctate fluorescence signal from calcein clearly identifies MPT.
Cell viability assays

Cell viability was assessed with CCK-8 (cell counting kit-8, Kumamoto, Japan) after irradiation. CCK-8 uses highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl) -5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt] to produce a water-soluble formazan dye upon reduction in the presence of an electron carrier. Being non-radioactive, CCK-8 allows sensitive determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give a yellow colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. The detection sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT, XTT, MTS, or WST-1. At the indicated time, CCK-8 was added to cells and incubated for 1.5 h. ODexp. the 90th absorbance value, was read with a 96-well plate reader (DG5032, Hua Dong, Nanjing, China). The value is directly proportional to the number of viable cells in a culture medium (Griffioen and Molema, 2000).

Imaging analysis of living cells

In order to image single cells, the confocal laser scanning microscope system (LSM510-ConfoCor2) (Zeiss) was used. The system is equipped with a krypton–argon air-cooled laser (30 mW) and a He–Ne laser (5 mW) for excitation illumination. The illumination power was reduced from 3% to 0.3% of the maximum power of the excitation lasers to avoid fluorescence saturation and bleaching. All images were acquired before and after laser irradiation with a Plan–Neofluar 100×/NA1.45, oil-immersed objective lens. Cells were also maintained at 37 °C, 5% CO₂ during imaging with the mini-type culture chamber with CO₂ supply.

The specific imaging process is as follows. pGFP-cyt c, DCF, calcein, and rhodamine 123 were excited using the krypton–argon air-cooled laser. The excitation wavelength was 488 nm, the main dichroic beam splitter was UV/488/543/633 nm, and the emission detection filter was band pass 500–550 nm. pCFP-Bax was excited using the krypton–argon air-cooled laser. The excitation wavelength was 458 nm, the main dichroic beam splitter was 458 nm, and the emission detection filter was band pass 470–500 nm. TMRE was excited using the He–Ne laser. The excitation wavelength was 543 nm, the main dichroic beam splitter was UV/488/543/633 nm, and the emission detection filter was band pass 565–615 IR. pDsRed-mt was excited using the He–Ne laser. The excitation wavelength was 543 nm, the main dichroic beam splitter was UV/488/543/633 nm, and the emission detection filter was long pass 560 nm.

For intracellular measurements, the desired area was chosen in the confocal image. To quantify the results, the fluorescence emission intensities (including the background fluorescence) were obtained with Zeiss Rel 3.2 image processing software (Zeiss).

Statistics

MATLAB software was used for data analysis. For fluorescence emission intensity analysis, a background subtraction was performed for all the data. For the analysis of cytochrome c release, images were analyzed with MATLAB 6.5 software by drawing regions around individual cells and then computing standard deviations of the intensity of the pixels (punctate/diffuse) and integrated brightness (total brightness) (Muñoz-Pinedo et al., 2006).

Unless otherwise indicated, data were analyzed using one-way or two-way ANOVA. Comparisons of individual groups were performed using tuckey honest test. All results showed in our studies were repeated for at least five times in different cells in independent experiments.

Results

HF-LPLI-induced MPT

To determine the relationship between HF-LPLI-induced cell apoptosis and MPT, a technique based on calcein AM loading and Co²⁺ quenching was used. Non-irradiated cells under the same experimental procedures were used as control. Ca²⁺ overload was used as a positive experiment control. CaCl₂ was added and sequential images were acquired at various time points. As shown in Figure 1A, in control cells, calcein fluorescence remained constant (Fig. 1A, control), while cells treated with CaCl₂ had a significant fluorescence reduction (Fig. 1A, Ca²⁺ overload). Data showed that the calcein fluorescence emission intensities from mitochondria were decreased sharply 60 min post-irradiation (Fig. 1A, HF-LPLI), indicating a long-lasting MPT, which was blocked by CsA, a specific inhibitor of MPT, in our experiment (Fig. 1A, HF-LPLI + CsA).

To further confirm our presumption that HF-LPLI induces MPT, we used CCK-8 method to determine the protective role of CsA. ASTC-a-1 cells were exposed to CsA 2 h before irradiation. Non-irradiated cells under the same experimental procedures were used as control. Cell viabilities were assessed 6 and 10 h after irradiation. Cell death was significantly prevented when CsA was added (91.36% cell viability with CsA vs. 26.36% cell viability without CsA), as shown in Figure 1B. However, 10 h after HF-LPLI treatment in the presence of CsA, cell viability was reduced to only 30.66%, indicating that CsA only delayed, but not completely prevented, cell death.

ROS mediated cell death caused by HF-LPLI

To monitor ROS generation caused by HF-LPLI, the fluorescent products DCF were determined by confocal microscopy. ASTC-a-1 cells were labeled with H₂DCFDA for 30 min and imaged after irradiation. Non-irradiated cells under the same experimental procedures were used as control. An increased production of ROS in cells was observed after irradiation (Fig. 2A, HF-LPLI) and it reached a plateau at about 1 h 40 min (Fig. 2B, HF-LPLI) in comparison to the control cells (Fig. 2B, control). Moreover, the antioxidant DHA scavenged ROS induced by HF-LPLI treatment (Fig. 2A, B, HF-LPLI + DHA).

To determine whether ROS generation was a key step in HF-LPLI-induced cell apoptosis, we used CCK-8 method to determine the protective role of DHA on cell death under HF-LPLI treatment. ASTC-a-1 cells were exposed to DHA 30 min before irradiation. Cell viability was assessed 6 h post-irradiation. The data revealed that cell death was significantly prevented when DHA was added, as shown in Figure 2C (93.36% cell viability with DHA vs. 25.33% cell viability without DHA), and ROS generation mediated cell death.

DHA prevented MPT under HF-LPLI treatment

In order to study the relationship between irradiation induced “triggering” ROS generation and MPT, experiments were performed in the presence of DHA. MPT was monitored by calcein–Co²⁺ method. Non-irradiated cells under the same experimental procedures were used as control. In cells treated with DHA, calcein fluorescence decrease was prevented, suggesting the inhibition of MPT (Fig. 3). Based on the fact that DHA prevents MPT, it is reasonable to speculate that the ROS is crucial from the standpoint of MPT induction.
CsA delayed mitochondrial depolarization under HF-LPLI treatment

In order to monitor the effect of HF-LPLI on $\Delta \psi _{m}$, ASTC-a-1 cells loaded with rhodamine 123 for at least 20 min were treated by HF-LPLI and imaged by confocal microscopy.

Non-irradiated cells under the same experimental procedures were used as control. Under the normal conditions, mitochondria maintained $\Delta \psi _{m}$, as revealed by the accumulation of a potential-sensitive dye, rhodamine 123, determined from images during a 5 h recording period (Fig. 4A, control). In comparison, HF-LPLI caused a significant
reduction of rhodamine 123 signal (Fig. 4A, HF-LPLI); it fell below 50% of its initial level within 20 min (Fig. 4B, HF-LPLI).

To explore the relationship between HF-LPLI-induced MPT and mitochondrial depolarization, we examined the effects of CsA on ΔΨm. HF-LPLI treatment plus CsA exposure delayed the decrease of rhodamine 123 fluorescence emission intensity (reached 50% of its initial level in about 70 min) for about 50 min in comparison with HF-LPLI treatment alone (reached 50% of its initial level in about 20 min), as shown in Figure 4A,B. The data demonstrated that CsA delayed mitochondrial depolarization under HF-LPLI treatment.

**CsA prevented cytochrome c release under HF-LPLI treatment**

We assessed the subcellular location of cytochrome c in response to HF-LPLI treatment. ASTC-a-1 cells were transfected with pDsRed-mit for localizing mitochondria and pGFP-cyt c for monitoring the dynamics of cytochrome c. Non-irradiated cells under the same experimental procedures were used as control. Under the normal conditions, cytochrome c was relatively unchanged in the mitochondria (Fig. 5A, control). The data in Figure 5B were obtained using five cells in which cytochrome c was released from mitochondria into cytosol at about 50 min post-treatment. The cytochrome c was then translocated and assembled at the end-stage of apoptosis (Fig. 6B). The GFP-cyt c punctate/diffuse index at different times under the normal condition, HF-LPLI treatment or HF-LPLI treatment in the presence of CsA are shown in Figure 5B. These data indicated that HF-LPLI induced cytochrome c release and CsA prevented the process.

**Activation of Bax caused by HF-LPLI treatment**

To investigate the activity of Bax under HF-LPLI treatment, ASTC-a-1 cells transfected with pGFP-Bax for localizing Bax and then stained by TMRM for monitoring ΔΨm were treated with HF-LPLI and imaged by confocal microscopy. In control cells, GFP-Bax was largely cytosolic or loosely associated with mitochondria, which kept a constant ΔΨm, as shown in Figure 6A. Under HF-LPLI treatment, Bax translocated and assembled at the end-stage of apoptosis (Fig. 6B). The GFP-cyt c punctate/diffuse index began to increase 2 h 20 min post-irradiation (Fig. 6C, GFP-Bax).

To explore the relationship between Bax translocation and cytochrome c release under HF-LPLI treatment, ASTC-a-1 cells doubly transfected with pCFP-Bax and pGFP-cyt c were treated with HF-LPLI and imaged by confocal microscopy. Figure 6D shows the CFP-Bax and GFP-cyt c fluorescence emission intensities under the HF-LPLI treatment. After irradiation, GFP-cyt c punctate/diffuse index kept unchanged for 50 min and then decreased sharply. At about 1 h 40 min,
COLOR

Fig. 5. CsA prevented cytochrome c release under HF-LPLI treatment. Time sequence of cytochrome c subcellular location under HF-LPLI treatment. ASTC-a-1 cells were transfected with pDsRed-mit (red emission) for mitochondrial localization and pGFP-cyt c (green emission), and treated with HF-LPLI or HF-LPLI in the presence of CsA. Fluorescence images were acquired by confocal microscopy. Cells no treatment were control. Bar = 10 μm.

Discussion

In light of previous studies demonstrating HF-LPLI induced cell apoptosis via the mitochondria/caspase-3 signaling pathway (Wang et al., 2005; Wu et al., 2007), the working hypothesis of this study was that the induced apoptosis was resulted from MPT which was mediated by a high level of intracellular ROS generation.

In our experiments, two main lines of evidence led to the conclusion that HF-LPLI induced MPT in ASTC-a-1 cells. Firstly, calcein (~620 Da), a mitochondrial membrane impermeant fluorescent molecule, was permeable between mitochondria and cytosol under HF-LPLI treatment (Fig. 1A). Secondly, CsA, a specific inhibitor of MPT, prevented the permeabilization of calcein, and delayed cell death under HF-LPLI treatment (Fig. 1A,B). Therefore, these results demonstrated that HF-LPLI induced MPT, which acted in a CsA sensitive manner.

Mitochondrial functions such as protein import, ATP generation, and lipid biogenesis depend on the maintenance of ΔΨm; the loss of ΔΨm during apoptosis likely causes cell death through disabling these functions (Brenner and Kroemer, 2000). It appears that pre-apoptotic ΔΨm disruption is mediated by the MPT pores (Kroemer et al., 2006), that is, regulated mega-channels that allow the dissipation of inner transmembrane ion gradients. This was confirmed in cells treated with HF-LPLI, in which CsA had a significant inhibitive effect on mitochondrial depolarization (Fig. 4). In addition, HF-LPLI-induced mitochondrial depolarization may also be due to the direct photodamage on inner mitochondrial membrane. This was revealed by the fact that CsA only delayed mitochondrial depolarization but not completely blocking it (Fig. 4). Based on our experiments that CsA delayed the ΔΨm dissipation (Fig. 4B, HF-LPLI + CsA), we speculate that photodamage on inner mitochondrial membrane and subsequently triggered MPT co-affected ΔΨm dissipation. Specifically, after irradiation the possible photoacceptors in the inner mitochondrial membrane absorb light of certain wavelength (Karu, 1999; Krasnovsky et al., 2003; Karu et al., 2004, 2005), and then the primary ROS are generated. The ROS generation causes damages on inner membrane, which partly result in ΔΨm decrease. Otherwise, when the primary ROS causes the subsequently ROS generation, it may trigger the opening of the transition pores which lead to acute ΔΨm collapse. When cells were treated with CsA, the MPT pores were blocked (Fig. 1A). ΔΨm decrease was inhibited (Fig. 4), and in turn cytochrome c release was prevented (Fig. 5). These phenomena indicated that the decrease of ΔΨm caused by direct photodamage was unable to cause outer mitochondrial permeabilization. In addition, the function of the redox system in mitochondria could not be ruled out, which could scavenge some ROS induced by HF-LPLI treatment (Balaban et al., 2005).

HF-LPLI triggered a high-level intracellular ROS generation and this was monitored in our early studies (Wu et al., 2007). As shown in Figure 2A, immediately after irradiation, mitochondria had a higher level of ROS production than that of cytosol; as the time increased, all cellular space showed high level ROS. These results indicated that mitochondria were the major ROS...
generation site and the first damage site in cells immediately after irradiation. This speculation was confirmed by the result that HF-LPLI could induce MPT in a short time (less than 1 h) post-irradiation (Fig. 1A). This phenomenon was similar to that under photodynamic therapy (PDT) treatment. The intracellular localization of the sensitizer in PDT coincides with the primary site of photodamage, mainly because of the limited diffusion of the short-lived singlet oxygen (half-life: <0.04 μs, radius of action: <0.02 μm), which is thought to be the predominant oxidant in PDT (Dougherty et al., 1998;
found that Bax was still activated (Fig. 6F), demonstrating that Bax signaling pathway was indeed independent of MPT under HF-LPLI treatment. Previous research has indicated that intracellular oxidative stress can trigger the activation of glycogen synthase kinase (GSK3) (Shin et al., 2004; King and Jope, 2005; Beurel and Jope, 2006). In addition, Lineman et al. (2004) reported that GSK3 β phosphorylated Bax and promoted its mitochondrial localization during neuronal apoptosis. Therefore, we concluded that Bax activation was via the LPLI/ROS/GSK3 β/Bax signaling pathway.

Comparing the data shown in Figures 5A and 6F, we found that in the presence of CsA, cytochrome c release was inhibited even 7 h post-irradiation, but Bax was activated at about 6 h, indicating that Bax activation was independent of cytochrome c release and that Bax translocation failed to induce cytochrome c release. This is an interesting phenomenon, because it was established that Bax translocation to mitochondria could induce mitochondrial membrane permeabilization, and subsequently release of proapoptotic proteins, such as cytochrome c (Adams and Cory, 2007). Why would Bax translocation fail to induce cytochrome c release under these conditions? One possible explanation was that Bax activation was involved in ER stress, finally induced cell apoptosis. The mechanism by which apoptosis is induced in response to ER stress has been examined in multiple cell types. Bax and Bak are important in this pathway, as mouse embryonic fibroblasts (MEFs) doubly deficient in Bax and Bak are resistant to ER stress-induced apoptosis (Scorrano et al., 2003; Zong et al., 2003). In addition to the established roles of Bax and Bak in the mitochondria, both are also found to be localized on the ER membrane and both play an important role in Ca2þ regulation and release in response to ER stress (Nutt et al., 2002; Scorrano et al., 2003; Zong et al., 2003). The discovery of an ER-localized caspase, caspase-12, has brought into question the mechanism by which ER stress activates caspases to induce apoptosis (Nakagawa et al., 2000). In vitro, activated caspase-12 is able to cleave caspase-9 directly, which subsequently activates caspase-3, potentially eliminating the requirement of the mitochondria and apoptosome to carry out ER stress-induced apoptosis (Rao et al., 2002). The lack of apoptosome involvement in ER stress-induced apoptosis is consistent with the observation that Sak2 cells, which are deficient in Apaf-1, are capable of activating caspases when treated with ER stress-inducing agents thapsigargin (TG) and brefeldin A (Rao et al., 2002). Likewise, C2C12 cells treated with tunicamycin (TU) or TG show caspase activation without any detectable release of cytochrome c (Morishima et al., 2002).

Recent studies suggested that the mitochondrial signaling pathway was significant in either inducing or amplifying ER stress-induced apoptosis. First, in contrast to the data from C2C12 cells, cytochrome c was released in response to ER stress in a variety of other cell types (Reimertz et al., 2003; Di Sano et al., 2006). Second, a recent study found that the Apaf-1-deficient ETNA (murine embryonic telencephalic naive) cell line and Apaf-1-deficient MEFs and embryonic cortical cells were resistant to ER stress-induced apoptosis (Di Sano et al., 2006). This may be revealed by our results that HF-LPLI-induced Bax translocation was faster than under the conditions when MPT was blocked (Fig. 6B,F).

Finally, for the first time we provided the evidence that a long-lasting, CsA-sensitive MPT occurred in the early phase of apoptotic cell death induced by HF-LPLI, and induced mitochondrial ROS generation played key roles in the MPT induction. We also showed another signaling pathway, which was mediated by Bax translocation, occurred at the last stage of cell apoptotic process. Results shown in Figure 7 demonstrated that MPT signaling pathway was dominant and Bax signaling pathway was secondary; however, both pathways were controlled by the intracellular ROS generation.

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Fig. 7. The effect of DHA, CsA, and Bax siRNA on cell viability under HF-LPLI treatment. CCK-8 assayed the viability of ASTC-a-1 cells treated with HF-LPLI, HF-LPLI in the presence of CsA, HF-LPLI in the presence of CsA, and HF-LPLI in the presence of Bax siRNA. Cells with no treatment were control. Cell viability was assessed 6 and 10 h after irradiation. The data represent mean ± SEM of five independent experiments.
Acknowledgments

pGFP-Bax was a gift from Prof. Richard J. Youle (Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892). pDsRed-mit was presented by Prof. Yuikko Gotoh (the Institute of Molecular and Cellular Bioscience, University of Tokyo). pCFP-Bax was presented by Prof. Charles and Andrew (The School of Biological Science, University of Manchester). We also thank Dr. G.J. Gores (Center for Basic Research in Digestive Diseases, Molecular Medicine Program, Mayo Clinic, Rochester Minnesota) for kindly providing the pGFP-cyt c.

Literature Cited


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AUTHOR(S) Shengnan Wu, Da Xing, Xuejuan Gao, and Wei, R. Chen

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