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# Mitochondrial oxidative stress causes mitochondrial fragmentation via differential modulation of mitochondrial fission-fusion proteins

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#### Keywords

dynamin-related protein 1 (Drp1); fission; high-fluence low-power laser irradiation (HF-LPLI); mitofusin 2 (Mfn2); oxidative stress

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Mitochondria are dynamic organelles that undergo continual fusion and fission to maintain their morphology and functions, but the mechanism involved is still not clear. Here, we investigated the effect of mitochondrial oxidative stress triggered by high-fluence low-power laser irradiation (HF-LPLI) on mitochondrial dynamics in human lung adenocarcinoma cells (ASTC-a-1) and African green monkey SV40-transformed kidney fibroblast cells (COS-7). Upon HF-LPLI-triggered oxidative stress, mitochondria displayed a fragmented structure, which was abolished by exposure to dehydroascorbic acid, a reactive oxygen species scavenger, indicating that oxidative stress can induce mitochondrial fragmentation. Further study revealed that HF-LPLI caused mitochondrial fragmentation by inhibiting fusion and enhancing fission. Mitochondrial translocation of the profission protein dynamin-related protein 1 (Drp1) was observed following HF-LPLI, demonstrating apoptosis-related activation of Drp1. Notably, overexpression of Drp1 increased mitochondrial fragmentation and promoted HF-LPLIinduced apoptosis through promoting cytochrome c release and caspase-9 activation, whereas overexpression of mitofusin 2 (Mfn2), a profusion protein, caused the opposite effects. Also, neither Drp1 overexpression nor Mfn2 overexpression affected mitochondrial reactive oxygen species generation, mitochondrial depolarization, or Bax activation. We conclude that mitochondrial oxidative stress mediated through Drp1 and Mfn2 causes an imbalance in mitochondrial fission-fusion, resulting in mitochondrial fragmentation, which contributes to mitochondrial and cell dysfunction.

#### Introduction

Mitochondria are dynamic organelles that frequently move, undergo fission and fuse with one another to maintain their structure and functions [1]. Aside from influencing mitochondrial morphology and the degree of connectivity of mitochondrial networks, mitochondrial fission-fusion can contribute to the repair of

#### Abbreviations

CFP, cyan fluorescent protein; COX IV, cytochrome *c* oxidase subunit IV; DCF, 2,7-dichlorofluorescein; Drp1, dynamin-related protein; FCM, flow cytometry; FITC, fluorescein isothiocyanate; FRAP, fluorescence recovery after photobleaching; FRET, Förster resonance energy transfer; HF-LPLI, high-fluence low-power laser irradiation; H2DCFDA, dichlorodihydrofluorescein diacetate; Mfn2, mitoflusin 2; MitoTracker, MitoTracker Deeper Red 633; MMP, mitochondrial membrane potential; PI, propidium iodide; RNAi, RNA interference; ROS, reactive oxygen species; shRNA, short hairpin RNA; STS, staurosporine; TMRM, tetramethylrhodamine methyl ester; YFP, yellow fluorescent protein.

defective mitochondria, the proper segregation of mitochondria into daughter cells during cell division, the efficiency of oxidative phosphorylation, and intramitochondrial calcium signal propagation [1]. Imbalanced fission-fusion is involved in various pathological processes [2,3], including neuronal injury [4-6], cellular senescence [7], ischemia-reperfusion [8], and muscle atrophy [9]. During apoptosis, mitochondria often fragment into smaller units, and it remains unresolved whether this event has a significant impact on the rate of cell death, or merely accompanies apoptosis as an epiphenomenon [10]. Many apoptosis stimuli, such as staurosporine (STS), have been reported to caus mitochondria to fragment during the apoptotic process [10]. Recent experiments have demonstrated that mitochondrial morphology is an important determinant of mitochondrial function [11].

Mitochondrial shape depends on the balance between fission and fusion, and is controlled by multiple proteins that mediate remodeling of the outer and inner mitochondrial membranes [12]. Many of the gene products mediating the fission and fusion processes have been identified in yeast screens, and most are conserved in mammals, including the fission mediators dynamin-related protein (Drp1) (Dnm1 in yeast) and Fis1, the fusion mediators mitofusin 1 and mitofusin 2 (Mfn2) (Fzo1 in yeast), and optic atrophy 1 (Mgm1 in yeast) [12]. Unbalanced fusion leads to mitochondrial elongation, and unbalanced fission leads to excessive mitochondrial fragmentation, both of which impair mitochondrial function [12]. However, it is still unclear how extracellular stimuli modulate intracellular signaling processes to control mitochondrial dynamics and morphology.

Previous studies have demonstrated that high-fluence low-power laser irradiation (HF-LPLI) can induce mitochondrial oxidative stress in human lung adenocarcinoma cells (ASTC-a-1) and SV40-transformed African green monkey kidney fibroblast cells (COS-7) cells through selectively exciting the endogenous photoacceptor (cytochrome c oxidase) by laser irradiation (632.8 nm) [13,14]. The oxidative stress causes mitochondrial permeability transition pores to open for a long time, causes mitochondrial depolarization, cytochrome c release, and caspase-3 activation, and finally results in cell apoptosis [13–15].

However, whether HF-LPLI-induced mitochondrial oxidative stress can induce mitochondrial morphological changes is still not clear. Thus, in the present study, we investigated the regulatory pathways involved in mitochondrial dynamics following HF-LPLI, using fluorescent imaging, western blot and flow cytometry techniques in ASTC-a-1 cells and COS-7 cells.

#### Results

#### Mitochondrial oxidative stress caused by HF-LPLI

Dichlorodihydrofluorescein diacetate (H2DCFDA) is a reactive oxygen species (ROS)-sensitive probe that can be used to detect ROS production in living cells. It passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, releasing the corresponding dichlorodihvdrofluorescein derivative. Dichlorodihydrofluorescein oxidation yields a fluorescent adduct, 2,7-dichlorofluorescein (DCF), that is trapped inside the cell. Thus, we used DCF to label ROS and monitor the changes in ROS in ASTC-a-1 cells under various treatments. The ROS level correlates positively with the DCF fluorescence emission intensity. As is known, the recording laser used in confocal microscopy, such as 488 nm for DCF, can probably cause an artefactual ROS signal through photosensitization and consequent in situ photo-oxidation of the dye. Therefore, we tried to increase the recording interval time and decrease the power intensity of the recording laser to avoid this problem in control studies, and then applied the same set of experimental parameters in the following studies (Fig. 1A, upper panel). As shown in Fig. 1A (upper panel), cells treated with HF-LPLI showed a significant increase in DCF fluorescence immediately after the treatment, as opposed to the poor increase observed in control cells. As shown in Fig. 1B, for quantitative analysis of the DCF signal, we normalized the initial fluorescence intensity of each term as 100 a.u., and then made a comparison between different experimental groups, as the ability to take up H2DCFDA varies slightly between cells, even in the same cell line. Quantitative analysis of DCF fluorescence emission intensities (Fig. 1B) gave similar results as those in Fig. 1A. Also, the highest DCF signal caused by HF-LPLI was found in mitochondria, as clearly shown by overlapping of the spatial mappings of fluorescence from mitochondria and the ROSspecific probes MitoTracker Deeper Red 633 (Mito-Tracker) and DCF, respectively (Fig. 1A, lower panel). In addition, dehydroascorbic acid (vitamin C, a ROS scavenger) pretreatment totally inhibited ROS generation caused by HF-LPLI (Fig. 1A,B). These data dem-HF-LPLI triggers onstrate that mitochondrial oxidative stress.

## Mitochondrial fragmentation through oxidative stress caused by HF-LPLI

Mitochondrial shapes were examined in two independent cell lines, ASTC-a-1 cells and COS-7 cells. Cells Fig. 1. HF-LPLI causes mitochondrial ROS generation. (A) Representative sequential images of ASTC-a-1 cells stained with H2DCFDA under various treatments (upper panel). The increase in DCF fluorescence represents the generation of ROS. HF-LPLI caused intracellular ROS generation. Vitamin C (Vit C) pretreatment totally inhibited HF-LPLI-induced ROS generation. Representative images are shown of cells doubly stained with DCF (green emission) and MitoTracker (red emission) to label ROS and mitochondria, respectively, 15 min after HF-LPLI treatment (lower panel). ROS were mainly generated in mitochondria after HF-LPLI treatment. Scale bar: 10 µm. (B) Quantitative analysis of relative DCF fluorescence emission intensities (directly related to ROS generation) from ASTC-a-1 cells after various treatments. Control groups received no treatment. Data represent the mean ± standard error of the mean of at least five independent experiments (\*P < 0.05, Student's t-test).



were transiently transfected with pDsRed-mit to localize mitochondria, and the morphological changes of mitochondria in positively transfected cells were monitored by confocal microscopy. Most control cells (~ 98%) showed normal, short, tubular mitochondria (Fig. 2A,B). By contrast, under HF-LPLI treatment at a fluence of 120 J·cm<sup>-2</sup>, only  $\leq 25\%$  cells displayed the normal tubular mitochondria seen in control cells, and  $\geq 75\%$  of the HF-LPLI-treated cells had mitochondria with a fragmented, punctiform morphology (Fig. 2A,B). These data demonstrate that HF-LPLI induces mitochondrial fragmentation by triggering oxidative stress.

We also investigated the correlation between the laser fluence of HF-LPLI and the severity of mitochondrial fragmentation in the two cell lines. STS was used as a positive control to induce mitochondrial fragmentation (Fig. 2B). Cells were irradiated at various laser fluences in the range from 60 to 240 J·cm<sup>-2</sup>. A significant positive correlation was found between laser fluence and the percentage of cells with fragmented mitochondria (Fig. 2B). Moreover, HF-LPLI-induced mitochondrial fragmentation was completely prevented by vitamin C pretreatment (Fig. 2A,B), demonstrating that the changes were mediated by oxidative stress caused by HF-LPLI.

#### **HF-LPLI** inhibits mitochondrial fusion

Given the alterations in mitochondrial morphology under HF-LPLI treatment, it is likely that an impaired fission-fusion balance is involved. To measure the



Fig. 2. HF-LPLI causes mitochondrial fragmentation through ROS generation (A) ASTC-a-1 cells and COS-7 cells were transiently transfected with pDsRed-mit, and, 48 h after transfection, cells expressing DsRed-mit were subjected to various treatments. Scale bars: 10 µm. Representative confocal microscopic images show two types of mitochondrial morphology under normal conditions and HF-LPLI treatment (120 J·cm<sup>-2</sup>): normal tubular and fragmented mitochondria, respectively. Vitamin C (Vit C) pretreatment totally prevented mitochondrial fragmentation caused by HF-LPLI. (B) ASTCa-1 cells and COS-7 cells expressing DsRedmit were treated with HF-LPLI at a fluence of 60–240  $\mathrm{J}{\cdot}\mathrm{cm}^{-2}$  with or without vitamin C. Quantitative analysis of the percentage of cells with fragmented mitochondria reveals that HF-LPLI increases fragmentation of mitochondria, and that the fragmentation correlates with the laser fluence and is prevented by vitamin C pretreatment. STS was used as a positive control to identify mitochondrial fragmentation. Data represent the mean ± standard error of the mean of at least five independent experiments (\*P < 0.05, Student's t-test).

occurrence of mitochondrial fission-fusion events under HF-LPLI treatment (120 J·cm<sup>-2</sup>), ASTC-a-1 cells were labeled with the mitochondria-targeted fluorescent probe MitoTracker. Cells with similar shapes were chosen and monitored by time-lapse confocal microscopic imaging. Mitochondrial behavior in the entire cell was monitored for the following 25 min, with or without HF-LPLI treatment (Fig. 3). It took 20 min for the mitochondria to complete the fissionfusion cycle under normal conditions (Fig. 3, upper panel). However, we did not observe mitochondrial fusion caused by HF-LPLI even with a longer period of treatment (25 min) (Fig. 3, lower panel). Because the two-dimensional picture did not convincingly demonstrate the mitochondrial fission-fusion events, we obtained the three-dimensional picture with the use of Z-stack software and confocal microscopy to confirm

the occurrence of fission-fusion events in each mitochondrial morphological study (data not shown). These data demonstrated that HF-LPLI inhibits or delays mitochondrial fusion.

## Recruitment of Drp1 to mitochondria caused by HF-LPLI

We explored the involvement of Drp1 in HF-LPLI-induced apoptosis at a fluence of 120 J·cm<sup>-2</sup> in ASTC-a-1 cells. Both control cells and HF-LPLI-treated cells were labeled with MitoTracker, and endogenous Drp1 in the cells was detected by immunofluorescence. Images were obtained by confocal microscopy. As shown in Fig. 4A, HF-LPLI resulted in an obvious increase in the mitochondrial accumulation of Drp1. Vitamin C pretreatment totally prevented HF-LPLI-induced mitochondrial





accumulation of Drp1 (Fig. 4A). Also, cells were transiently cotransfected with pYFP-Drp1 and pDsRed-mit, and 48 h after transfection, cells coexpressing the two plasmids were subjected to HF-LPLI treatment. Yellow fluorescent protein (YFP)-Drp1 was mainly localized in the cytoplasm, but a small amount was associated with mitochondria (Fig. 4B, upper panel). The subcellular locations of YFP-Drp1 were changed from partial association with mitochondria to complete recruitment to mitochondria in response to HF-LPLI (Fig. 4B, lower panel). We also assessed the cycling properties of YFP-Drp1 at 150 min after HF-LPLI treatment, using fluorescence recovery after photobleaching (FRAP). As expected, we observed almost complete inhibition of the fluorescence recovery of YFP-Drp1 in cells treated with HF-LPLI (Fig. 4C), indicating the stable association of Drp1 with mitochondria induced by HF-LPLI. Western blotting analysis of the levels of Drp1 in the mitochondrial and cytosolic fractions also demonstrated the recruitment of Drp1 to mitochondria under HF-LPLI treatment (Fig. 4D). These data demonstrate that Drp1 is activated and probably involved in HF-LPLI-induced mitochondrial fragmentation.

## Effects of Drp1/Mfn2 overexpression on mitochondrial dynamics in cells under normal conditions

To visualize mitochondria, ASTC-a-1 cells were transiently cotransfected with pDsRed-mit and pYFP-Drp1/ YFP-Mfn2. The efficiency of transient transfection was demonstrated by flow cytometry (FCM) analysis (Fig. 5A). Forty-eight hours after transfection, the positively transfected cells were evaluated by confocal microscopy (Fig. 5B). At steady state, most control cells (i.e. only pDsRed-mit positively transfected cells) (~99%) showed normal, short, tubular mitochondria (Fig. 5B,C). Conversely, among Drp1-overexpressing cells, ~9% displayed normal mitochondria as seen in control cells (Fig. 5B,C), but ~90% had mitochondria with a fragmented, punctiform structure (Fig. 5B,C). Also, Mfn2-overexpressing cells showed a large population (~92%) of mitochondria with an elongated, net-like structure (Fig. 5B,C). These data demonstrate that Drp1 overexpression causes mitochondrial fission and Mfn2 overexpression causes mitochondrial elongation in ASTC-a-1 cells.

#### Effects of Drp1 and Mfn2 on mitochondrial dysfunction under HF-LPLI treatment

Because mitochondrial morphology is critical for mitochondrial function, we investigated the effect of Drp1/Mfn2 overexpression on mitochondrial dysfunction under HF-LPLI treatment (60-240 J·cm<sup>-2</sup>). ASTC-a-1 cells were transiently transfected with pDrp1/Mfn2, and 48 h after transfection the transfectants were selected by growth in medium containing G418 for the next 24 h. Then, overexpression was confirmed by western blot analysis (Fig. 6A). ROS levels (as indicated by the DCF fluorescent signal) were significantly increased in HF-LPLI-treated cells as compared with control cells (Fig. 6B). Notably, transient overexpression of either Drp1 or Mfn2 did not change the ROS levels in HF-LPLI-treated cells. These data demonstrate that neither Drp1 nor Mfn2 overexpression affects mitochondrial oxidative stress caused by HF-LPLI.



**Fig. 4.** HF-LPLI causes recruitment of Drp1 to mitochondria. (A) Mitochondria in ASTC-a-1 cells were stained with MitoTracker (red emission), and endogenous Drp1 was detected by immunofluorescence with antibody against Drp1 (green emission) 170 min after HF-LPLI treatment (120 J·cm<sup>-2</sup>). The merged image clearly shows the recruitment of Drp1 to mitochondria in response to HF-LPLI treatment. Vitamin C (Vit C) pretreatment totally prevented mitochondrial translocation of Drp1 induced by HF-LPLI. Data are representative confocal microscopic images of at least five independent experiments. Scale bars: 10  $\mu$ m. (B) ASTC-a-1 cells were transiently cotransfected with pYFP-Drp1 and pDsRed-mit. Forty-eight hours after transfection, cells coexpressing YFP-Drp1 and DsRed-mit were treated with HF-LPLI at a fluence of 120 J·cm<sup>-2</sup>. The control group received no treatment. Representative confocal microscopic images reveal increased association of YFP-Drp1 with mitochondria in response to HF-LPLI (n = 5). Scale bars: 10  $\mu$ m. (C) ASTC-a-1 cells were transiently transfected with pYFP-Drp1. Forty-eight hours after transfection, relative fluorescence emission intensities of YFP-Drp1 recorded during photobleaching protocols were plotted as a function of time. YFP-Drp1 cycling between cytosol and mitochondria completely ceased after HF-LPLI treatment. Data represent the mean  $\pm$  standard error of the mean of at least five independent experiments. (D) Western blot analysis was employed to study the translocation of Drp1 to mitochondria in response to HF-LPLI treatment. The levels of Drp1 in the cytosolic fraction decreased by 150 min post-HF-LPLI treatment. Drp1 was activated by HF-LPLI.

We also measured mitochondrial membrane potential (MMP) with the fluorescent dye tetramethylrhodamine methyl ester (TMRM), and the level of the decrease in MMP in HF-LPLI-treated cells was not changed by either Drp1 or Mfn2 overexpression (Fig. 6C,D). Also, vitamin C pretreatment totally prevented the change in MMP caused by HF-LPLI (Fig. 6D). These data demonstrate that neither Drp1 nor Mfn2 overexpression affects mitochondrial depolarization caused by HF-LPLI.



**Fig. 5.** Effects of Drp1 and Mfn2 on mitochondrial morphology (A) ASTC-a-1 cells were transiently cotransfected with pYFP-Drp1/YFP-Mfn2 and pDsRed-mit. The transfection efficiencies of YFP-Drp1 and YFP-Mfn2 were detected by FCM 48 h after transfection. Mitochondrial morphology was monitored in cells coexpressing YFP-Drp1/YFP-Mfn2 and DsRed-mit. Representative confocal microscopic images (B) and quantification analysis (C) reveal that transient overexpression of Drp1 promotes mitochondrial fragmentation, whereas overexpression of Mfn2 causes elongated mitochondrial morphology. Data represent the mean ± standard error of the mean of at least five independent experiments (\**P* < 0.05, Student's *t*-test). Scale bars: 10 μm.



To explore the role of Drp1 in mitochondrial proapoptotic functions under HF-LPLI treatment, we knocked down Drp1 expression with the short hairpin-

activated gene silencing system. To generate ASTC-a-1 cell lines that stably suppress the endogenous gene for Drp1, we transferred plasmids containing Drp1 short

Fig. 6. Effects of Drp1 and Mfn2 on HF-LPLI-induced mitochondrial functional changes. ASTC-a-1 cells were transiently transfected with pDrp1/Mfn2. Forty-eight hours after transfection, the transfectants were selected by growth in medium containing G418 for the next 24 h. Transfection efficiencies were examined by western blot analysis with antibody against Drp1/Mfn2 (A). Relative DCF (B) and TMRM (C) fluorescence emission intensities in cells after HF-LPLI treatment were measured as described in Experimental procedures. Data represent the mean ± standard error of the mean of at least five independent experiments (\*P < 0.05, Student's t-test). (D) Quantitative analysis of TMRM fluorescence emission intensities over time after various treatments. Data represent the mean ± standard error of the mean of at least five independent experiments (\*P < 0.05, Student's t-test). Neither Drp1 nor Mfn2 overexpression had an effect on mitochondrial depolarization and ROS generation caused by HF-LPLI (120 J·cm<sup>-2</sup>). Vitamin C (Vit C) pretreatment totally prevented mitochondrial depolarization caused by HF-LPLI. (E) Both normal cells and Drp1 RNAi cells were treated with HF-LPLI (120 J·cm<sup>-2</sup>), fractionated into cytosol and mitochondria, and analyzed for the distribution of Bax and Drp1 by western blot analysis. The fractionation quality was verified by the distribution of specific subcellular markers: COX IV for mitochondria and β-actin for cytosol. Drp1 knockdown did not affect translocation of Bax to mitochondria following HF-LPLI treatment. (F) Immunoblotting of ASTC-a-1 cells treated with HF-LPLI with or without Drp1/Mfn2 overexpression was performed to detect the level of cytochrome c, with COX IV and β-actin as markers of the amounts of mitochondrial and cytosolic proteins in cells, respectively. Cells without any treatment were set as control groups. (G) Spectrofluorometric analysis of caspase-9 activation after HF-LPLI treatment (120 J·cm<sup>-2</sup>) in living cells. The cells were excited at the wavelength of CFP (434 ± 5 nm), resulting in a CFP emission peak (476 nm) and FRET emission peak (528 nm) caused by FRET from CFP. The fluorescence emission spectra were obtained with a luminescence spectrometer. Data represent the mean ± standard error of the mean of at least five independent experiments (\*P < 0.05 versus control group; #P < 0.05 versus the indicated group; Student's t-test). Cytochrome c release and caspase-9 activation were both enhanced by Drp1 overexpression, but inhibited by Mfn2 overexpression. (H) Representative cell death analysis by FCM based on annexin V-FITC/PI double staining was performed in cells under various treatments. (I) Quantitative analysis of the FCM data shown in (H). Drp1 overexpression promotes cell apoptosis caused by HF-LPLI, whereas Mfn2 overexpression inhibits the apoptosis. Vitamin C pretreatment totally prevented the apoptosis caused by HF-LPLI. Z-VAD-fmk pretreatment also inhibited the apoptosis caused by HF-LPLI. Data represent the mean ± standard error of the mean of at least five independent experiments (\*P < 0.05 versus control group; Student's t-test).

hairpin RNA (shRNA) into cells with G418 as a selective marker. Selection of the transfected cells with nearly complete depletion of Drp1 required  $\sim$  1 week of growth in the presence of G418. Bax activation was monitored under HF-LPLI treatment by western blot analysis (Fig. 6E). In control cells, Bax was detected mainly in the cytosolic fraction (Fig. 6E). Following HF-LPLI treatment, Bax levels decreased in the cytosolic fraction and increased in the mitochondrial fraction, suggesting activation of Bax caused by HF-LPLI. However, the levels of Bax in the two sections were not affected by Drp1 RNA interference (RNAi) (Fig. 6E), demonstrating that Drp1 is not required for Bax activation in response to HF-LPLI.

Cytochrome c release was also monitored under HF-LPLI treatment, by western blot analysis. The results are shown in Fig. 6F. In control cells, cytochrome cwas detected mainly in the mitochondrial fraction (Fig. 6F). Following HF-LPLI treatment, cytochrome c levels decreased in the mitochondrial fraction (Fig. 6F). Drp1 overexpression accelerated the release of cytochrome c to the cytosol, whereas Mfn2 overexpression had an opposite effect (Fig. 6F).

To examine the effect of Drp1/Mfn2 on caspase-9 activation following HF-LPLI treatment, spectrofluorometric analysis, a technique for monitoring the overall profile of Förster resonance energy transfer (FRET) fluorescence emission from a group of cells, was applied to measure the activation of caspase-9. ASTCa-1 cells stably expressing SCAT9 were transiently transfected with pDrp1/Mfn2. Forty-eight hours after transfection, the samples were treated with HF-LPLI at a fluence of 120 J·cm<sup>-2</sup>. Six hours later, all groups were investigated with a luminescence spectrometer for fluorescence emission spectra. The FRET/cyan fluorescent protein (CFP) ratio is inversely proportional to the caspase-9 activity. Under HF-LPLI treatment, caspase-9 activity in the cells overexpressing Drp1 was much lower than that in non-overexpressing cells, whereas the activity of caspase-9 in Mfn2-overexpressing cells was higher than that in non-overexpressing cells (Fig. 6G). These data suggest that Drp1 overexpression promotes caspase-9 activation caused by HF-LPLI, whereas Mfn2 overexpression inhibits this activation.

Analysis of the results of FCM based on annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining was used to determine the role of Drp1/Mfn2 overexpression in cell apoptosis 6 h after HF-LPLI treatment. The data in Fig. 6H,I show that cell apoptosis caused by HF-LPLI was significantly promoted by Drp1 overexpression, whereas Mfn2 over-expression had the opposite effect. Vitamin C pre-treatment totally prevented the apoptosis (Fig. 6H,I), demonstrating that ROS generation is a determinant of HF-LPLI-induced apoptosis. Also, HF-LPLI-induced apoptosis could be inhibited by the caspase inhibitor z-VAD-fmk (Fig. 6H,I), demonstrating that the apoptosis was caspase-dependent. Experiments performed in COS-7 cells gave similar results (Fig. 6I).

#### Discussion

Here, we have presented a method to expose mitochondria in ASTC-a-1 cells and COS-7 cells to incremental doses of ROS by photoexcitation of endogenous photoacceptor (cytochrome c oxidase) in the mitochondrial respiratory chain [16-18] by HF-LPLI, the result that has been clearly identified in our earlier studies [13,14]. This method of localized excitation caused the generation of 'triggering' ROS (presumably singlet oxygen) inside mitochondria (Fig. 1), enabling us to demonstrate the induction of mitochondrial fragmentation caused by mitochondrial oxidative stress in living cells, as the ROS scavenger prevented the fragmentation (Fig. 2). In this study, we have shown that HF-LPLI results in perturbed mitochondrial dynamics and causes mitochondrial fragmentation that impacts on mitochondrial function and cell function. It is likely that HF-LPLI affects mitochondrial dynamics through the differential modulation of mitochondrial fission and fusion proteins, causing an impaired mitochondrial fission-fusion balance, because manipulation of Drp1 and Mfn2 expression changed the effects of HF-LPLI.

One major observation of this study was that HF-LPLI-induced oxidative stress caused mitochondrial fragmentation in ASTC-a-1 cells and COS-7 cells, as shown by confocal microscopic imaging (Fig. 2). Because mitochondrial morphology is tightly controlled by the balance between mitochondrial fission and fusion [12], we hypothesize that HF-LPLI-induced mitochondrial fragmentation is caused by enhanced fission, reduced fusion, or both. In support of this notion, using the mitochondria-targeted fluorescent probe MitoTracker, we were able to demonstrate that mitochondria in HF-LPLI-treated cells were nearly unable to fuse as compared with control mitochondria (Fig. 3). At the molecular level, we found that HF-LPLI-triggered ROS resulted in Drp1 activation, as indicated by a significantly increased association with mitochondria (Fig. 4). Although the ROS originate predominantly from mitochondria, owing to photoexcitation of cytochrome c oxidase, they may diffuse out to the cytosol, as shown in Fig. 1A. Also, the added vitamin C can be easily taken up by the cytosol, whereas it can be taken up only slowly by mitochondria. Therefore, the inhibition of Drp1 mitochondrial translocation by vitamin C may occur primarily through changes in cytosolic ROS. The activation of Drp1 may contribute to the increased fission rate in our models, as it has been reported that a cell line with an endogenous loss of activity mutation in Drp1 displays resistance to hydrogen peroxide-induced cell death [19]. The change in Drp1 activity caused by HF-LPLI is highly likely to be involved in a mechanism of calcineurin-dependent translocation of the Drp1 to mitochondria in dysfunction-induced fragmentation [20,21]. In detail, when mitochondrial depolarization is associated with a sustained cytosolic Ca<sup>2+</sup> rise, it activates the cytosolic phosphatase calcineurin, which normally interacts with Drp1 [20]. Calcineurin-dependent dephosphorylation of Drp1, and in particular of its conserved Ser637, regulates its translocation to mitochondria, as substantiated by site-directed mutagenesis [20]. Our results support this point of view, because ROS generation in response to HF-LPLI can induce obvious and severe mitochondrial depolarization [13,14], and increased  $Ca^{2+}$  levels are also seen after HF-LPLI (data not shown). On the other hand, opinion on the relationship between apoptosis and fission is divided about whether this phenomenon of fragmentation is simply a consequence of apoptosis or plays a more active role in the process. Some investigators have suggested that mitochondrial fission may promote cytochrome c release and therefore act to drive caspase activation during apoptosis [22,23]. However, other data suggest that apoptosis-associated mitochondrial fission is a consequence rather than a cause of apoptosis, and reflects events involving some hitherto unrecognized connection between members of the Bcl-2 family and the mitochondrial morphogenesis machinery [24-27]. Therefore, it is probable that oxidative stress is impacting directly on cell death without affecting mitochondrial fragmentation.

It is known that changes in mitochondrial morphology often affect mitochondrial function [28,29]. In this regard, it is important to note that aspects of mitochondrial dysfunction following HF-LPLI, i.e. increased ROS levels, reduced MMP, Bax activation, and cytochrome c release (Fig. 6B–F), were all observed in the cells characterized by mitochondrial fragmentation (Fig. 2). Therefore, it is likely that mitochondrial fragmentation contributes to HF-LPLIinduced mitochondrial dysfunction. In support of this, Drp1 overexpression accelerated cytochrome c release and caspase-9 activation, and thus promoted cell apoptosis, under HF-LPLI treatment, whereas Mfn2 overexpression inhibited these processes (Fig. 6F-I). It has been reported that, upon apoptotic stimulation, Drp1 is recruited to the mitochondrial outer membrane [22,30-32], where it colocalizes with Bax and Mfn2 at fission sites [24,33]. Drp1 function is required for apoptotic mitochondrial fission, as expression of a dominant negative mutant (Drp1K38A) or downregulation of Drp1 by RNAi delays mitochondrial fragmentation, cytochrome c release, caspase activation, and cell death [22,30,34,35]. In the present study, Drp1 knockdown had no effect on Bax activation by HF-LPLI (Fig. 6E). The data suggest that the modulation of cytochrome c release by Drp1 is a downstream event of Bax activation.

On the other hand, Drp1/Mfn2 overexpression modulated the release of cytochrome c (Fig. 6F), whereas it had no effect on HF-LPLI-induced generation of ROS and collapse of MMP (Fig. 6B-D), indicating that apoptosis-associated fission was a relatively downstream event and quite close to mitochondrial outer membrane permeabilization. It is known that HF-LPLI triggers mitochondrial ROS generation through excitation of the endogenous photoacceptor cytochrome c oxidase in the mitochondrial respiratory chain [13,14]. The process may not be affected by Drp1/Mfn2, as there has been no report indicating a correlation between Drp1/Mfn2 and the complexes in the respiratory chain. Previously, we have demonstrated that HF-LPLI-induced MMP is caused by the ROS-induced opening of mitochondrial permeability transition pores, but not the direct permeabilization of the outer mitochondrial membrane [14]. This may explain why Drp1/Mfn2 can modulate cytochrome c release but not mitochondrial depolarization. Nevertheless, given the presence of HF-LPLI-triggered ROS in mitochondria and the mitochondrial locations of other mitochondrial dynamic-regulated proteins, the possibility that HF-LPLI may also induce mitochondrial dysfunction through other pathways cannot be ruled out.

Taken together, the findings presented here suggest that abnormal mitochondrial dynamics are involved in mitochondrial dysfunction after HF-LPLI stimulation. The data indicate a balance tipped towards mitochondrial fission that facilitates HF-LPLI-induced apoptosis. Given the study on mitochondrial fission performed in ASTC-a-1 cells and COS-7 cells, it is very likely that abnormal mitochondrial dynamics may be a common pathway leading to cellular dysfunction that is critical to apoptosis in various cells.

#### **Experimental procedures**

#### Cell culture and transfection

ASTC-a-1 cells and COS-7 cells were grown on 22-mm culture glasses, in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (Gibco, Grand Island, NY, USA), 50 units·mL<sup>-1</sup> penicillin, and 50  $\mu$ g·mL<sup>-1</sup> streptomycin, in 5% CO<sub>2</sub>/95% air at 37 °C in a humidified incubator. In all experiments, 70– 85% confluent cultures were used. Downregulation of Drp1 levels in ASTC-a-1 cells was achieved by RNAi, using a vector-based shRNA approach, with the target sequence for Drp1 (a gift from J.-C. Martinou, University of Geneva, Switzerland). The shRNA sequences were transfected into cells with Lipofectamine 2000 reagent (Invitrogen Life Technologies, Grand Island, NY, USA), according to the manufacturer's protocol. Forty-eight hours after transfection, the cells were washed once with NaCl/Tris and grown in fresh medium supplemented with 800  $\mu$ g·mL<sup>-1</sup> G418 (Sigma-Aldrich, St Louis, MO, USA) for another 24 h to select the transfectants. The cultures were then washed with NaCl/P<sub>i</sub>, and incubated in fresh growth medium in order to start the experiment.

#### Chemicals

The following fluorescent probes were used in our experiments: MitoTracker (100 nM) was used to label mitochondria; H2DCFDA (10  $\mu$ M) was used to detect the generation of ROS; and TMRM (100 nM) was used to monitor MMP. All of these probes were purchased from Molecular Probes (Eugene, OR, USA). The optimal incubation time for each probe was determined experimentally.

STS (1 µM) was purchased from Sigma-Aldrich (St Louis, MO, USA). zVAD-fmk (20 µM) was purchased from BD PharMingen (San Diego, CA, USA). Dehvdroascorbic acid. an oxidized form of ascorbic acid (vitamin C; Sigma-Aldrich, St Louis, MO, USA) (100, 200 and 400 µM for 60, 120 and 240 J·cm<sup>-2</sup> HF-LPLI treatments, respectively), was added to the cell culture medium 30 min before laser irradiation in order to scavenge ROS. Monoclonal antibodies against cytochrome c and cytochrome c oxidase subunit IV (COX IV) were purchased from BD PharMingen. Monoclonal antibodies against Drp1, Mfn2 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies against Bax were purchased from Cell Signaling Technology (Beverly, MA, USA). Mitochondrial and cytosolic fractions were obtained with the Mitochondria Isolation Kit (Cat: KGA 827; KeyGEN, Nanjing, China).

#### HF-LPLI treatment for single-cell analysis

For irradiation of cells, a 633-nm He–Ne laser inside a confocal laser scanning microscope (LSM510-ConfoCor2) (Zeiss, Jena, Germany) was used in HF-LPLI treatment. Laser irradiation was performed through the objective lens of the microscope. In this setup, only the cells under observation were irradiated by the laser. A minitype culture chamber with a CO<sub>2</sub> supply (Tempcontrol 37-2 digital; Zeiss) was used in order to keep cells under normal culture conditions (37 °C, 5% CO<sub>2</sub>) during irradiation. Under the HF-LPLI treatment, the cells in selected area were irradiated for 10 min with a laser fluence of 60, 120 or 240 J·cm<sup>-2</sup>.

#### **Detection of ROS generation and MMP decrease**

Cells grown on 96-well plates were labeled with H2DCFDA or TMRM and then irradiated with an He–Ne laser (635 nm, semiconductor laser, NL-FBA-2.0-635; nLight Photonics Corporation, Vancouver, WA, USA) at fluence of 60, 120 or 240 J·cm<sup>-2</sup> in the dark. The interval wells were filled with ink in order to minimize the scattered or reflected light. Immediately after irradiation, fluorescence emission intensity was recorded at the indicated time with an Infinite 2000 plate reader (Tecan, Mönnedorf, Switzerland).

#### Immunofluorescence microscopy

Cells grown on 22-mm culture glasses were first labeled with MitoTracker, and then fixed for 10 min in 4% paraformaldehyde; this was followed by permeabilization with 0.15% Triton X-100 in NaCl/P<sub>i</sub> for 15 min. The cells were then incubated for 1 h in blocking buffer (2% BSA in NaCl/P<sub>i</sub>), and overnight with a rabbit monoclonal antibody against Drp1 (dilution range 1 : 50 to 1 : 500) antibody. Cells were washed three times for 10 min each in blocking buffer, and then incubated for 2 h with secondary antibody [Alexa Fluor 488 dye-conjugated goat anti-(rabbit IgG)] (argon ion laser, excitation 488 nm, emission bandpass 500–550 nm) (Molecular Probes, Eugene, OR, USA). Images were acquired with a LSM510-ConfoCor2 microscope (Zeiss) through a 40× oil fluorescence objective (LSM510-ConfoCor2; Zeiss, Jena, Germany).

#### Imaging analysis of living cells

In order to obtain images of single cells, the confocal microscope was used. Cell images before and after laser irradiation were acquired with a Plan-Neofluar 100×/NA1.3, oil-immersed objective lens. Cells were maintained at 37 °C and 5% CO<sub>2</sub> during imaging, in a minitype culture chamber with a CO<sub>2</sub> supply. The following specific settings were used for light excitation and emissions. Mito-Tracker: He-Ne laser; excitation, 633 nm; emission (long pass), 650 nm. DCF: argon ion laser; excitation, 488 nm; emission (bandpass), 500-530 nm. TMRM: He-Ne laser; excitation, 543 nm, emission (bandpass), 565-615 nm. DsRed: argon ion laser; excitation, 488 nm; emission (long pass), 560 nm. YFP: argon ion laser; excitation, 514 nm; emission (bandpass), 530-550 nm. For intracellular measurements, the desired areas were chosen in the confocal image. Quantitative analysis of mitochondrial shape changes was performed by evaluating which cells displayed fragmented mitochondria after addition of the inducer at the indicated time. Organelles were classified as fragmented when 50% of the total cellular mitochondria displayed a major axis of  $< 2 \mu m$  in ASTC-a-1 cells and COS-7 cells [36].

#### FRAP

FRAP analysis was performed with an LSM510-ConfoCor2 confocal laser-scanning microscope. Briefly, ASTC-a-1 cells coexpressing YFP-Drp1 and DsRed-mit were treated without or with HF-LPLI (120 J·cm<sup>-2</sup>) for 10 min. Then, each mitochondrial region of interest was photobleached by scanning for 50 s with an argon laser at the highest power. Decrease in fluorescence in the region after photobleaching and recovery of fluorescence in the region after photobleaching were then analyzed by confocal microscopy with low laser power at the indicated times after photobleaching. For all of the images, the noise levels were reduced by line scan averaging.

#### FCM

For FCM analysis, an annexin V–FITC Apoptosis Detection Kit (annexin V–FITC conjugate, PI dyes, and binding buffer) (BD PharMingen) was used. FCM was performed on a FACScanto II flow cytometer (Becton Dickinson, Mountain View, CA, USA) with excitation at 488 nm. Fluorescent emission of FITC was measured at bandpass 515–545 nm, and that of DNA–PI complexes at 564– 606 nm. Cell debris was excluded from analysis by an appropriate forward light scatter threshold setting. Compensation was used wherever necessary. The number of events considered in FCM was 10 000 per independent experiment.

#### Western blot analysis

Cells were harvested in 300 µL of lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 50 mM  $\beta$ -glycerophosphate, 1% Triton X-100, 100 mM phenylmethanesulfonyl fluoride). The resulting lysates were resolved by 4–2% SDS/PAGE, and transferred to pure nitrocellulose blotting membranes (BioTrace NT; Pall Life Science, Pensacola, FL, USA). The membranes were blocked in 10 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20 containing 5% nonfat milk, and then probed with different antibodies (Drp1, 1:1000; Mfn2, 1:1000; COX IV, 1:1000;  $\beta$ -actin, 1:2000; Bax, 1:1000; cytochrome *c*, 1:1000). Proteins were detected with an Odyssey two-color infrared imaging system (LI-COR; Lincoln, NE, USA). Mitochondrial isolation was performed with a mitochondrial isolation kit (Cat: KGA 827; KeyGEN).

#### Spectrofluorometric analysis

ASTC-a-1 cells stably expressing SCAT9, a FRET reporter of caspase-9 activity [15], were transiently transfected with pDrp1/Mfn2, cultured for 48 h, and then treated with HF-LPLI (120 J·cm<sup>-2</sup>). The cells were transferred into a 96-well flat-bottomed microplate. The microplate was then

placed inside the sample chamber of a luminescence spectrometer (LS55; PerkinElmer, Wellesley, MA, USA), and the fluorescence emission spectra were then acquired. The step length of the scanning spectra was 2 nm. The excitation wavelength of SCAT9 was  $434 \pm 5$  nm, and the emission fluorescence channel was bandpass 454-600 nm.

#### Statistics

Unless otherwise indicated, data were analyzed with oneway ANOVA. All results shown were from at least five independent experiments.

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