Infrared Radiation Affects the Mitochondrial Pathway of Apoptosis in Human Fibroblasts

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We have previously observed that near-infrared (IR) pre-irradiation protects normal human dermal fibroblasts from ultraviolet (UV) cytotoxicity *in vitro*. Here, we show that IR pre-irradiation of human fibroblasts inhibited UVB activation of caspase-9 and -3, leading us to study early events in the mitochondrial apoptotic pathway after IR irradiation. IR irradiation led to a partial release of cytochrome *c* and Smac/Diablo but not apoptosis-inducing factor (AIF). This was accompanied by a slight but transient decrease in the mitochondrial membrane potential ($\Delta\psi_m$) and by the insertion of Bax into mitochondrial membrane. Early apoptotic events in the mitochondrial pathway thus occurred after IR irradiation despite a lack of caspase-9 and -3 activation. This could be explained by the induction by IR of the expression of heat shock protein Hsp27, which is known to prevent apoptosome assembly. Furthermore, the balance between pro-apoptotic (i.e., Bax) and anti-apoptotic (i.e., Bcl-2 or Bcl-x_L) proteins, which was rather pro-apoptotic after IR exposure, became anti-apoptotic 24 h later, suggesting a protective effect. Together, these actions could also contribute to prepare the cell to resist UVB-triggered apoptosis. Finally, isolated rat liver mitochondria-released cytochrome *c* in response to IR, demonstrating that mitochondria were a primary target of IR radiation.

Key words: apoptosis/cytochrome *c*/infrared irradiation/mitochondria
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Infrared (IR) irradiation is used for therapeutic purposes including surgery (Kaufmann *et al*, 1994), or promotion of wound healing (Horwitz *et al*, 1999; Danno *et al*, 2001; Toyokawa *et al*, 2003). This laser medicine is based on the mechanism of photoenergy conversion in heating, but has also been suggested to activate photoacceptors such as cytochrome *c* oxidase, pointing to a particular role for mitochondria (Karu, 1999).

In addition, despite the fact that IR (700–4000 nm) accounts for 40% of the solar radiation reaching the earth's surface, very little is known about its biological effects. Nevertheless, several mechanisms of action of non-coherent IR radiation have been proposed. Mitogen-activated protein kinases (MAPK) have been shown to be involved in IR induction of matrix metalloproteinase 1, or collagenase (Schieke *et al*, 2002). Ferritin has also been proposed as a possible cell defense system induced by IR (Applegate *et al*, 2000).

Furthermore, most of our knowledge on solar radiation is based on monochromatic ultraviolet (UV) and interactions between the different solar wavelengths have been overlooked. Nevertheless, we have previously shown that prior treatment with naturally occurring doses of IR protects normal human dermal fibroblasts from UV toxicity, *in vitro* (Menezes *et al*, 1998). This effect was observed under temperature-controlled conditions, independently of heat shock protein induction (Hsp72–70) (Menezes *et al,* 1998; Schieke *et al*, 2002), and was independent of cell division (Menezes *et al*, 1998).

In order to better understand how IR prevents UV cytotoxicity (Menezes *et al*, 1998), we evaluated the effect of IR radiation on UVB-induced apoptosis and investigated more particularly the influence of IR on early apoptotic events involving mitochondria, which contain IR chromophores such as cytochrome *c* oxidase as previously suggested (Karu, 1999).

In fact, mitochondria play a key role in type II apoptosis, by releasing pro-apoptotic factors, such as cytochrome *c*, Smac/Diablo and AIF (apoptosis-inducing factor), from the intermembrane space into the cytoplasm. Cytochrome *c*, when associated with Apaf-1 (apoptosis protease activating factor 1), activates caspase-9 (Li *et al*, 1997), which in turn activates caspase-3 (Li *et al*, 1997; Green, 1998). Smac/Diablo binds to XIAP (X chromosome inhibitor of apoptosis protein) and blocks its inhibitory activity, indirectly leading to caspase activation (Du *et al*, 2000). AIF, when translocated to the nucleus, triggers apoptosis through a caspase-independent pathway, by inducing chromatin condensation and DNA fragmentation (Susin *et al*, 1999).

In addition, a reduction in mitochondrial transmembrane potential ($\Delta\psi_m$) is also detected during the early stages of the apoptotic process (Zamzami *et al*, 1996), when cells are not irreversibly committed to death (Vayssiere *et al*, 1994). These early events, mitochondrial protein release and re-

Abbreviations: AIF, apoptosis-inducing factor; Hsp, heat shock protein; IR, infrared; $\Delta\psi_m$, mitochondrial transmembrane potential

duction in $\Delta\psi_m$, are regulated by pro-apoptotic proteins such as Bax (Marzo *et al*, 1998; Finucane *et al*, 1999) and anti-apoptotic proteins such as Bcl-2 and Bcl-x_L (Shimizu *et al*, 1996; Kluck *et al*, 1997; Yang *et al*, 1997; Gross *et al*, 1998; Vander Heiden and Thompson, 1999; Sun *et al*, 2002). The ratio of Bcl-2 to Bax determines whether a cell survives or dies following an apoptotic stimulus (Oltvai *et al*, 1993).

Finally, other anti-apoptotic molecules such as the small Hsp27 protect cells against apoptosis triggered by hyperthermia (Landry *et al*, 1989) and by a variety of other stimuli, including oxidative stress (Mehlen *et al*, 1993), staurosporine, Fas ligand, (Mehlen *et al*, 1996b), or tumor necrosis factor-alpha (TNF)-α (Mehlen *et al*, 1996a). Interestingly, Hsp27 can act both upstream of mitochondria, by inhibiting the release of cytochrome *c* (Paul *et al*, 2002), and downstream of mitochondria, by preventing caspase-3 and -9 activation (Garrido *et al*, 1999; Pandey *et al*, 2000).

In the present work, we show that IR inhibited UVB-induced caspase-9 and -3 activation, and focus on the effects of IR on mitochondria, by analyzing the release of the pro-apoptotic molecules such as cytochrome *c*, Smac/Diablo, or AIF, and the changes in $\Delta\psi_m$. The importance of the role of mitochondria in the action of IR is confirmed by studying cytochrome *c* release by isolated rat liver mitochondria. Finally, to determine how IR impaired caspase activation, we studied the effects of IR on the balance of pro- (Bax) and anti- (Bcl-2, Bcl-x_L, Hsp27) apoptotic molecules modulating the $\Delta\psi_m$ and the release of mitochondrial proteins.

Results

IR pre-irradiation inhibits UVB activation of caspase-9 and -3 Caspase activation was studied using the most efficient IR protocol found to prevent UV-induced cytotoxicity (Menezes *et al*, 1998), i.e., three 30 min sessions (3×810 kJ per m^2) followed 24 h later, by UVB irradiation. Caspase activation was analyzed 4 d later.

A significant increase in both caspase-9 and -3 activity was found in UVB-irradiated cells (1500 J per m^2). IR pre-irradiation significantly reduced UVB-induced caspase activation. No activation of either caspase-9 or 3, however, was detected after IR irradiation only (Fig 1). These results showed that IR pre-irradiation caused an inhibition of UVB-induced caspase activation.

IR induces cytochrome *c* release from mitochondria Early apoptotic events involving the mitochondrial pathway were studied after a single 60 min IR irradiation (1620 kJ per m^2). The cellular localization of cytochrome *c* was examined using laser confocal microscopy. In non-irradiated cells, cytochrome *c* staining showed a punctuate cytoplasmic pattern in keeping with its mitochondrial localization. In contrast, IR-irradiated cells exhibited a diffuse cytochrome *c* staining, suggesting a translocation from mitochondria to the cytoplasm (Fig 2).

A time-course study of cytochrome *c* release showed the presence of a cytosolic fraction of cytochrome *c* as early as 3 h after IR irradiation, a further increase was observed at 6 h (Fig 2), which persisted until 24 h. The pattern of cytochrome *c* staining resembled control cells 72 h after IR treatment (not shown). Staining with an anti-cytochrome

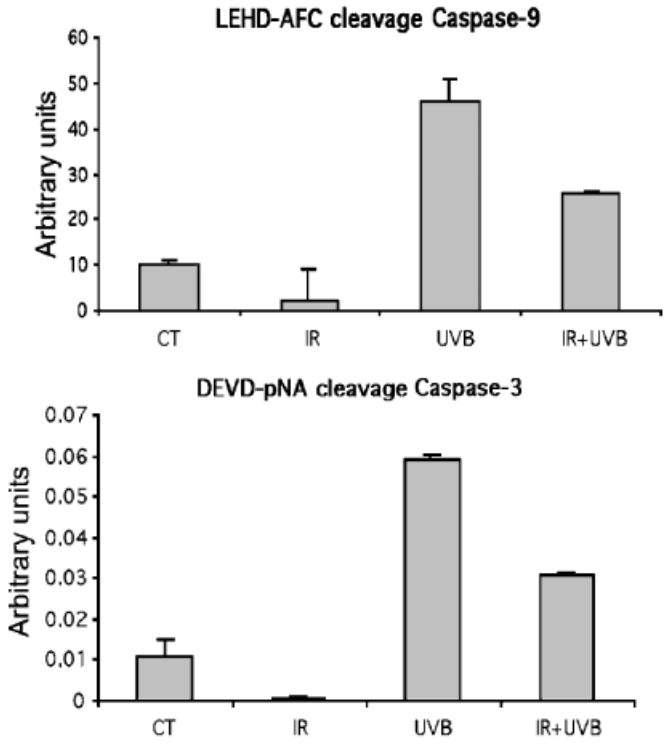

Figure 1
Infrared (IR) pre-irradiation inhibits ultaviolet B (UVB) activation of caspase-9 and -3. Fibroblasts were irradiated using the protocol most efficient for preventing UVB-induced cytotoxicity, i.e., three sessions of 30 min each (3×810 kJ per m^2), with UVB irradiation the following day (1500 J per m^2). DEVD and LEHD activities were measured 4 d after UVB irradiation, as described in Materials and Methods. (For each caspase, one representative experiment among at least 4.)

oxidase subunit IV antibody (a marker of the inner mitochondrial membrane) showed the particular mitochondrial pattern (Fig 2). Similar results were obtained when a Schott (Schott France, Clichy, France) RG 715 filter was used, indicating that cytochrome *c* release was induced at wavelengths between 700 and 2000 nm (Fig 2).

An evaluation of the release of cytochrome *c* was determined by analyzing the degree of co-localization of F1-ATPase (inner mitochondrial membrane) and cytochrome *c* using Metamorph 4.6 software (Universal Imaging, Roper Scientific, Evry, France) after laser confocal microscopy analysis. At 6 h after IR treatment, an extra-mitochondrial fraction of cytochrome *c* was clearly detectable in cells after IR irradiation, whereas a marked co-localization was found in control fibroblasts. IR was estimated to induce the release of 30% of mitochondrial cytochrome *c* (Fig 3).

To confirm this cytochrome *c* release, fibroblast subcellular fractionation was performed 6 and 18 h after IR irradiation. The localization of cytochrome *c* was analyzed by immunoblotting of the mitochondrial and cytosolic fractions. As shown in Fig 4, a band corresponding to cytochrome *c* was clearly visible in the cytosolic fraction of irradiated cells. Cytochrome *c* was still present in the mitochondrial fraction of both irradiated and control cells, confirming the partial release suggested by confocal analysis.

A release of Smac/Diablo was also observed in IR-irradiated cells. AIF, another pro-apoptotic protein, however, was not detected in the cytosolic fraction at either 6 or 18 h

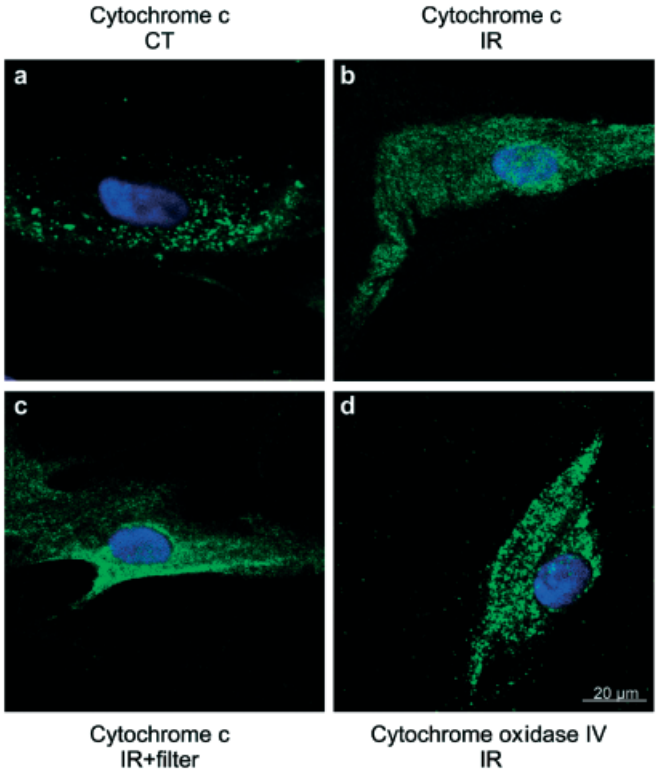

Figure 2
Infrared (IR) irradiation induces cytochrome *c* release from mitochondria. Fibroblasts were infrared (IR) irradiated for 60 min (1620 kJ per m^2), then immunostained 6 h later, and observed under confocal immunofluorescence microscopy as described in Materials and Methods. Compared to non-irradiated fibroblasts (*panel a*), cytochrome *c* labelling was more diffuse after IR, both without (*panel b*) and with a Schott RG 715 filter (*panel c*). After IR irradiation, cytochrome oxidase subunit IV labelling was punctuate, suggesting mitochondrial integrity (*panel d*). (Each image is representative of at least three experiments.)

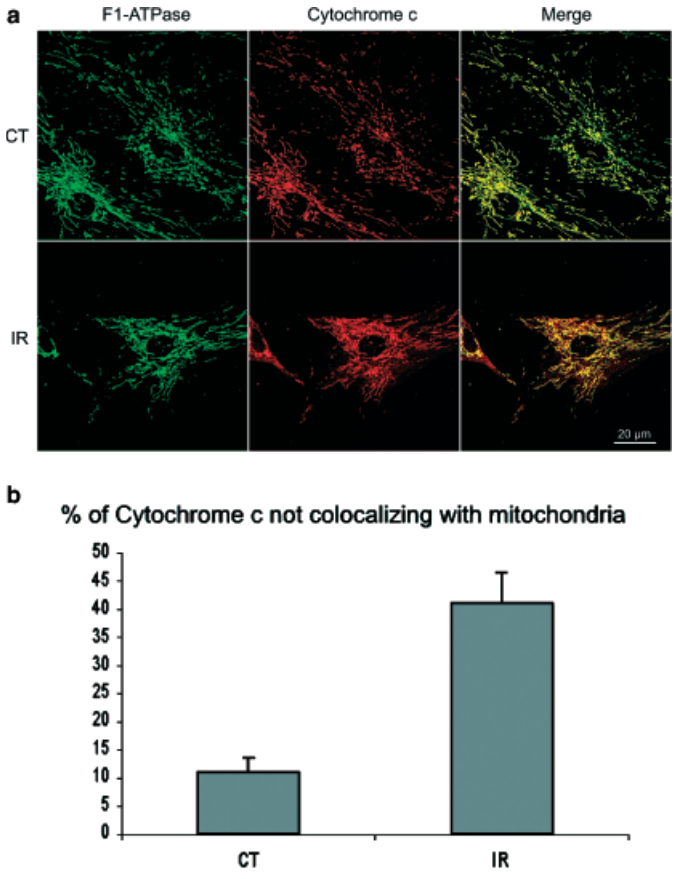

Figure 3
Co-localization of cytochrome *c* and F1-ATPase. (*a*) Double-labelling using F1-ATPase (green) and cytochrome *c* (red) antibodies on fibroblasts 6 h post-infrared (IR) irradiated (1620 kJ per m^2), as described in Materials and Methods. Observations by laser confocal microscopy showed only partial overlay of the two antibody staining (yellow) in IR-irradiated fibroblasts. (*b*) Co-localization based on the comparison of each staining on a pixel-by-pixel basis, using Metamorph 4.6 software. The graph represents the percentage of cytochrome *c* not co-localizing to the mitochondria (F1-ATPase). (Co-localization was measured in six different microscopic fields.)

after IR-irradiation (Fig 4). As a control, we examined the distribution of porin (Fig 4), a mitochondrial transmembrane protein, also known as VDAC (voltage-dependent anion channel) and Hsp60 (Fig S1), a soluble mitochondrial matrix protein. As expected, those proteins were only found in the mitochondrial fraction of both irradiated and control fibroblasts showing that the mitochondria were not fragmented during cell fractionation.

Finally, IR irradiation did not reduce the amount of procaspase-3 present in the cells nor induce the proteolytic activation of procaspase-3 (Figs 1 and 4).

IR induces cytochrome *c* release by isolated mitochondria To determine whether cytochrome *c* release was due to a direct action of IR on mitochondria, isolated rat liver mitochondria were resuspended in mitochondrial isolation buffer (MIB), then IR irradiated and the presence of cytochrome *c* was determined in the mitochondrial pellet and in the supernatant. Cytochrome *c* was detected in the supernatant immediately after IR irradiation (1620 kJ per m^2), and also 30 min later (Fig 5). The absence of porin in the buffer indicated that the mitochondria were still intact and that the release of cytochrome *c* was not due to a disruption of the mitochondrial outer membrane (Fig 5).

The use of a Schott RG 715 filter did not affect the results underlining the fact that the efficient wavelengths were between 700 and 2000 nm (Fig 5). Thus, IR-irradiated mitochondria release cytochrome *c*, even in the absence of cytosolic components, demonstrating a direct effect of IR on this organelle.

IR transiently reduces mitochondrial transmembrane potential A decrease in $\Delta\psi_m$ is another early event in apoptosis and is often associated with cytochrome *c* release. We thus analyzed $DiOC_6$ fluorescence by flow cytometry. No change in $\Delta\psi_m$ was detected immediately after a 60 min IR irradiation (1620 kJ per m^2) as compared to control cultures. In contrast, depending on the experiment and on the strain of fibroblast, a 10%–30% decrease was always detected between 2 and 3 h (Fig 6). This decrease sometimes persisted 5 h after irradiation; however, the $\Delta\psi_m$ always returned to the control level after 18 h. Propidium iodide nuclear staining of IR-irradiated fibroblasts was always negative in keeping with the absence of cell death.

This decrease in $\Delta\psi_m$ further supports the notion that mitochondria are targets of IR. In addition, the recovery of $\Delta\psi_m$ is compatible with the absence of apoptosis after IR irradiation.

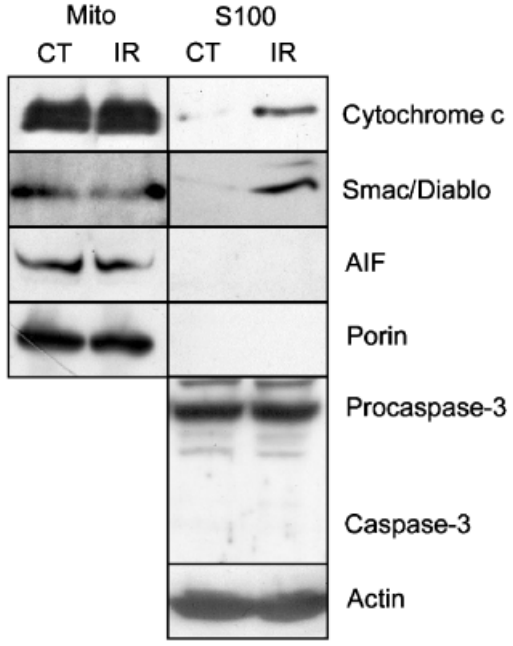

Figure 4
Infrared (IR) induces the release of cytochrome *c* and Smac/Diablo, but not apoptosis-inducing factor (AIF). Fibroblasts were fractionated 18 h after IR irradiation (1620 kJ per m^2) The cytosolic (S100) and mitochondrial fraction (Mito) were analyzed by western blotting using antibodies against cytochrome *c*, Smac/Diablo, AIF, caspase-3, actin, and porin, as described in Materials and Methods. Actin was used as a cytosolic marker and as a control for protein loading. Porin, a mitochondrial transmembrane protein, was used as an indicator of the absence of mitochondrial contamination of the cytosolic fraction. (Representative of three independent experiments.)

IR activates Bax translocation In healthy cells, Bax is predominantly localized to the cytosol in a inactive form. After apoptotic stimuli, however, Bax is translocated into mitochondria (Wolter *et al*, 1997).

By immunohistochemistry, using an antibody that recognizes active Bax, we showed a punctuated distribution of Bax 6 and 24 h after IR irradiation (1620 kJ per m^2), which was consistent with mitochondrial localization, whereas in non-irradiated cells, the staining was absent (Fig 7). Bax translocation after IR irradiation could thus contribute to the permeability of the outer mitochondrial membrane observed.

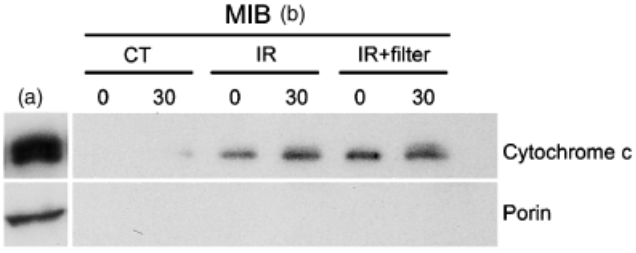

Figure 5
Infrared (IR) irradiation induces cytochrome *c* release from isolated mitochondria. Isolated rat liver mitochondria (100 μg) in mitochondrial isolation buffer (MIB) buffer were infrared (IR) irradiated (1620 kJ per m^2) or sham-irradiated for 60 min with or without a Schott RG 715 filter. Cytochrome *c* and porin were analyzed in the MIB buffer, immediately (0) or 30 min (30) later, as described in Materials and Methods. The presence of porin solely in the mitochondrial fraction (*a*) showed the absence of mitochondrial contamination in the MIB buffer (*b*). (Representative of three independent experiments.)

IR induces Hsp27 To identify the mechanisms leading to the inhibition of caspase activation despite cytochrome *c* and Smac/Diablo release, we analyzed by Western blot the expression of Hsp27, a protein known to inhibit cytochrome *c*-induced caspase-9 activation. The data presented in Fig 8 show that Hsp27 was induced 48h after IR irradiation (1620 kJ per m^2) and this induction persisted 72 h after IR irradiation.

Whatever the conditions, Hsp27 was always induced after 48h by IR irradiation; however, depending on the fibroblast strain, this induction could be detected earlier. Similar results were obtained with or without a Schott RG 715 filter.

IR modulates the expression of proteins of the Bcl-2 family The expression of Bcl-2, Bcl-x_L (anti-apoptotic proteins), and Bax (pro-apoptotic protein) was determined by western blots. Bax expression increased immediately after a 60 min IR irradiation (1620 kJ per m^2). Sometime this induction persisted for 24 h after IR irradiation, then Bax expression decreased gradually until 72 h (Fig 9). Depending on the fibroblast strain, Bcl-2 and Bcl-x_L could be detected immediately after IR exposure and continued to accumulate over the following 72 h. Similar results were obtained with and without a Schott RG 715 filter.

Thus, the balance between Bax, Bcl-2, and Bcl-x_L was pro-apoptotic during the first 24 h after IR exposure, before becoming anti-apoptotic.

Discussion

This study demonstrates that IR induces mitochondrial release of cytochrome *c* and Smac/Diablo, showing that early events in the mitochondrial apoptotic pathway occur despite the lack of caspase activation after IR exposure, and of course despite the absence of apoptotic cells. Thus, the apoptotic process initiated by IR is rapidly stopped after the release of pro-apoptotic molecules from mitochondria. Once in the cytoplasm, cytochrome *c* interacts with its adapter molecule Apaf-1 and contributes to the processing and activation of procaspase-9 in the presence of dATP (Li *et al*, 1997). In turn, caspase-9 cleaves and activates procaspase-3, thereby initiating the final phase of apoptosis (Li *et al*, 1997). These steps do not occur after IR exposure. Smac/Diablo normally potentiates caspase activation by binding to inhibitors of apoptotic proteins (IAP) and blocking their inhibitory activity (Du *et al*, 2000). After IR exposure, however, we found that neither Smac/Diablo nor cytochrome *c* generated active caspase-9 or -3. We also found that IR did not induce the release of AIF, known to lead to DNA fragmentation (Susin *et al*, 1999). The lack of AIF release from the mitochondrial intermembrane space to the cytoplasm agrees with the normal DAPI nuclear staining observed 24 and 72 h after IR exposure, reflecting a lack of DNA fragmentation (data not shown).

We thus demonstrate that IR induces selective release of pro-apoptotic factors from mitochondria, leading to the release of caspase-dependent cell death proteins such as cytochrome *c* and Smac/Diablo but not of caspase-independent cell death proteins such as AIF. These findings are consistent with results from Arnoult *et al* (2002, 2003), who reported that pro-apoptotic Bcl-2 proteins caused cytosolic

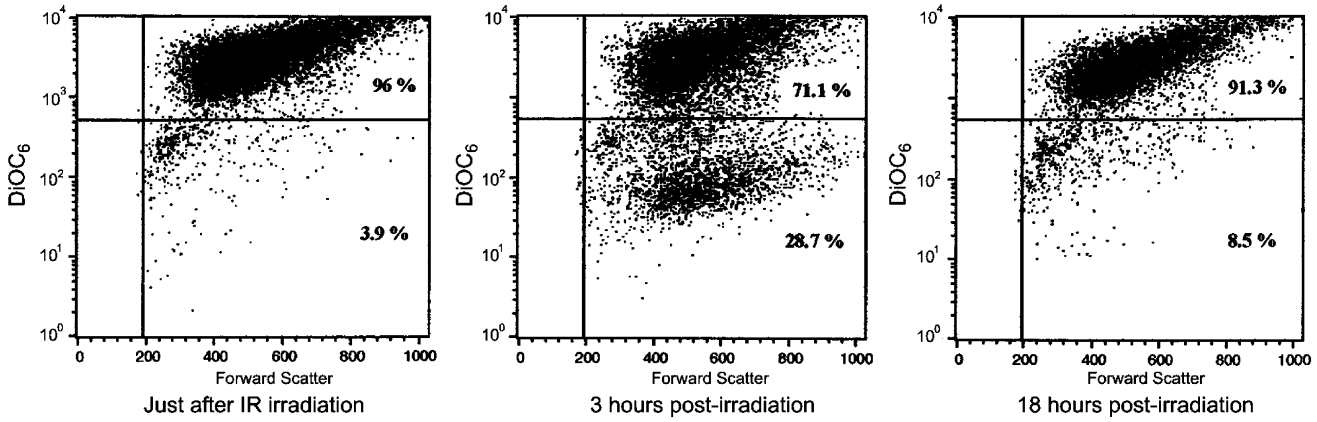

Figure 6
Infrared (IR) induces a transient fall in $\Delta\psi_m$. Fibroblasts were IR irradiated for 60 min (1620 kJ per m^2) then harvested by trypsinization at the indicated time points (0, 3, and 18 h). The $\Delta\psi_m$ was assessed by flow cytometry using $DiOC_6$ fluorescent staining, as described in materials and methods. IR-induced changes in the fibroblast population distribution, according to $DiOC_6$ fluorescence, are shown as cytograms (abscissa: forward scatter; ordinate: $DioC_6$ fluorescence). A clear transient increase in the proportion of fibroblasts with a decrease in $\Delta\psi_m$ is seen in the lower right quadrant, the upper right quadrant representing cells with high $\Delta\psi_m$. (Representative of four independent experiments.)

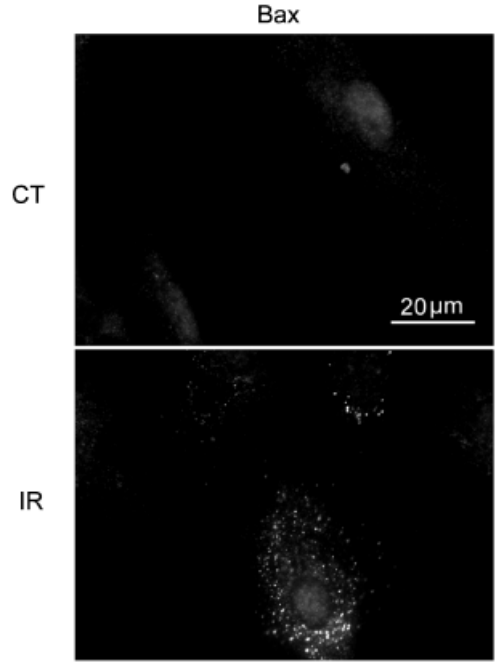

Figure 7
Infrared (IR) activates Bax translocation. Fibroblasts were IR irradiated (1620 kJ per m^2) or sham-irradiated for 60 min, then immunostained 6 h later with an anti-active Bax antibody, and observed under immunofluorescence microscopy as described in Materials and Methods. (Representative of three independent experiments.)

release of cytochrome *c* and Smac/Diablo, but that subsequent caspase activation was required to translocate AIF to the cytosol, suggesting a hierarchical ordering of effectors involved in cell death induction.

Furthermore, cytochrome *c* release after IR exposure is only partial, whereas it is generally complete in cells reaching apoptosis (Goldstein *et al*, 2000). This partial release could correspond to IR mobilization of cytochrome *c* in loosely bound conformation. Indeed, Ott *et al* (2002) suggested the existence of two distinct pools of cytochrome *c*. The first pool is sensitive to electrostatic alterations

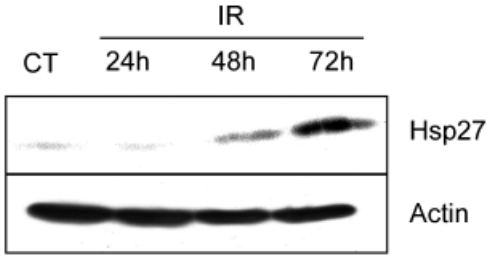

Figure 8
Infrared (IR) induces heat shock protein (Hsp)27. Fibroblasts were IR irradiated (1620 kJ per m^2) or sham-irradiated for 60 min in the presence of a Schott RG 715 filter, then Hsp27 was assayed in the total cytosolic extract by Western blotting, immediately, 24 or 48 h after irradiation, as described in materials and methods. Actin was used as a control for protein loading. (Representative of at least three independent experiments.)

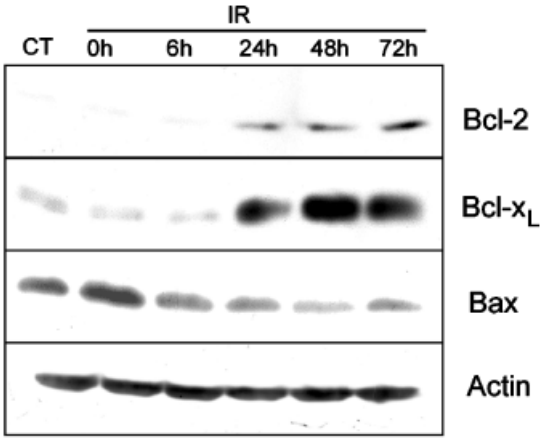

Figure 9
Infrared (IR) modulates the expression of proteins of the Bcl-2 family. Fibroblasts were IR irradiated (1620 kJ per m^2) or sham-irradiated for 60 min in the presence of a Schott RG 715 filter, then the total cytosolic extract was analyzed by western blotting, immediately, 24, 48, or 72 h later, using antibodies against Bax, Bcl-2, Bcl-x_L, and actin (control for protein loading) as described in Materials and Methods. (Representative of at least three independent experiments.)

(Rytömaa *et al*, 1992, 1995), and thus most likely reflects cytochrome *c* in the loosely bound conformation. The second pool can be mobilized by oxidative modification of mitochondrial lipids (specifically cardiolipin), and therefore, likely represents tightly bound cytochrome *c* that is detached by disturbances in membrane structure. Our previous observation that IR does not induce lipid peroxidation (Menezes *et al*, 1998) favours preferential IR mobilization of loosely bound cytochrome *c*.

Interestingly, the effects of IR on mitochondria leading to cytochrome *c* release appears to be a general phenomenon, as we obtained similar results with several human dermal fibroblasts strains, the human lung fibroblast cell line WI-26 (Knaup *et al*, 1978), and the non-adherent promyelocytic leukemia cell line HL60 (Gallagher *et al*, 1979).

This study demonstrates the key role of mitochondria in the cellular effects of IR. Indeed, IR induces the release of cytochrome *c* from isolated mitochondria, in the absence of cytosolic components, demonstrating that mitochondria are a primary target of IR. Furthermore, the use of a long-wave pass sharp-cut filter shows that wavelengths between 700 and 2000 nm (i.e., near infrared) are involved in these effects. Even if other targets within the cell cannot be excluded, the latter results reinforce the idea that mitochondrial cytochromes are good candidates as IR chromophores. Mitochondria have been shown to respond to laser IR irradiation by initiating a cascade of events altering the redox properties of the respiratory chain components (Karu, 1999). Here, we show that mitochondria are also sensitive to naturally occurring IR doses and energies, and contribute to the modulation of important cellular functions.

The mechanisms by which pro-apoptotic molecules are released from mitochondria remain controversial.

One proposed model involves pro-apoptotic Bcl-2 proteins like Bax/Bak, which induce permeabilization of outer membrane through the formation of channels or pores (Eskes *et al*, 1998; Desagher and Martinou, 2000; Martinou and Green, 2001). Here, we show that Bax is activated and translocated to the mitochondria under IR treatment and could thus be responsible for the selective release of pro-apoptotic proteins observed.

According to another model, the opening of the permeability transition pore (PTP) leads to $\Delta\psi_m$ collapse, mitochondrial swelling and rupture of the mitochondrial outer membrane (Marzo *et al*, 1998; Vander Heiden and Thompson, 1999; Zamzami and Kroemer, 2001). In fact, the PTP can operate in two modes: a high-conductance state associated with persistent pore opening and consequently, with a long-term reduction in $\Delta\psi_m$, and a low-conductance state associated with transient pore opening and mitochondrial depolarization spikes (Ichas *et al*, 1997). It has also been speculated that cytochrome *c* release into the cytoplasm may not require a complete, irreversible collapse of $\Delta\psi_m$, but could be related to transient PTP opening (Bossy-Wetzel *et al*, 1998). This low-conductance mode could account for our observations. Indeed, in our case, i.e., absence of apoptosis, $\Delta\psi_m$ fell transiently, returning to normal the day after IR exposure, and this could explain the partial release of cytochrome *c*.

It has also been reported that in various cells types, the reduction in $\Delta\psi_m$ occurs downstream of cytochrome *c* release and is dependent on caspase activation (Bossy-Wetzel *et al*, 1998; Waterhouse *et al*, 2001; Arnoult *et al*, 2003). Our study was not designed to determine whether cytochrome *c* release preceded the decrease in $\Delta\psi_m$, but we did find that the reduction in $\Delta\psi_m$ induced by IR was independent of caspase activation. Interestingly, the recovery and maintenance of $\Delta\psi_m$ observed here after IR exposure could be related to the fact that mitochondria can use cytoplasmic-released cytochrome *c* when caspase activity is inhibited (Waterhouse *et al*, 2001).

Multiple mechanisms of resistance to apoptosis have recently been identified. For example, Ferguson *et al* (2003) observed caspase-9 unresponsiveness after cytochrome *c* release in MCF-7 breast cancer cells and suggest the presence of a cytoplasmic inhibitor of cytochrome *c*.

To understand the mechanisms that could lead to a lack of caspase activation despite cytochrome *c* and Smac/Diablo release, we analyzed the effects of IR on the expression of Hsp27, a small Hsp that protects against apoptotic cell death triggered by various stimuli (Landry *et al*, 1989; Mehlen *et al*, 1993, 1996a, b). We irradiated fibroblast cultures under temperature-controlled conditions avoiding Hsp72 induction (Menezes *et al*, 1998; Schieke *et al*, 2002). Nevertheless, IR exposure induced Hsp27 that could contribute to the interruption of the apoptotic process induced by IR, firstly by inhibiting cytochrome *c* (Paul *et al*, 2002) and Smac/Diablo release (Chauhan *et al*, 2003), and secondly by preventing cytochrome *c*-dependent activation of procaspase-9 (Garrido *et al*, 1999), by inhibiting apoptosome formation (Bruey *et al*, 2000). In addition, Hsp27 appears to bind procaspase-3 and repress its activation (Pandey *et al*, 2000).

The effects of IR on mitochondria-mediated apoptosis can also be modulated by the balance between pro- and anti-apoptotic proteins. We have shown that the level of Bax protein increases during the first 24 h, confirming that IR initially induces pro-apoptotic events. Then, 24 to 72 h after IR irradiation, the Bax protein level decreases, whereas Bcl-2 and Bcl-x_L continue to accumulate in the cell. This could explain the interruption of the apoptotic process induced by IR. Indeed, Bcl-2 and Bcl-x_L have been shown to prevent apoptosis by inhibiting cytochrome c translocation (Kluck *et al*, 1997; Yang *et al*, 1997; Finucane *et al*, 1999), Smac/Diablo release (Sun *et al*, 2002), a decrease in $\Delta\psi_m$ (Shimizu *et al*, 1996) and Bax activation following a death signal (Gross *et al*, 1998).

The balance between Bcl-2 and Bax that determines survival or death following an apoptotic stimulus (Oltvai *et al*, 1993) favors an inhibition of the apoptotic process, days after IR exposure. Furthermore, the Bcl-2 protein level can increase immediately after IR exposure. It has been demonstrated that cells over-expressing both Bcl-2 and Bax have no signs of caspase activation and survive with significant amounts of cytochrome *c* in their cytoplasm (Rossé *et al*, 1998). The protective functions of Bcl-2 and Hsp27 only partially overlap (Guenal *et al*, 1997). After IR exposure they could, therefore, have complementary roles at different levels, to suppress programmed cell death.

In parallel, the level of XIAP after IR irradiation was examined in the fibroblasts. Over-expression of XIAP after IR treatment was weak or absent showing that XIAP is not a

major actor in the blocking of apoptosis after IR irradiation (data not shown).

Naturally occurring doses of IR radiation thus induces early apoptotic events, including partial cytochrome *c* release, release of Smac/Diablo, a transient reduction in $\Delta\psi_m$, and Bax protein accumulation with mitochondrial translocation for up to 24 h. The "apoptotic" process then ceases, as shown by the absence of caspase-9 and -3 activation. The $\Delta\psi$m returns to normal, anti-apoptotic proteins (Hsp27, Bcl-2, and Bcl-x_L) accumulate, and the Bax protein level falls. These mechanisms could help prepare the cell to resist UVB-triggered apoptosis. Indeed, we also show that a pre-irradiation of IR partially inhibits UVB activation of caspase-9 and -3. The latter results, although preliminary, give some insight into the mechanism of inhibition of IR-irradiation on UVB-induced cytotoxicity previously analyzed by counting cells (Menezes *et al*, 1998).

In conclusion, this work underscores that mitochondria is a key element in the effect of IR and reinforces the fact that at least one IR chromophore is located within mitochondria. This study also indicate that IR exposure affects various steps in the mitochondrial apoptotic pathway. Further work needs to be made on IR-UVB interaction as well as on mutagenesis and DNA repair to evaluate the IR beneficial effects to prevent apoptosis induced by UVB.

Materials and Methods

Cell culture Normal human dermal fibroblasts were obtained from healthy, plastic breast surgery donors (18–50 y old) as previously described (Menezes *et al*, 1998) and cultured in Eagle's minimal essential medium (EMEM, Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS, Invitrogen), 100 IU per mL penicillin (Invitrogen), 100 μg per mL streptomycin (Invitrogen), and 2.5 μg per mL amphotericin B (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2-95% air. Experiments were generally performed on fibroblast cultures between passages 4 and 8. Cells were seeded in Petri dishes (60 or 100 mm diameter, 4×10^5 or 9×10^5 cells per dish, respectively), and irradiated when pre-confluent.

The human fibroblast cell line WI-26 was also used (Knaup *et al*, 1978), notably for fractionation experiments requiring large cell numbers (at least 10^7 cells per condition).

Irradiation We used a 250-W GE 27 IR lamp operating at a distance of 42 cm, with an emission spectrum in the range 400–2000 nm, as stated by the manufacturer (Eurosep Instruments, Cergy St Christophe, France), and a 45 mW per cm^2 irradiance (Menezes *et al*, 1998). When specified, a long-wave pass sharp-cut filter (RG 715) was interposed between the lamp and the samples to block wavelengths below 700 nm. The culture medium (about 4 mm in height) was left in the dishes and the dishes were placed on a cooled-plate connected to 20°C thermostatic-bath (Ministat Huber, Fisher, Elancourt, France), thus avoiding an increase in temperature by maintaining the culture medium below 35°C. IR irradiation usually lasted 60 min, corresponding to a total dose of 1620 kJ per m^2.

When IR irradiation was followed by UVB irradiation, the protocol was similar to that we used previously to study IR inhibition of UVB toxicity (Menezes *et al*, 1998). Briefly, cells were IR irradiated 3×30 min (3×810 kJ per m^2). Between irradiation sessions, plates were kept for 3 h in a CO_2 incubator at 37°C. Twenty-four hours after IR treatment, fibroblasts were irradiated with UVB (1500 J per m^2), using an UVB illuminating table (TFX-35.M, Vilbert-Lourmat, Marne la Vallée, France) equipped with six 15-W lamps (312 nm). Culture medium was removed, identified to each dish and kept in sterile conditions and reused after irradiations, thus avoiding growth stimulation by fresh medium. The covers were removed during UVB irradiation.

Preparation of total cytosolic extracts Immediately and at various times (up to 72 h) after IR treatment, fibroblasts were lysed and cytosolic extracts were prepared as previously described (Laboureau *et al*, 2004).

Cell fractionation Six or 18 h after IR treatment, fibroblasts were scraped free in their medium and pelleted by centrifugation at $800 \times g$ for 10 min at 4°C. Cell fractionation was performed as previously described (Oliver *et al*, 2000), except that the supernatants were finally centrifuged at $100{,}000 \times g$ for 1 h in an ultracentrifuge (Optima TM TLX equipped with a TLA-100 rotor, Beckman Coulter, Roissy, France). The resulting cytosolic fraction was further referred as the S100 fraction. The distribution of porin and Hsp60 was examined to verify the absence of mitochondrial damage.

Isolated mitochondria Mitochondria were purified from normal rat liver as described elsewhere (Vallette *et al*, 1994) except that a percoll gradient was added in the procedure.

Isolated mitochondria (5 mg protein per mL) were seeded in 96-microwell plates in 20 μL MIB (210 mM mannitol, 60 mM sucrose, 10 mM KCl, 10 mM Na-succinate, 5 mM EGTA, 1 mM ADP, 0.5 mM DTT, 20 mM Hepes-KOH pH 7.5 (all from Sigma Saint Quentin Fallavier, France), complete inhibitor cocktail (Roche Diagnostics, Meylan, France). The plates were irradiated with IR for 60 min (1620 kJ per m^2), or sham-irradiated, and kept at 30°C until mitochondria were collected. Immediately or 30 min after IR treatment, mitochondria were pelleted by centrifugation at $8000 \times g$ for 10 min at room temperature. Supernatants and pellets were analyzed by SDS-PAGE and immunoblotting to evaluate cytochrome *c* release.

Western blotting SDS-PAGE and immunoblots were performed as described elsewhere (Laboureau *et al*, 2004). The following primary antibodies were used for immunoblotting: anti-cytochrome *c* monoclonal (1:1000; 7H8.2C12), anti-caspase-3 (1:1000; (CPP32) antibody, (Pharmingen, Becton Dickinson, Le Pont de Claix, France), anti-porin (1:200; 31HL; Calbiochem, VWR International, Fontenay-sous-Bois, France), anti-Smac/Diablo antiserum (1:500; Alexis Biochemicals, Coger, Paris, France), anti-Hsp27 (1:500; Euromedex, Souffelweyersheim, France), anti-Hsp60 (1:2000; Stressgen Biotechnologies, Tebu, Le Perray-en-Yvelines, France), anti-AIF (1:40; Ab-2; Oncogene Research Products, VWR International), anti-Bcl-$x_{S/L}$ (1:500; antibody (S-18): sc-634), anti-Bcl-2 (1:500; antibody (100): sc-509) (Santa Cruz, Tebu), anti-Bax (1:1000; antibody YTH-2D2), and anti-actin (1:5000; ICN chemical credential, Orsay, France).

Immunocytochemistry

Cytochrome c and Bax Fibroblasts were seeded in eight-well Labteck chamber slides (VWR International) at a density of 8.4×10^3 per well and were cultured for 48 h. Fibroblasts were irradiated with IR for 60 min (1620 kJ per m^2) and then fixed with methanol–acetone (1:1) for 10 min at −20°C, 1, 3, 6, 24, or 72 h after IR treatment for cytochrome *c* analysis, or 6 and 24 h after IR treatment for Bax analysis.

Cytochrome *c* immunostaining was performed as described by Bossy-Wetzel *et al* (1998). Briefly, fixed cells were labelled with anti-cytochrome *c* antibody (6H2B4 Pharmingen; diluted 1:150) or with anti-cytochrome oxidase subunit IV mouse monoclonal antibody (20E8-C12, Molecular Probes, Interchim, Asnières, France; diluted 1:100). A high-fluorescence FITC-labelled goat anti-mouse antibody (Vector Laboratories; Coger, diluted 1:50) was used as secondary antibody.

For active Bax immunostaining, cells were labelled with anti-Bax antibody (1:100; 6A7, Pharmingen). The secondary antibody was Alexa Fluor 568 goat anti-mouse (Molecular Probes).

Finally, glass coverslips were mounted on Labteck slides using Vectashield Mounting Medium containing DAPI (4′,6-diamidino-2-phenylindole) fluorochrome (Vector Laboratories). Cytochrome *c* images were acquired with a BIO-RAD MRC 1024 laser scanning confocal microscope. Bax images were acquired with an epifluorescence Zeiss microscope.

Co-localization studies of cytochrome c and mitochondria Fibroblasts were seeded at 2×10^4 cells per mL on glass coverslips and placed in 24-well plates 48 h before IR treatment. Fibroblasts were IR-irradiated for 60 min (1620 kJ per m^2) and then fixed 6 h later with 4% paraformaldehyde–0.19% picric acid in PBS for 30 min at room temperature. After permeabilization in 0.1% SDS in PBS and saturation with 3% bovine serum albumin (BSA) in PBS, cells were incubated at 4°C overnight with F1-ATPase antibody (1:500, kindly provided by Pr J. Lunardi, CNRS Grenoble, France). The secondary antibody Alexa Fluor 488 goat anti-rabbit (1:400, Molecular Probes) was added at room temperature for 1 h. Fibroblasts were then incubated with monoclonal anti-cytochrome *c* (1:500, 6H2B4, Pharmingen), for 1 h at room temperature, followed by another 1 h with the secondary antibody Alexa Fluor 568 goat anti-mouse (Molecular Probes). Images were collected on a Leica TCS NT microscope (Leica, Rueil-Malmaison, France), and co-localization was measured with Metamorph 4.6 software.

Measurement of caspase activity Caspase activities were measured with commercial kits. Four days after UVB irradiation, fibroblasts were scraped free in the provided lysis buffer and total cytosolic extracts were analyzed as recommended by the manufacturer.

Caspase-9 activity was determined using the Caspase 9 Activity Assay Fluorometric kit (Oncogene Research Products) by measuring the fluorescence of the cleaved substrate Leucine glutamic acid nistidine aspartic acid-7-amino-4-trifluoromethyl coumarin (LEHD-AFC).

Caspase-3 activity was determined using the BIOMOL Quantizyme Assay System Caspase-3 Cellular Activity assay kit plus (Tebu), by measuring, colorimetrically, the formation of *p*-nitroanilide aspartic acid, glutamic acid, valine, asp (DEVD-*p*NA) or the fluorescence of the cleaved substrate (DEVD-AMC).

Caspase activities were normalized with respect to protein content. Non-specific hydrolysis of DEVD-pNA, DEVD-AMC, and LEHD-AFC was evaluated by using the inhibitors DEVD-CHO and Z-LEHD-FMK (included in the kits).

Measurement of $\Delta\psi_m$ by flow cytometry Fibroblasts were harvested by trypsinization, immediately, then every hour for 6 h, and 18 h after IR treatment, and washed with culture medium.

Fibroblasts (5×10^5 to 1×10^6) were incubated with $DiOC_6$ (3,3′-diethyloxacarbocyanine, 0.1 μM; Molecular Probes) for 30 min in culture medium at 37°C. Fibroblasts were collected by centrifugation at $500 \times g$ for 10 min then resuspended in culture medium containing propidium iodide (10 μg per mL; Sigma Chemical) and analyzed immediately by flow cytometry with laser excitation at 488 nm. At least 15,000 events were recorded per sample. $DiOC_6$-stained cells were detected in channel FL1-H. Side scatter versus forward scatter served to gate on the cell population of interest, excluding debris. Flow cytometry analysis was carried out on a FACSCalibur using CellQuest software (Becton Dickinson).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/JID/JID23472/JID23472sm.htm

Figure S1
Validation of the absence of mitochondrial contamination of the cytosolic fraction using heat shock protein (Hsp)60 and porin. Fibroblasts were fractionated 18 h after infrared (IR) irradiation (1620 kJ per m^2) The cytosolic (S100) and mitochondrial fraction (Mito) were analyzed by western blotting using antibodies against cytochrome *c*, Smac/Diablo, actin, porin, and Hsp60 as described in materials and methods. Actin was used as a cytosolic marker and as a control for protein loading. Porin and Hsp60, mitochondrial transmembrane proteins, were used as an indicator of the absence of mitochondrial contamination of the cytosolic fraction. (Representative of four independent experiments.)

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