Chapter

Radiation-Generated ROS Induce Apoptosis via Mitochondrial

Sandra Claro, Alice Teixeira Ferreira and Maria Etsuko Miyamoto Oshiro

Abstract

Ionizing radiation (IR) causes an increase in intracellular calcium, alters contractility, and triggers apoptosis via the activation of PKCα and -ε in irradiated smooth muscle cells. The present study investigated the role of the mitochondria in these processes and characterized the proteins involved in IR-induced apoptosis. Intestinal smooth muscle cells were exposed to 10–50 Gy from a γ-source. ROS and H_2O_2 levels were measured with colourimetry and a DCFH-DA probe, and protein expression was analyzed by immunoblotting and immunofluorescence. The IR-induced generation of ROS was inhibited by glutathione, and apoptosis was mediated by the mitochondria via BAX, cytochrome c, and caspase 3. IR increased the expression of the cyclins A, B2, and E, and led to unbalanced cellular growth in an absorption dose-dependent manner. However, radiation did not induce alterations in the mitochondrial ultrastructure or in $K\Psi_{mito}$. In contrast, IR increased the nuclear expression of BAG-1, TNFα, PKCα, and -ε and cyclins A and E. In conclusion, IR triggers the activation of antiapoptotic proteins and enhances the risk of a second type of cancer in patients undergoing radiotherapy. In addition to increasing the radioresistance of cells, antiapoptotic proteins can also stimulate uncontrolled cell proliferation that culminates in mutagenesis.

Keywords: ROS, apoptosis, mitochondria, cyclins, smooth muscle

1. Introduction

The molecular pathways that induce and regulate apoptosis have been extensively studied [1, 2]. Apoptosis is characterized by the condensation of nuclear chromatin and blebbing of nuclear and cytoplasmic membranes, a process that leads to the formation of membrane-bound apoptotic bodies [3]. The proteolytic caspase cascade plays a central role in the apoptotic response, and proteins of the BCL-2 family are key checkpoints in the regulation of apoptosis [4, 5]. In healthy cells, the BCL-2 family is kept in an inactive form, with a complex distribution in the mitochondrial outer membrane (MOM), sarco/endoplasmic reticulum (SER), cytosol, and nuclear envelope [6].

The mitochondria also play a key role in Ca2+ homeostasis and oxidative stress [7]. Elevated intracellular calcium concentrations ($[Ca2+]_i$) do not seem to inhibit mitochondrial motility [8] but can lead to the opening of the mitochondrial transition pore (MTP) complex during the process of swelling, which is responsible in turn for the permeability of the MOM to large molecules and the collapse of the mitochondrial transmembrane electric potential ($K\Psi_{mito}$) [9]. Several studies have used tumor cells to investigate the molecular pathways involved in the regulation and triggering of apoptosis by ionizing radiation (IR) [10, 11], but IR is more effective in normal than neoplasic tissue; so it is important to minimize the exposure in it and to clarify the mechanisms involved in the cellular damage [12]. In addition, damage to healthy tissues due to IR used in cancer treatment is frequently associated with the appearance of a second cancer occurring in the radiated field or in its vicinity [13]. This event could be explained by remodeling of the molecular and cellular processes triggering a number of inter- and intracellular signaling cascades that regulate the progression of the cell cycle and cell survival [14–16].

The apoptotic pathway activated by IR is different from the extrinsic pathway activated by ligands and involves the generation of reactive oxygen species (ROS) and H_2O_2 [10, 17]. According to Orrenius [18], the enhanced ROS production regulates cellular metabolism, for the execution of the suicide program, by proteins released from the mitochondria. One of the factors involved in ROS-induced cell death is tumor necrosis factor alpha (TNFα) [15, 19], and mitochondria appear to participate in the production of this mediator. A number of hypotheses have been put forward to explain the mechanism by which TNFα cytotoxicity induces the intrinsic pathway [11]. Nevertheless, the mechanisms regulated by ROS is not totally clear, but our previous results described an increase in $[Ca2+]_i$ [20] and the activation of protein kinase C (PKCα and -ε) [21]. IR has not been directly demonstrated to affect proteins, including cyclins, cyclin-dependent kinases (CDKs), retinoblastoma protein (Rb), and E2F complex proteins [22–24], involved in the orchestration of the cell cycle. The goals of this study were to examine the extrinsic and intrinsic mechanisms involved in the apoptosis, and to investigate ROS and H_2O_2 generation and the mitochondria role under IR of intestinal smooth muscle cells from the guinea pig ileum.

1.1 Tissues and cell culture

Fragments of the longitudinal smooth muscle layer of the guinea pig ileum (LSMLGPI) were prepared as described previously [20, 21], and the IR exposure in tissue fragments and confluent cell cultures from the LSMLGPI were exposed to single dose of 10–50 Gy, emitted by a 60Co γ-source [25]. The samples were radiated with a total dose of 10–50 Gy, and were then maintained for 3 days in Dulbecco's Modified Eagle Medium (DMEM).

1.2 Colourimetry

The ROS level was measured in the homogenates using the fluorescent method described by Yagi [26].

The H_2O_2-induced lipid peroxidation (LP) was measured through the oxidation of Fe2+ in the presence of xylenol orange in a spectrophotometer [27].

1.3 Immunofluorescence analysis

a. The data were acquired and analyzed using a FACS Calibur flow cytometer and CellQuest software.

b. The cell death study was measured at 585/542 nm using the log or linear model in the FL-2 channel [20, 28].

c. To test if the generation of ROS contributes to apoptosis, some cultured cells were incubated with glutathione (GSH), 10^{-3} M reduced glutathione, and yeast glutathione reductase type II (0.08 units/mg protein) and then fixed and stained as described in section *b* [29].

d. The generation of H_2O_2 was measured with 2′7′-dichlorofluorescein diacetate (DCFH-DA, as described by Hasui [30]) in live cultured cells. The cells were suspended in PBS, mixed with 0.3 mM DCFH-DA at 37°C to allow the conversion to DCF, and analyzed at 570/530 nm in the FL-1 channel.

e. To measure the degree of unbalanced growth, cultured cells were detached and stained with acridine orange (AO) for the evaluation of the ratio of RNA content, according to Traganos [31].

f. The proteins involved in apoptosis were measured by immunofluorescence by specific antibodies, anti-: caspase 3, cyclin A, cyclin B2, cyclin E, PKCα, PKCε, TNFα, BAX, cytochrome c, BAG-1, BCL-2, and BCL-xL ([20, 32]).

g. The cyclins A, B2, and E, and the DNA content were analyzed by MODFIT 3.0 software as described by Gong [33].

1.4 Western blot analysis

The experimental procedure was performed as previously described [21] using LSMLGPI homogenates. The following antibodies were used, anti-: caspase 3, cyclin A and cyclin B2, cyclin E, BCL-xL, BAX, cytochrome c, and BCL-2.

1.5 Confocal microscopy

LSMLGPI cells were seeded onto glass coverslips and exposed to IR. The mitochondria were stained with a probe as described by Claro [20] in living cells.

For analysis of the $K\Psi_{mito}$, 0.5 μM $DiOC_6(3)$ was used in DMEM, in vivo. The fluorescence was measured between 546/500 nm. To confirm the mitochondrial accumulation of $DiOC_6(3)$, the cells were incubated with $K\Psi_{mito}$ inhibitors [34] for different periods of time.

1.6 Electron micrography

The cells were seeded as described by Claro [21], and were then radiated and fixed before being analyzed with a transmission electron microscope (1200 EXII, JEOL, Tokyo, Japan).

1.7 Fluorescence microscopy

Living cells were incubated with 2 μg/ml bisbenzimides diluted in DMEM and were analyzed between 461 and 350 nm, for DNA labeling.

1.8 Statistical analysis

Differences between irradiated and nonradiated groups were identified using the analysis of variance (ANOVA) of the unpaired Newman-Keuls tests (GraphPad Prism 5 software). Statistical significance was set at $P < 0.05$.

2. Results

We tested if LSMLGPI cells die by apoptosis in response to IR and observed that the maximum number of apoptotic bodies appeared 72 h following radiation with 10–50 Gy [21, 28]. The first step was to evaluate the effects of IR on the expression of cell-cycle proteins in LSMLGPI cells (**Figure 1**). In contrast to the cyclins B2 and E, the expression of cyclin A was unchanged at 24, 48, and 72 h postradiation. Subsequently, all proteins were analyzed at 24 h postradiation.

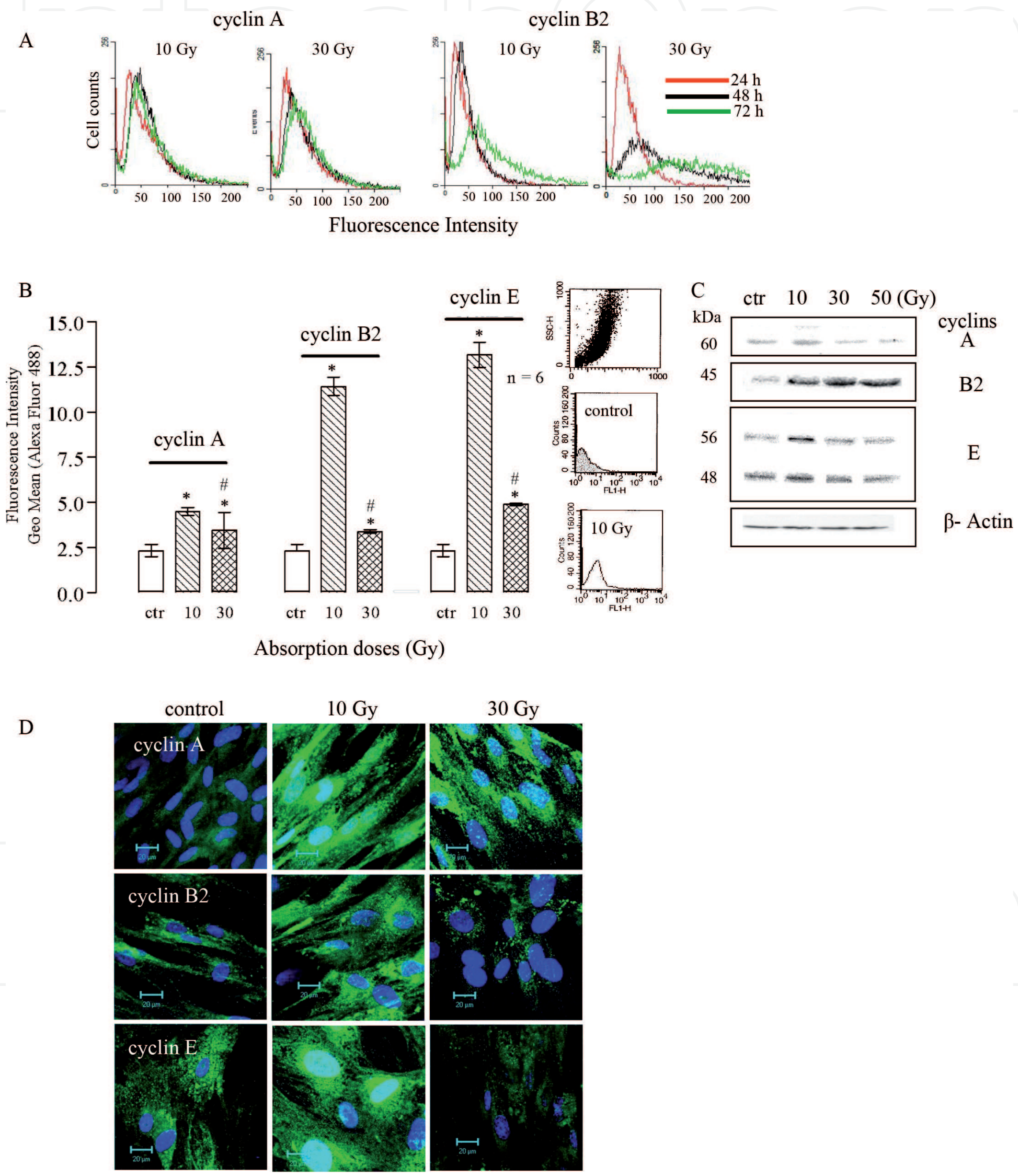

Figure 1.
*Effects of IR on the expression and localisation of cell cycle proteins. Cell cultures from LSMLGPI were fixed and labeled with specific primary and secondary antibodies. (A) Representative time-course histograms of activation of cyclins by IR. (B) Quantification of cells resuspended in PBS 24 h postradiation; besides, representative histograms of the acquisition data of relative cell size and analysis of fluorescence intensity distribution are shown. *P < 0.01 compared to control, #P < 0.01 compared to 10 Gy, Newman-Keuls test. Error bars indicate SEM. (C) Western blot analysis in whole-cell lysates demonstrating expression of cyclin a, B2 and E detected with appropriate antibodies. (D) Images of irradiated cells are representative of three independent experiments. Cyclin a and E co-localized with nucleus are light blue. Nuclear staining was done using DAPI (blue). Scale bar indicates 20 μm.*

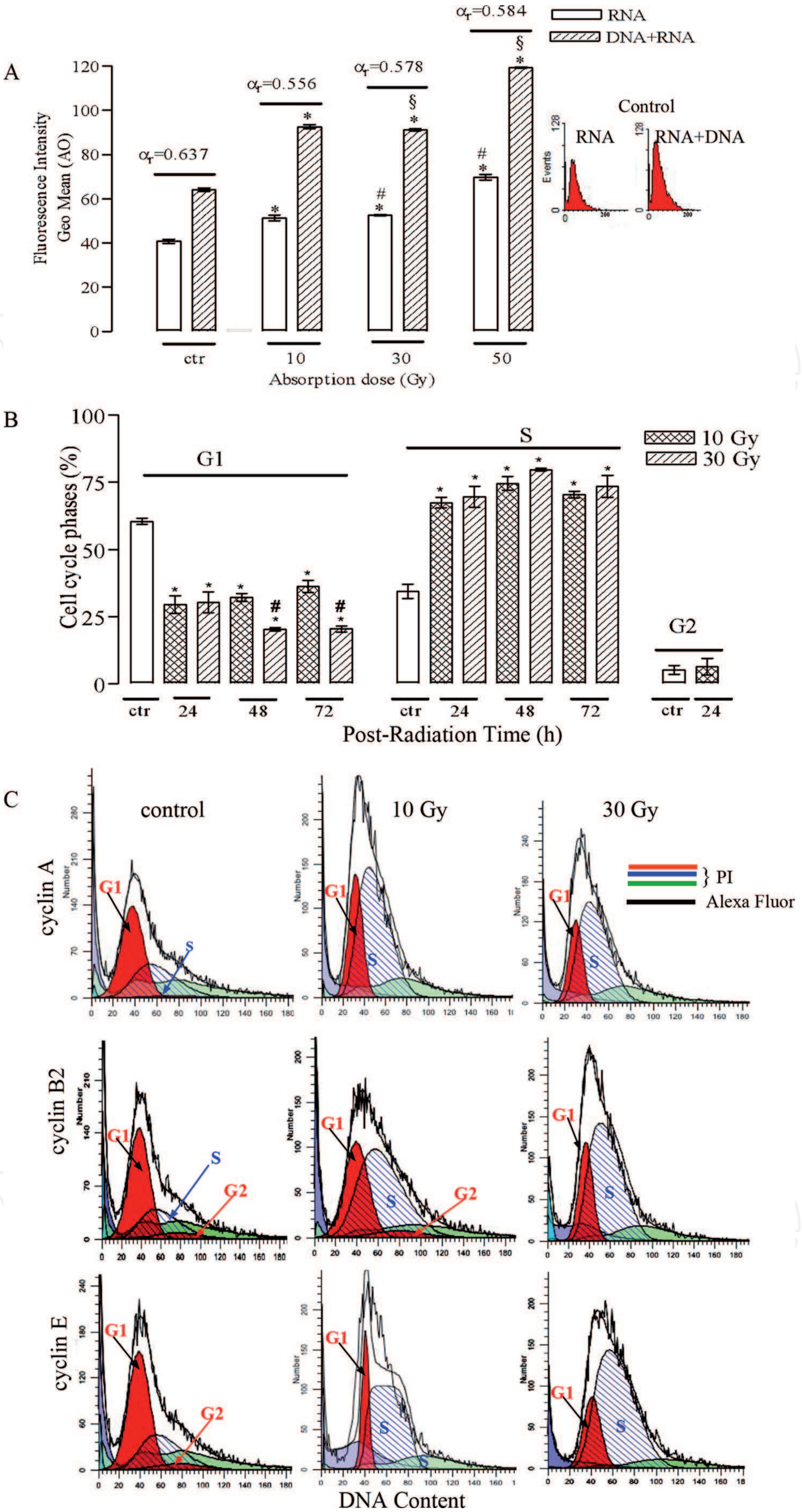

Figure 2.
Effects of IR the on synthesis of RNA and DNA and on the cyclins in the cell cycle. (A) DNA, RNA and α_r RNA/ (DNA + RNA) distribution 24 h postradiation; besides, representative histograms are shown. (B) Quantification of cell cycle phases by DNA content and analysis of G1, S, and G2 phases of cell cycle at different times of postradiation. (C) Scheme illustrating the analysis performed to estimate the cells expressing cyclins versus cell-cycle phases in measurements of cellular DNA content (PI) and the intensity of cyclins associated Alexa Fluor immunofluorescence analyzed by MODFIT 3.0 software. $^{*}P < 0.01$ compared to control, $^{\#}$and $^{\S}P < 0.01$ compared to 10 and 30 Gy, respectively. Newman-Keuls test. Error bars indicate SEM.

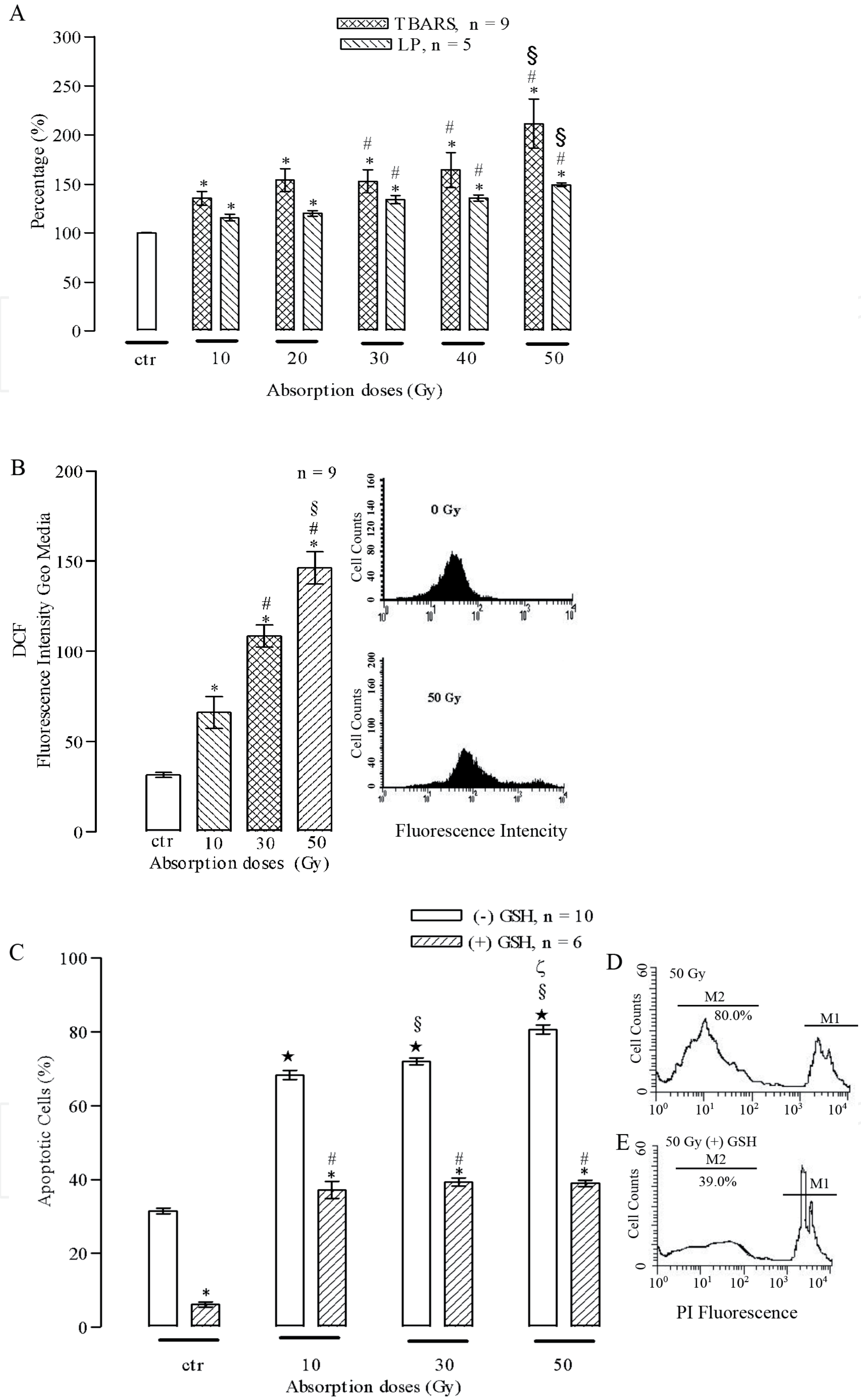

Figure 3.

Measurements of IR-generated-ROS and H_2O_2. (A) TBARS and lipid peroxidation measured in homogenate of LSMLGPI via colourimetric assays. (B) Detection of intracellular H_2O_2 using DCFH-DA probe analyzed at flow cytometer, and (C) the representative histograms. $^*P < 0.01$ compared to 0 Gy, $^\#$and $^\S P < 0.01$ compared to 10 and 30 Gy respectively. (D) Effect of glutathione on irradiated cells and fixed in 50% ethanol, and loaded with PI in the presence (+) or absence (−) of GSH, measured 72 h postradiation using flow cytometry and (E) representative histograms. $^*P < 0.01$ indicates statistical difference between GSH-treated and untreated cells, $^\#P < 0.01$ compared to GSH-untreated control. *, § and $^\zeta P < 0.01$ indicate statistical difference between untreated cells compared to control, 10 and 30 Gy, respectively. Newman-Keuls test. Error bars indicate SEM.

Figure 2 correlates the changes in cyclin expression and the alteration of the cell cycle caused by IR. The α_r ratio of RNA to total nucleic acid content decreased in an absorption dose-dependent manner, and it visualizes nuclear content. The radiated population of cells did not divide because the G2 phase was arrested despite a significant increase in the accumulation of RNA and DNA during the S phase. Cyclins were continuously expressed during the cell cycle, however it was observed the G2 phase.

Figure 3 indicates that IR caused dose-dependent increases in the generation of thiobarbituric acid reactive substances (TBARS) and H_2O_2 with maximal ROS generation and a decrease in ROS levels. IR effects were suppressed by GSH, with a reduction in the number of cells in the M2 region. GSH reduced cell death independent on the dose of radiation, resulting in levels similar to those in control cells.

Apoptosis was assessed 24 h later by the binding of antibodies specific for BAX, cytochrome c, and caspase 3 (**Figure 4**). IR also increased the expression levels of BCL-xL and BCL-2, suggesting that these oncoproteins attempted to promote cell proliferation.

Figure 5 shows the stained apoptotic bodies and the localization of Bax, caspase 3, cytochrome c, Bcl-2, and BCL-xL.

Figure 6 proves that mitochondria presented no evidence of damage other than the appearance of several lysosomes. To prove that the mitochondria were healthy, various agents known to reduce the $K\Psi_{mito}$ were incubated with $DiOC_6(3)$, in living cells.

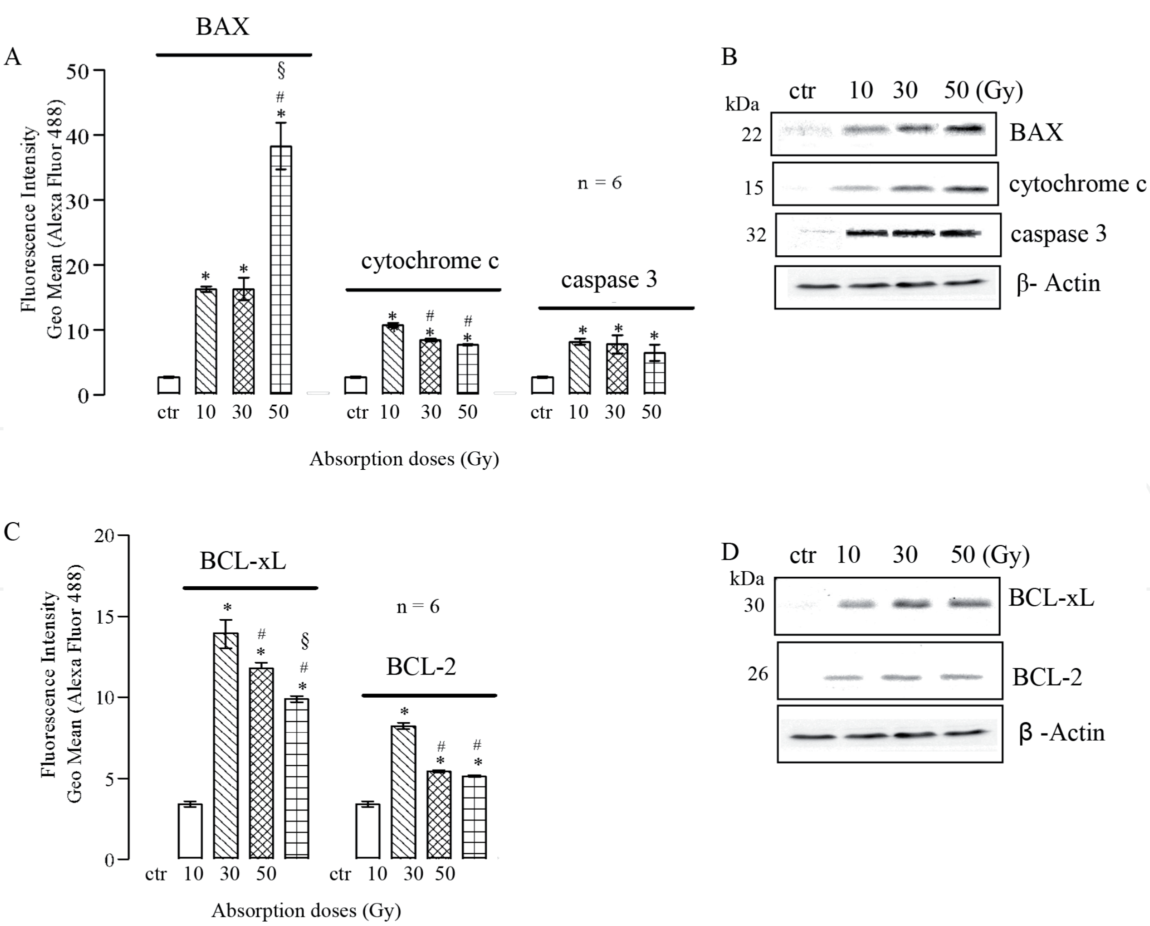

Figure 4.
Effects of IR on (A) pro- and (C) antiapoptotic proteins of LSMLGPI cells measured in the flow cytometer 24 h postradiation. Cells were fixed, permeabilised and incubated with specific primary and secondary antibodies and resuspended in PBS. $^*P < 0.001$ *compared to control,* $^{\#}$*and* $^{\$}P < 0.01$ *compared to 10 and 30 Gy, respectively, Newman-Keuls test. Error bars indicate SEM. (B) and (D) western blot analyses demonstrating BAX, cytochrome c, caspase 3, BCL-xL, and BCL-2 expression, respectively.*

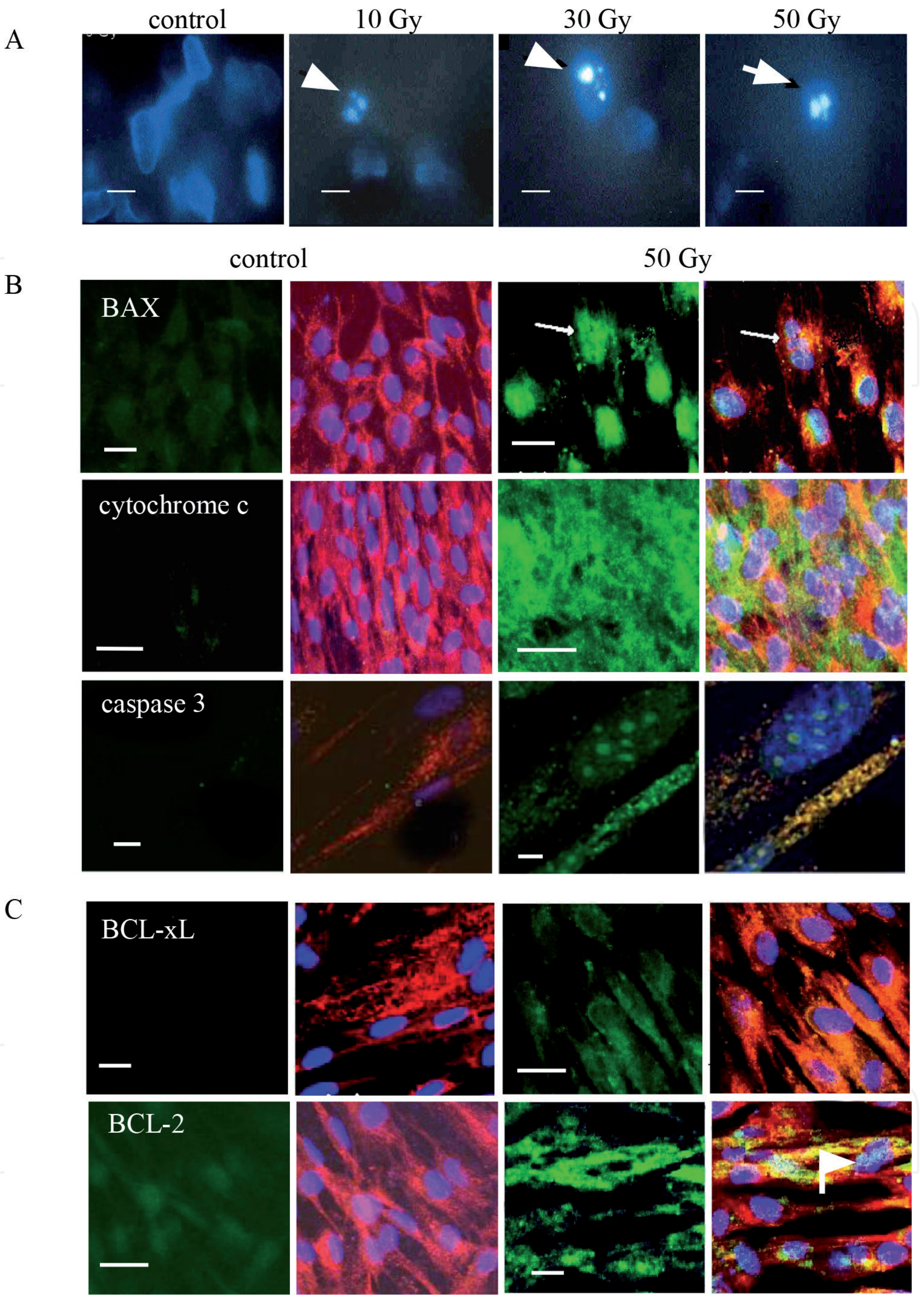

Figure 5.
Effects of IR on apoptotic proteins localisation 24 h postradiation. (A) Cell death by apoptosis is shown by apoptotic bodies formation in irradiated living cells labeled with 2 μg/ml Hoechst 33342 resuspended in cultured medium DMEM maintained at 37°C. Control cells exhibit low blue fluorescence, while irradiated cells exhibit high blue fluorescence and some apoptotic bodies (arrows). Images of irradiated cells present (B) proapoptotic and (C) antiapoptotic proteins with mitochondria stained with Mitotracker (red), and cells incubated with specific primary and secondary antibodies. Nuclear staining was done using DAPI (blue). Proteins co-localized with mitochondria are yellow. Arrows indicate apoptotic bodies. Images are representative of three independent experiments observed in confocal microscope. Scale bar indicates 20 μm.

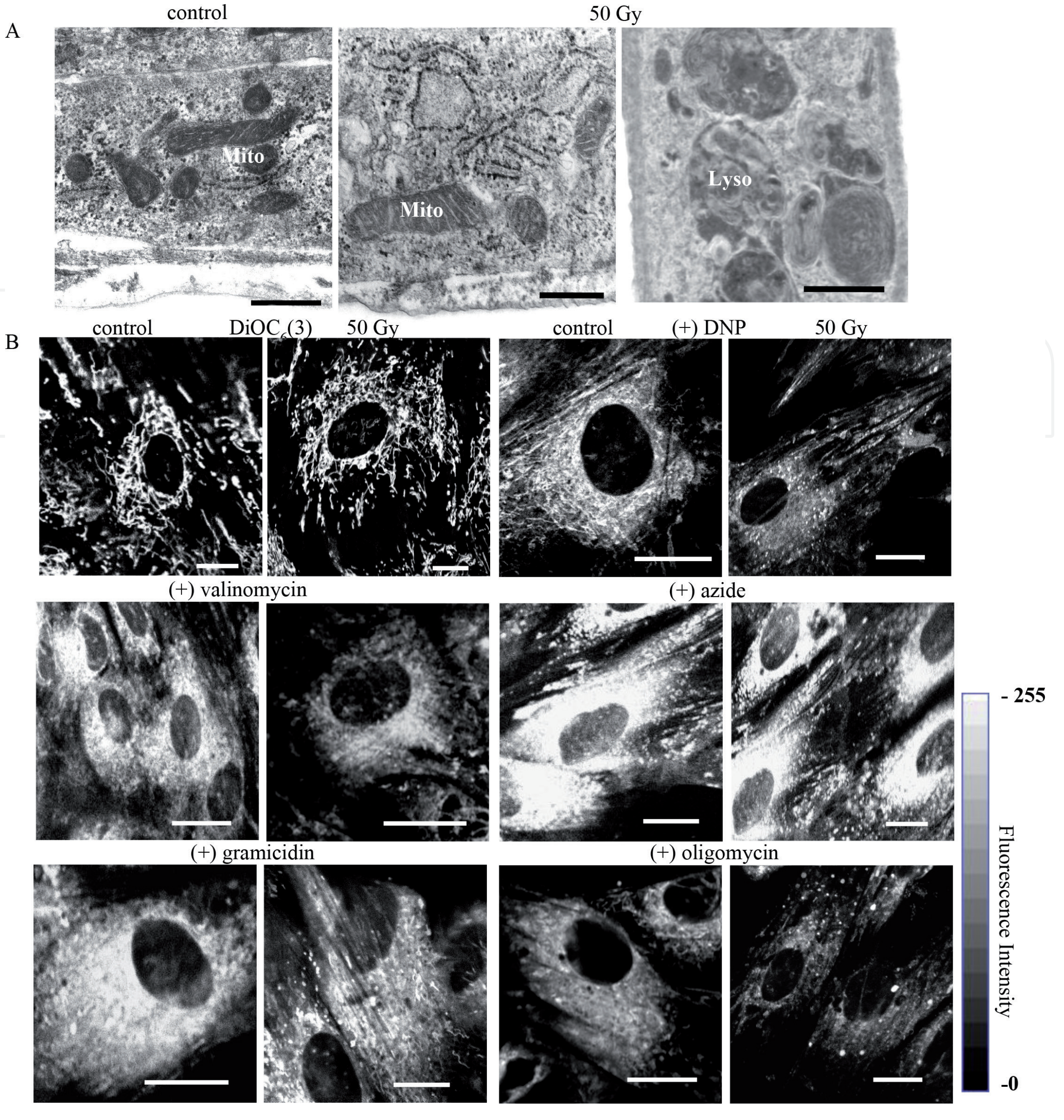

Figure 6.
Effect of IR on mitochondria in LSMLGPI cells cultures 72 h postradiation. (A) Electron microscopic analysis showing the mitochondria (Mito) with normal morphology scattered in the cytosol of control and lysosomes (Lyso); scale bar indicates 0.5 μm. (B) Confocal microscopy images in living cells loaded with $DiOC_6(3)$ and kept at 37°C. Cells were photographed before administration of ionophores, and after exposure to 4.5 nM valinomycin, 1 μM gramicidin, 1 mM DNP, 10 mM sodium azide, and 6.5 μM oligomycin; scale bar indicates 50 Zm. The figures are representative of three independent experiments.

The increased levels and activation/translocation of PKCα and -ε to the nucleus induced IR. Similarly, a large part of the TNFα was internalized and BAG-1 immunofluorescence appears next to the nucleus (**Figure 7**).

3. Discussion

IR generates ROS and H_2O_2 and promotes changes related to the expression and localization of cyclins, and in the cellular cycle phase distributions in a dose-dependent manner in LSMLGPI. Cyclins were continuously expressed during the cell cycle after treatment with IR; however, an arrest of the G2 phase and enhanced DNA replication at the initiation of the S phase occurred. The G2 phase is known

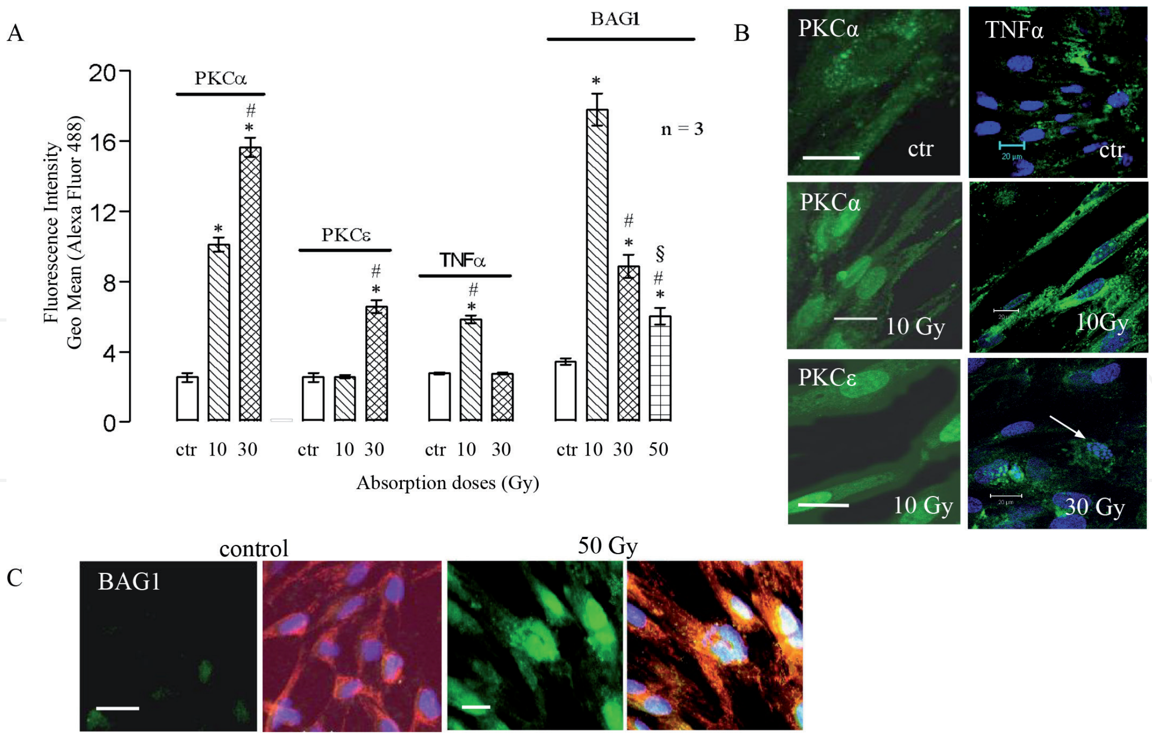

Figure 7.
Effects of IR on the expression and localisation of TNFα and BAG1, PKCα, and -ε, of LSMLGPI cell cultures 24 h postradiation. Cells were fixed and incubated with specific primary and secondary antibodies. (A) Quantification using flow cytometry in cells resuspended in PBS. $^{*}P < 0.01$ *compared to control,* $^{\#}$*and* $^{\S}P < 0.01$ *compared to 10 and 30 Gy, respectively, Newman-Keuls test. Error bars indicate SEM. Figures are representative of three independent experiments and present enhanced green fluorescence of (B) PKCα, PKCε, TNFα, and (C) BAG-1 co-localized with mitochondria that are yellow and with nucleus that are light blue (arrow shows apoptotic bodies). Nuclear staining was done using DAPI (blue). Scale bar indicates 20 μm.*

to be the most radiosensitive phase of the cell cycle, followed by the G1 phase [35]; thus, cells in the G2 phase did not continue to synthesize RNA or DNA. IR induced an excess of DNA in relation to RNA content. These results demonstrate that IR interferes in the cell-cycle distribution, but it does not cause cyclins degradation.

Cell death was effectively triggered by the activation and translocation of BAX to the mitochondria, resulting in cytochrome c release into the cytosol in an absorption dose-dependent manner. Ultrastructural changes and DNA fragmentation characteristics of apoptosis were also identified in vitro [21] and it was confirmed by Hoechst which stained the apoptotic bodies in living cells.

The BAX fluorescence intensity was increased next to the perinuclear region, with some co-localization with the MOM (yellow). Caspase 3 was overexpressed in the nucleus and co-localized with the mitochondria (yellow), and possible retention in the intermembrane space. We also observed caspase 3 localization in the nucleolus which is an atypical form. As cytochrome c mediates the activation of caspases via BAX disruption, we hypothesized that it might also induce the activation of antiapoptotic proteins. According to Edlich [36], activation of BCL-xL and BCL-2 increased the cellular resistance to death and could also cause the retrotranslocation of BAX to the cytosol, confirming our results. Our results demonstrated that there is more than one type of cellular response to IR, namely death or survival. The mitochondrial ultrastructure and function appeared normal in IR-induced apoptosis.

We have shown that IR causes apoptosis which is preceded by the activation of PKCα and -ε and suggests a role for the PKC-mediated pathway [21] and caspase 12 translocation to the cytosol [20]. We and other authors have shown that single absorption doses induce early reactions in normal smooth muscle cells, including protein breakage and the degradation of membrane phospholipids. However, ROS and H_2O_2 also cause DNA fragmentation and prevent the repair mechanisms elicited by sublethal damage [20, 21, 37]. ROS and H_2O_2 have been implicated in several mechanisms of cellular injury, including peroxidation of membrane phospholipids, which increases membrane permeability and leads to apoptosis ([38], pp. 196–208). In the present study, however, we observed that up to 50 Gy of IR led to cell death by apoptosis, despite the preservation of the plasma membrane. It is possible that H_2O_2, rather than ROS, can cross cell membranes rapidly and cause LP in small, discrete sites on smooth muscle membranes ([38], pp. 79–80). In contrast, ROS can mediate necrosis in neurons by the MTP pathway [18]. H_2O_2 is a weak oxidizing agent but can form hydroxyl radicals. These findings suggest that IR-generated ROS or H_2O_2 favors the internalization of TNFα. Several mechanisms may have protected the cells against injury in the presence of GSH, including the prevention of protein oxidation, the accumulation of H_2O_2 through its transformation in water ([38], pp. 10–21), the provision of a substrate for glutathione peroxidase, and the scavenging of hydroxyl radicals. Nevertheless, the most remarkable effect of GSH appears to be protection against alterations in the cell cycle ([38], pp. 247–251).

In fact, here, we show that high concentrations of ROS or H_2O_2 generated by IR were followed by the release of cytochrome c from the mitochondria into the cytosol. Several models of cytochrome c release have been proposed [2, 5], such as release through the MTP mega channel [39].

The mechanisms involving BAX, which is inserted into the MOM, may include the formation of channels, by oligomerization, and the preservation of mitochondrial membrane integrity [40]. Although we cannot discount the possible involvement of heterodimers among activated BCL-2, BCL-xL and BAG-1 proteins, there is no clear evidence that any of these have pore-forming activity [41].

The mitochondrial membranes were maintained intact in radiated cells, with similar fluorescence as the control cells, in which the electronegativity of the probe allowed its retention in the mitochondrial interior [34], $K\Psi_{mito}$ was maintained.

Our data indicate an intrinsic mechanism of IR-induced apoptosis. Moreover, this mechanism may be different in different types of mitochondria [15, 37].

Another potential repair mechanism is the decrease in the cellular ROS or H_2O_2 levels induced by BCL-2 [42]. This mechanism may also be activated by increased levels of antiapoptotic proteins BCL-xL and BAG-1. However, it has been suggested that BCL-2 survival factors are characteristic of cancer cell metabolism [43].

In addition to this survival pathways, that prevented cell death, we observed that BCL-2, BCL-xL, and BAG-1 were activated by direct IR and/or indirect via ROS or H_2O_2 action [44, 45]. Besides, the mitochondrial pattern can vary on different cells and it causes apoptosis that could be independent on the mitochondrial pathway [15, 37]. The radioresistance of mitochondria may be due to the action of natural antioxidants ([37, 38], pp. 97–98) and/or other compounds [46].

Increases in $[Ca2+]_i$ can potentiate the effects of ROS by enhancing LP [8, 14, 47]. ROS and increased $[Ca2+]_i$ have been shown to induce opening of the MTP, which triggers the mitochondrial of cell death [47]. It is noteworthy that mitochondria are located close to the SER, which sequestrates part of the Ca2+ released by these organelles, and this may affect the release of apoptotic and antiapoptotic factors from the SER [48–52]. The mitochondrial morphology may be altered by Ca2+ overload, with an increase in the MOM permeability culminating in the release of proapoptotic factors [8, 11]. However, our data demonstrated that the mitochondrial motility was maintained even in elevated $[Ca2+]_i$ after IR [20]. Increases of $[Ca2+]_i$ can also inhibit DNA and protein synthesis as well as nuclear transport, resulting in an accumulation of cells in the quiescent state (G0) [23]. In addition, $[Ca2+]_i$ up to 500 nM has been implicated in the regulation of the mammalian cell cycle during the early G1 phase and in the transition from the G1 to S phase [53]. Ca2+/calmodulin may also modulate the activity of cyclin-dependent kinases (CDK) and/or cyclin E [54]. In previous studies [20], we observed an increase in basal $[Ca^{2}+]_i$ cells was observed and it was suggested that IR causes modifications in the plasma membrane and/or in the sarco/endoplasmic reticulum, but the capacitative Ca2+ entry into radiated cells was reduced [55].

The cyclins A and E are constitutively nuclear proteins when involved in mitosis [14, 16]; nevertheless, in radiated cells, they leaked from the nucleus to the cytosol. The cyclin B2 complex appears to be localized predominantly in the SER [14, 16, 22, 23]. At the start of mitosis, cyclin B2 is rapidly transported into the nucleus [14]. An important fact to consider is that IR induced unbalanced growth [31]. Similar mechanism to Polavarapu [56] could be explained is the penetration of TNFα in the intestinal smooth muscle. According to our results, TNFα may penetrate the intracellular compartment through damage caused by lipid peroxidation in small, discrete sites of plasma membrane, since there is an ability of TNFα to form pores in biomembranes, or through the conventional receptor/lysosome route [46]. Also, activated TNFα can contribute to the apoptosis, as caused by ROS or H_2O_2. The increased TNFα expression in the cytosol could be explained by the presence of lysosomes in radiated cells, and we can infer that the TNFα was not subject to lysosomal autodigestion, since the mitochondrial membranes were preserved. TNFα can induce cell survival by the polymerization and depolymerization of actin filaments, which prevent the nuclear translocation of proapoptotic molecules and subsequently inhibit caspase 3 [57]. The activation involving ROS or H_2O_2 has been associated with the triggering of cell death modulated by TNFα [10, 15], through the activation of BAX or the protease cascade [58]. TNFα can also be involved in cell survival similar to IR models with higher doses [41]. In addition, we can infer that caspase 3 may enter into the MOM through membrane openings caused by activated BAX or TNFα [39, 59].

IR induces the formation of apoptotic bodies which will remain in the medium of cultured cells or they will be phagocytosed and digested by adjacent cells in the tissue [60]. Although DNA lesions induced by IR are lethal if not properly repaired, it is clear that membrane events may also contribute to radiation-induced apoptosis [61].

Our experiments demonstrated that radiation induced atypical activation of PKCα and -ε, and there is evidence that this may be related to a conservative regulation of cell cycle events, which act as a molecular link connecting signal transduction pathways and constituents of the cell-cycle machinery [62]. PKC participate in the control of G1 and G2/M, and PKCα and -ε may be regulators of the G1 phase and cause a delay in the G1/S transition, thereby halting DNA synthesis and contributing to cellular differentiation or death. In addition, we suggest that PKCα and -ε trigger cyclin activation and translocation to the nucleus, which occur through the C-terminal region [63]. The mechanism involved in the nuclear localization of PKCα and -ε after IR could be similar to that of PKCγ [63] but still remains to be determined. In contrast, the activation of PKCα and -ε may also have been induced by TNFα, with apoptosis triggered via activation of the TNF-receptor, in addition to elevated calcium, ROS and H_2O_2 levels [10, 15, 54]. PKCα and -ε may interact with the cyclins A, B2, and E in the mechanism of cellular survival, similar as the CDKs and PKC which have domains that may activate serine/threonine protein kinases [64, 65], in an atypical fashion. The involvement of PKCα and -ε activation in apoptosis has already been suggested [21].

We can speculate that cyclin E modulates PKCα and -ε when involved in the apoptosis. This possible involvement of PKCε would constitute a new finding, as currently it has only been associated with oncogenesis [66, 67]. Similar to TNFα, PKCε also contains an actin binding site, and its direct interaction with actin is essential for the invasion and metastasis of tumors grown in vitro or in vivo in the regulatory domain [66–68].

An important outcome of the complex network of events triggered by IR is the activation of antiapoptotic proteins in patients with cancer, and radiation therapy may lead to an increased risk of a second cancer [13]. In addition to their maleficent role in increasing radioresistance in normal cells, antiapoptotic proteins can stimulate uncontrolled cellular proliferation that culminates in carcinogenesis and mutagenesis [43]. Takayama et al. [69] identified BAG-1 and BCL-2 heterodimers that suppress apoptosis. Furthermore, BAG-1 overexpression is an important prognostic indicator of malignant tumors and may help to identify the metastatic potential of tumoral cells in vivo [70]. BCL-2 can alter the distribution of intracellular BAG-1, thereby changing the cancer risk [70]. Therefore, the overexpression of BCL-2, BCL-xL, and BAG-1 in normal cells may be a predictive indicator of carcinogenesis [69, 70]. In addition, PKCε is an important signaling molecule that influences the levels/activation of antiapoptotic proteins of the BCL-2 family and may regulate mitochondrial integrity, which is associated with cancer [71, 72]. However, the mechanism by which proteins of the BCL-2 family regulate cell death remains controversial. Our data suggest that not only apoptosis but also cellular repair mechanisms are activated in smooth muscle cells subjected to a low absorption dose.

Additionally, the expression level and localization of these proteins may be an important survival indicator in irradiated normal cells and may inform the prognosis of cancer patients undergoing radiotherapy.

Acknowledgements

The authors would like to thank Fundação de Amparo e Pesquisa do Estado de São Paulo (FAPESP); Federal University of São Paulo (UNIFESP); Edgar Paredes-Gamero, Soraya Smaili, Gustavo José Pareira and Renato de Arruda Mortara.

Author details

Sandra Claro*, Alice Teixeira Ferreira and Maria Etsuko Miyamoto Oshiro
Departamento de Biofísica, Federal University of São Paulo, São Paulo, Brazil

*Address all correspondence to: claro.sandra@unifesp.br

References

[1] Andersen JL, Kornbluth S. The tangled circuitry of metabolism and apoptosis. Molecular Cell. 2013;**49**:399-410

[2] Youle RJ, Strasser A. The Bcl-2 protein family: Opposing activities that mediate cell death. Nature Reviews. Molecular Cell Biology. 2008;**9**:47-59

[3] Kerr JR, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wideranging implications in tissue kinetics. British Journal of Cancer. 1972;**26**:239-257

[4] Burlacu A. Regulation of apoptosis by BCL-2 family proteins. Journal of Cellular and Molecular Medicine. 2003;**7**:249-257

[5] Sato T, Iries S, Krajewski S, Reed JC. Cloning and sequencing of a DNA encoding the rat BCL-2 protein. Gene. 1994;**140**:291-292

[6] Zhong W-X, Li C, Hatzivassiliou G, Lindsten T, Yu QC, Yuan J, et al. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. Journal of Cell Biology. 2003;**162**:59-69

[7] Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ, Camello PJ. Mitochondrial reactive oxygen species and Ca2+ signaling. American Journal of Physiology. Cell Physiology. 2004;**29**:C1082-C1088

[8] Yi M, Weaver D, Hajnoczky G. Control of mitochondrial motility and distribution by the calcium signal: A homeostatic circuit. Journal of Cell Biology. 2004;**167**:661-672

[9] Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulation in apoptosis and necrosis. Annual Review of Physiology. 1998;**60**:619-642

[10] Caputo F, Vegliante R, Ghibelli L. Redox modulation of the dna damage response. Biochemical Pharmacology. 2012;**84**:1292-1306

[11] Shareef MM, Cui N, Burikhanov R, Gupta S, Satishkumar S, Shajahan S, et al. Role of tumor factor-alpha and TRAIL in highdose radiation-induced bystander signalling in lung adenocarcinoma. Cancer Research. 2007;**67**:11811-11820

[12] Markus A, Belka C. A normal tissue dose response model of dynamic repair processes. Physics in Medicine and Biology. 2006;**51**:153-172

[13] Brenner DJ, Curtis RE, Hall EJ, Ron E. Second malignancies in prostate patients after radiotherapy compared with surgery. Cancer. 2000;**88**:398-406

[14] Pines J, Hunter T. The differential localization of human cyclins A and B is due to acytoplasmic retention signal in cyclin B. EMBO Journal. 1994;**13**:3772-3781

[15] Rodemann HP, Blaese MA. Responses of normal cells to ionizing radiation. Seminars in Radiation Oncology. 2007;**17**:8-88

[16] Ye X, Nalepa G, Welcker M, Kessler BM, Spooner E, Qin J, et al. Recognition of phosphodegron motifs in human cyclin E by the SCF (FBW7) ubiquitin ligase. Journal of Biological Chemistry. 2004;**279**:50110-50119

[17] Belka C, Jendrossek V, Pruschy M, Vink S, Verheij M, Budach W. Apoptosismodulating agents in combination with radiotherapy-current status outlook. International Journal of Radiation Oncology, Biology, Physics. 2004;**58**:542-554

[18] Orrenius S, Gogvadze V, Zhivotosky B. Mitochondrial Oxidative

Stress: Implications for cell death. Annual Review of Pharmacology and Toxicology. 2007;**47**:143-183

[19] Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YC, Mathison J, et al. Identity of tumor necrosis factor and macrophage-secreted factor cachectin. Nature. 1985;**316**:552-554

[20] Claro S, Oshiro ME, Freymuller E, Katchburian E, Kallas EG, Cerri PS, et al. γ-Radiation induces apoptosis via sarcoplasmatic reticulum in Guinea pig ileum smooth muscle cells. European Journal of Pharmacology. 2008;**590**:20-28

[21] Claro S, Kanashiro CA, Oshiro ME, Ferreira AT, Khalil RA. α- and ε-protein kinase C activity during smooth muscle cell apoptosis in response to γ-radiation. Journal of Pharmacology and Experimental Therapeutics. 2007;**322**:964-972

[22] Nurse P. A long twentieth century of the cell cycle and beyond. Cell. 2000;**100**:71-78

[23] Pines J. Cyclin and their associated cyclin-dependent kinases in the human cell cycle. Biochemical Society Transactions. 1993;**21**:921-925

[24] Zhao L, Bode AM, Cao Y, Dong Z. Regulatory mechanisms and clinical perspectives of miRNA in tumor radiosensitivity. Carcinogenesis. 2012;**33**:2220-2227

[25] Attix FH. Quantities for describing the interaction of ionizing radiation with matter. In: Wiley J, Wiley S, editors. Introduction to Radiological Physics and Radiation Dosimetry. New York: Interscience; 1986. pp. 20-37

[26] Yagi K. A simple fluorometric assay for lipoperoxide in blood plasma. Biochemical Medicine. 1976;**15**:212-216

[27] Jiang Z-Y, Woollard ACS, Wolf SP. Lipid hydroperoxide measurement by oxidation of Fe++ in the presence of xylenol orange. Lipids. 1991;**26**:853-856

[28] Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. The Journal of Cell Biology. 1975;**66**:188-193

[29] Kosower NS, Kosower EM. The glutathione status of cells. International Review of Cytology. 1978;**54**:109-160

[30] Hasui M, Hirabayashi Y, Kobayashi Y. Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxide production of neutrophils in whole blood. Journal of Immunological Methods. 1989;**117**:53-58

[31] Traganos F, Darzynkiewicz Z, Melamed MR. The ratio of RNA to total nucleic acid content as a quantitative measure of unbalanced cell growth. Cytometry. 1982;**2**:212-218

[32] Claro S, Oshiro MEM, Mortara RA, Paredes-Gamero EJ, da Silva Pereira GJ, Smaili SS, et al. Radiation-generated ROS induce apoptosis via mitochondrial and cell cycle alteration in smooth muscle. International Journal of Radiation Biology. 2013

[33] Gong J, Traganos F, Darzynkiewicz Z. Growth imbalance and altered expression of cyclins B1, A, E, and D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. Cell Growth & Differentiation. 1995;**6**:1485-1493

[34] Johnson L, Walsh ML, Bockus BJ, Chen LB. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. The Journal of Cell Biology. 1981;**88**:526-535

[35] Hall EJ. Radiobiology for the radiologist. In: For Students of Diagnostic Radiology, Nuclear Medicine, and Radiation Oncology.

5th ed. Philadelphia: Lippincott Williams & Wilkins; 2000. pp. 51-64

[36] Edlich F, Banerjee S, Suzuki M, Cleland MM, Arnoult D, Wang C, et al. Bcl-x(L) retrotranslocates Bax from the mitochondria into the cytosol. Cell. 2011;**145**:104-116

[37] Maity A, Kao GD, Muschel RJ, McKenna WG. Potential molecular targets for manipulating the radiation response. International Journal of Radiation Oncology, Biology, Physics. 1997;**37**:639-653

[38] Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. 2nd ed. Oxford: Oxford Univesrsity Press; 1993. pp. 10-21, 79-80, 97-98, 196-208, 247-251

[39] Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell. 2002;**111**:331-342

[40] Antonsson B, Montessuit S, Lauper S, Eskes R, Martinou JC. BAX oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c realese from mitochondria. Biochemical Journal. 2000;**345**:271-278

[41] Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? Trends in Cell Biology. 2008;**18**:157-164

[42] Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, et al. BCL-2 inhibition of neural death: Decreased generation of reactive oxygen species. Science. 1993;**262**:1274-1277

[43] Su ZZ, Lebedeva IV, Sarkar D, Emdad L, Gupta P, Kitada S, et al. Ionizing radiation enhances therapeutic activity of mda-7/IL-24: Overcoming radiation-and mda-7/IL-24-resistance in prostate cancer cells overexpressing the antiapoptotic proteins BCL-x(L) or BCL-2. Oncogene. 2006;**25**:2339-2348

[44] Chauhan D, Hideshima T, Anderson KC. Apoptotic signalling in multiple myeloma: Therapeutic implications. International Journal of Hematology. 2003;**78**:114-120

[45] Susnow N, Zeng L, Margineantu D, Hockenbery DM. BCL-2 family proteins as regulator of oxidative stress. Seminars in Cancer Biology. 2009;**19**:42-49

[46] Liddil JD, Dorr RT, Scuderi P. Association of lysosomal activity with sensitivity and resistance to tumor necrosis factor in murine L929 cells. Cancer Research. 1989;**49**:2722-2728

[47] Waring P. Redox active calcium ion channels and cell death. Archives of Biochemistry and Biophysics. 2005;**434**:33-42

[48] Hajnoczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, et al. Mitochondrial calcium signalling and cell death: Approaches for assessing the role of mitochondrial Ca^{2+} uptake in apoptosis. Cell Calcium. 2006;**4**:553-560

[49] Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL, Distelhorst CW. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. Proceedings of the National Academy of Sciences of the United States of America. 1994;**91**:6569-6573

[50] Pizzo P, Pozzan T. Mitochondria-endoplasmic reticulum choreography: Structure and signaling dynamics. Trends in Cell Biology. 2007;**17**:511-517

[51] Scorrano L, Korsmeyer SJ. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochemical and Biophysics Research Communications. 2003;**304**:437-444

[52] Touyz RM. Reactive oxygen species as mediators of calcium signaling by angiotensin implications in vascular physiology and pathophysiology. Antioxidants and Redox Signaling. 2005;**7**:1302-1304

[53] Kahl CR, Means AR. Regulation of cell cycle progression by calcium/calmodulin dependent pathways. Endocrine Reviews. 2003;**24**:719-736

[54] Choi J, Chiang A, Taulier N, Gros R, Pirani A, Husain M. A calmodulin-binding site on cyclin E mediates Ca2+–sensitive G1/S transition in vascular smooth muscle cells. Circulation Research. 2006;**98**:1273-1281

[55] Putney JW Jr. The capacitative model for receptor-activated calcium entry. In: August JT, Anders MW, Murad F, editors. Advances in Pharmacology. North Carolina: Academic Press; 1991. pp. 251-269

[56] Polavarapu R, Gongora MC, Winkles JA, Yepes M. Tumor necrosis factor-alpha weak inducer of apoptosis increases the permeability of the neurovascular unit nuclear factorkappa B pathway activation. Journal of Neuroscience. 2005;**25**:10094-100100

[57] Papakonstanti EA, Stournaras C. Tumor necrosis factor-alpha promotes survival of opossum kidney cells via Cdc42-induced phospholipase C-gamma activation and actin filament redistribution. Molecular Biology of the Cell. 2004;**15**:1273-1286

[58] Dumay A, Rincheval V, Trotot P, Mignotte B, Vayssiere JL. The superoxide dismutase inhibitor diethyldithiocarbamate has antagonistic effects on apoptosis by triggering both cytochrome c release and caspase inhibition. Free Radical Biology & Medicine. 2006;**40**:1377-1390

[59] Faleiro L, Lazebnik Y. Caspases disrupt the nuclear-cytoplasmic barrier. Journal of Cell Biology. 2000;**151**:951-959

[60] Kerr JF, Harmon BV. Definition and incidence of apoptosis: An historical perspective. In: Tomei LD, Cope FO, editors. Apoptosis: The Molecular Basis of Cell Death. Cold Spring Harbor. New York: Cold Spring Harbor Laboratory Press; 1991. pp. 5-29

[61] Cohen-Jonathan E, Bernhard EJ, McKenna WG. How does radiation kill cells? Current Opinion in Chemical Biology. 1999;**3**:77-88

[62] Livenh E, Fishman DD. Linking protein kinase C to cell-cycle control. European Journal of Biochemistry. 1997;**248**:1-9

[63] Baines CP, Song CX, Zheng YT, Wang GW, Zhang J, Wang OL, et al. Protein kinase C epsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. Circulation Research. 2003;**92**:873-880

[64] Dai Y, Pei XY, Rahmani M, Conrad DH, Dent P, Grant S. Interruption of the NFkappaB pathway by bay 11-7082 promotes UCN-01-mediated mitochondrial dysfunction and apoptosis in human myeloma cells. Blood. 2004;**103**:2761-2770

[65] Qiao LF, Xu YJ, Liu XS, Xie JG, Wang J, Du CL, et al. Role of protein kinase C alpha and cyclin D1 in the proliferation of airway smooth muscle in asthmatic rats. Chinese Medical Journal. 2008;**121**:2070-2076

[66] Kuwahara H, Nishizaki M, Kanazawa H. Nuclear localization signal and phosphorylation of Serine350 specify intracellular localization of DRAK2. Journal of Biochemistry. 2008;**143**:349-358

[67] Wu LW, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, et al. Utilization of distinct signaling

pathway by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation. Journal of Biological Chemistry. 2000;**275**:5096-5103

[68] Tachado SD, Mayhew MW, Wescott GG, Foreman TL, Goodwin CD, McJilton MA, et al. Regulation of tumor invasion and metastasis in protein kinase C epsilontransformed NIH3T3 fibroblasts. Journal of Cellular Biochemistry. 2002;**85**:785-797

[69] Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, et al. Cloning and functional analysis of BAG: A novel Bcl-2-binding protein with anti-cell death activity. Cell. 1995;**80**:279-284

[70] Takayama S, Krajewski S, Krajewska M, Kitada S, Zapata JM, Kochel K, et al. Expression and location of Hssp70/Hsc-binding anti-apoptotic protein BAG-1 and its variants in normal tissues and tumour cell lines. Cancer Research. 1998;**58**:3116-3131

[71] Gubina E, Rinaudo MS, Szallasi Z, Blumberg PM, Mufson RA. Overexpression of protein kinase C isoform epsilon but not delta in human interleukin-3-dependent cells suppresses apoptosis and induces bcl-2 expression. Blood. 1998;**91**:823-829

[72] Pardo OE, Wellbrock C, Khanzada UK, Aubert M, Arozarena I, Davidson S, et al. FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCeps, B-Raf and S6K2. EMBO Journal. 2006;**25**:3078-3088