IONOMYCIN-INDUCED Ca^{2+} OVERLOAD IS NOT ACCOMPANIED BY MITOCHONDRIAL MEMBRANE POTENTIAL DISSIPATION IN MURINE PRO-B CELLS

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Abstract: There are extremely few data concerning the involvement of Ca^{2+} fluxes in the apoptosis of the pro-B cell type Ba/F3. Thus, we aimed the characterization of ionomycin-induced effects on Ba/F3 cells *in vitro*. Our obtained data show that cytosolic Ca^{2+} increased in Ba/F3 cells by 1 μM ionomycin in the presence of 1 mM Ca^{2+} for 24 hours did not induced significant effects on the mitochondrial membrane potential as compared with control cells. The same effects were also associated by the higher concentrations of ionomycin, e.g. 10 μM, in contrast with that induced by the treatment with staurosporine 10 μM. Thus, Ba/F3 cells are resistant toward the collapsing of mitochondrial membrane potential induced by cytosolic Ca^{2+} overload, as shown by the use of JC-1 fluorescent dye and laser confocal microscopy.

INTRODUCTION

Apoptosis is an essential step in the development of tissues and organs. Early studies on apoptosis have been focused on the nucleus since the characteristic changes that occur at this level. However, it was easy to conclude that nuclear changes are far from being a trigger of cell death because they appear late, after employing cell apoptosis. Now it is known that key early events in apoptosis occur in the mitochondria and endoplasmic reticulum, the cytochrome c and calcium release in citosol being absolutely necessary to produce this phenomenon (Mattson & Chan, 2003).

Following the massive accumulation of Ca^{2+}, energized mitochondria may suffer a sudden increase in internal membrane permeability, a phenomenon that is called the permeability transition (PT). In the past, this way of permeability was considered devoid of specificity and that might represent a form of non-specific membrane damage. Recent findings indicate that PT would be mediated by opening at the mitochondrial membrane level of a non-selective channel, with increased conductance, the mitochondrial permeability transition pore (MPTP) (Bernardi et al., 1999). The role of mitochondrial permeability transition (MPT) in cell physiology and pathology is still a controversial topic in part because of lack of the appropriate methods for the direct demonstration of MTP in intact cells. Ca^{2+}, inorganic phosphate, alkaline pH and reactive oxygen species (ROS) are some of the agents that promote MPT, whereas cyclosporin A (CsA), Mg^{2+}, acid pH and phospholipase inhibitors such as trifluoperazine, dibucaine and quinacrine block MPTP opening (Kim et al., 2003). Downstream events of pore activation range from normal pore flicker to irreversible opening of the pore lead to programmed cell death, both associated with changes (dissipation) in mitochondrial membrane potential (Ψ_{mt}).

One of the mechanisms well known to induce apoptosis is the administration of ionomycin, a ionophore for Ca^{2+}, which results in strong and sustained growth of citosolic calcium, $[Ca^{2+}]_i$, e.g. in the case of thymocytes (Stasik et al., 2007). Cellular clone Ba/F3 was derived from murine bone marrow. Like similar clones, it expresses B-cell-specific surface glycoprotein B220 but has unrearranged immunoglobulin genes. It lacks T-cell (Thyl, Lyt), myeloid (MAC-1, Ia), and mature B-cell (Ig, Ia) antigens and strictly requires IL-3 for growth *in vitro* (Palacios & Steinmetz, 1985). Ba/F3 cells are immortalized pro-B lymphocytes presenting anti-apoptotic phenotype. There are extremely few data concerning the involvement of Ca^{2+} fluxes in the apoptosis of the pro-B cell type Ba/F3. Thus, we aimed the characterization of ionomycin-induced effects on Ba/F3 cells *in vitro*, using laser confocal microscopy.

MATERIALS AND METHODS

The IL-3-dependent mouse pro-B cell line Ba/F3 was maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum and 10% WEHI-3-conditioned media as a source of murine IL-3 (Royer et al., 2005), in an atmosphere with 5% CO_2 and at 37°C.

Cells were grown at a density of around 5 x 10^5 per ml before treatment. For some experiments (in triplicate) Ba/F3 cells were treated with 1 μM and 10 μM ionomycin and 1 mM Ca^{2+} for 24 hours. To compare, we used as control the effects of staurosporine 10 μM, a well known inducer of dissipation of mitochondrial membrane potential, also in triplicate. The control Ba/F3 cells received no treatment for 24 hours. After that, all batches of Ba/F3 cells were incubated in the presence of 1 μM JC-1 (Sigma-Aldrich) at 37°C for 30 minutes. JC-1 [5,5',6,6'-tetrachloro-1,1',3,3'-

tetraethylbenzimidazolyl-carbocyanine iodide or $CBIC_2(3)$] is a very sensitive marker for mitochondrial membrane potential. Ba/F3 cells were washed three times with phosphate-buffered saline (PBS, Sigma-Aldrich) at 300 x g for 5 minutes each and were plated in Nunc Lab-Tek II chamber slide systems (one well on glass, Sigma-Aldrich), being incubated for 20 minutes at 37°C. Non-adhered cells were carefully washed out with PBS and the adhered ones were covered with normal culture medium at room temperature. The Nunc chamber slide systems have been prepared by pretreatment for 24 hours with poly-L-lysine (0.1 mg/ml) at room temperature, washed three times with PBS, dried and exposed to UV for 30 minutes. The final concentration for dimethyl sulfoxide (DMSO) used as a drug solvent in the medium did not exceed 0.1%, having no cellular effects at this concentration.

For the laser confocal microscopy we used a Microradiance (Bio-Rad/Zeiss) setup, with an argon ion laser (488 and 514 nm), mounted on an inverted Nikon Eclipse TE-300 microscope. The x100 magnification images were generated using an x100 oil-immersion objective, CFI Plan Fluor (1.30 N.A.), and LaserSharp software. We used the HQ515/530 emission filter for 488 nm excitation and HQ530/560 emission filter for 514 nm excitation in a sequential mode. The laser power was of 3%. For the analysis of the collected images (resolution 1280 x 1024) we used ImageJ, a public domain, Java-based image processing program developed at the National Institutes of Health (U.S.A.). The red and green emissions of JC-1 were merged to obtain the final images.

RESULTS AND DISCUSSIONS

As figure no. 1 shows, 1 μM ionomycin and 1 mM Ca^{2+} treatment of Ba/F3 cells for 24 hours did not induced significant effects on the mitochondrial membrane potential as compared with control cells (figure no. 2). In both cases more than 90% of the cells are having high mitochondrial membrane potential (Ψ_{mt}), normal for live cells. This fact is re-

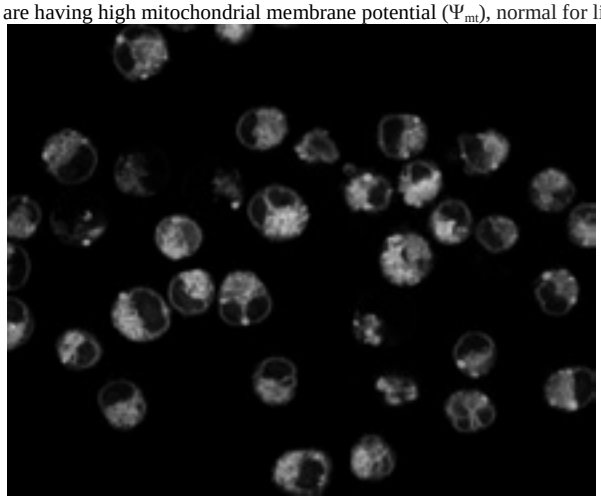

Figure no. 1: Laser confocal microscopy of Ba/F3 cells treated with ionomycin 1 μM for 24 hours in the presence of 1 mM Ca^{2+}. JC-1 [5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide or $CBIC_2(3)$], a very sensitive marker for mitochondrial membrane potential, is showing that more than 90% of the cells are having high mitochondrial membrane potential (Ψ_{mt}), normal for live cells. This is reflected in the high intensity of red emission of JC-1, the energized mitochondria being clearly distinct in the citosol. Image shown is representative of many acquired from three independent experiments (100x).

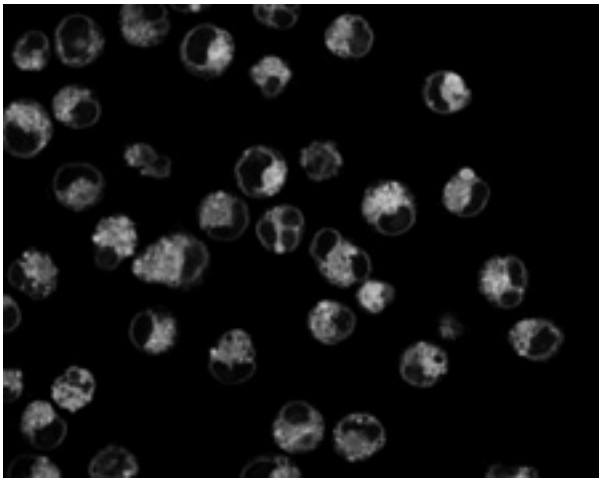

Figure no. 2: Ba/F3 control cells, non-treated, also have high mitochondrial membrane potential (Ψ_{mt}), normal for live cells, in proportion of more than 90%, when imaged by laser confocal microscopy with the help of JC-1. The rest of almost 10% of cells are usually apoptotic. Image shown is representative of many acquired from three independent experiments (100x).

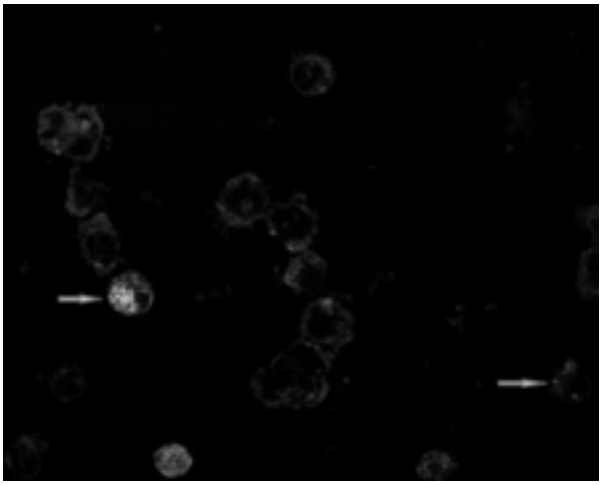

Figure no. 3: In the presence of staurosporine 10 μM, a well known inducer of dissipation of mitochondrial membrane potential, for 24 hours, extremely few Ba/F3 cells (less than 10%) are having high mitochondrial membrane potential (Ψ_{mt}), normal for live cells (arrows). On contrary, there is evident a large scale of dispersed green emission of JC-1shown by laser confocal microscopy. Image shown is representative of many acquired from three independent experiments (100x).

flected in the high intensity of red emission of JC-1, the energized mitochondria being clearly distinct in the citosol. On contrary, when Ba/F3 cells were treated with staurosporine 10 μM, a well known inducer of dissipation in mitochondrial membrane potential for 24 hours, there remained very few cells (less than 10%) alive, with distinct red energized mitochondria (figure no. 3).

JC-1 [5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide or $CBIC_2(3)$] is a cationic dye that exhibit potential-dependent accumulation in mitochondria, being a very sensitive marker for mitochondrial membrane potential. The JC-1 dye accumulates in the mitochondria of healthy cells as aggregates, which are fluorescent red in color. If the mitochondrial potential collapses, then the JC-1 dye can no longer accumulate in the mitochondria and remains in the cytoplasm in a monomeric form which fluoresces green. The differential distribution of the red and green forms of the dye is easily analyzed by fluorescence microscopy or laser confocal microscopy (Di Lisa et al., 1995). JC-1

is far more specific for mitochondrial versus plasma membrane potential, and more consistent in its response to depolarization, than other cationic dyes such as DiOC6(3) and rhodamine 123, giving at the same time very little background (Salvioli et al., 1997). The ratio of green to red fluorescence of JC-1 is dependent only on the membrane potential and not on other factors such as mitochondrial size, shape, and density that may influence single-component fluorescence signals.

The most widely implemented application of JC-1 is for detection of mitochondrial membrane potential dissipation (Jones et al., 2002). MPT, the result of the collapse of electrochemical gradient across the mitochondrial membrane, is one of the early events during cellular apoptosis. The mitochondrial voltage-dependent anion channel (VDAC), the physical supposed support for MPT, is increasingly involved in the control of apoptosis (Yuqi et al., 2009). Noxa, the BH3-only Bcl-2 family protein, was shown to be a key player in p53-induced cell death through the mitochondrial dysfunction. It was showed that the mitochondrial-targeting domain (MTD) of Noxa is a prodeath domain. Peptide containing MTD causes massive cell death *in vitro* through cytosolic calcium increase; it is released from the mitochondria by opening the mitochondrial permeability transition pore. MTD peptide-induced cell death can be inhibited by calcium chelator BAPTA-AM (Seo et al., 2009).

Single-channel currents were recorded from inner mitochondrial membranes of HepG2 hepatoma cells and of normal rat liver cells by means of patch-clamp techniques. Voltages of -40 mV and below closed the channels usually with a delay of about 2 minutes. Increasing Ca^{2+} concentrations activated the channels, whereas cyclosporin A (100 nM) blocked. Taken together the results indicate that the currents were recorded from the mitochondrial permeability transition pore (MPTP) (Loupatatzis et al., 2002). Ca^{2+} stimulates mitochondrial energy metabolism during spleen lymphocyte activation in response to the ascitic Walker 256 tumor in rats. Intracellular Ca^{2+} concentrations, phosphorylated protein kinase C (pPKC) levels, Bcl-2 protein contents, interleukin-2 (IL-2) levels, mitochondrial uncoupling protein-2 (UCP-2) contents and reactive oxygen species (ROS) were significantly elevated in these activated lymphocytes. Mitochondria of activated lymphocytes exhibited high free Ca^{2+} concentrations in the matrix and enhanced oligomycin-sensitive oxygen consumption, indicating an increased rate of oxidative phosphorylation. The production of ROS was largely decreased by diphenylene iodinium in the activated lymphocytes, suggesting that NADPH oxidase is the prevalent source of these species. Accumulation of UCP-2 and the anti-apoptotic protein Bcl-2 is probably important to prevent mitochondrial dysfunction and cell death elicited by the sustained high levels of intracellular Ca^{2+} and ROS and may explain the observed higher resistance from activated lymphocytes against the opening of the mitochondrial membrane permeability pore (MPT). All these changes were blocked by pretreatment of the rats with verapamil, an L-type Ca^{2+} channel antagonist. These data demonstrate a central role of Ca^{2+} in the control of mitochondrial bioenergetics in spleen lymphocytes during the immune response to cancer (Degasperi et al., 2006).

On the other hand, other data suggest a little bit different mechanism of mitochondria-triggered paraptotic cell death, induced by cytosolic Ca^{2+} overload through receptor-operated channel (vanilloid receptor subtype 1, VR_1), in Jurkat cells (Jambrina et al., 2003). Ca^{2+} uptake through the VR_1 channel, but not capacitative Ca^{2+} influx stimulated by the muscarinic type 1 receptor, induced sustained intracellular [Ca^{2+}] rises, exposure of phosphatidylserine, and cell death. Ca^{2+} influx was necessary and sufficient to induce mitochondrial damage, as assessed by opening of the permeability transition pore and collapse of the mitochondrial membrane potential. Ca^{2+}-induced cell death was inhibited by ruthenium red, protonophore carbonyl cyanide m-chlorophenylhydrazone, or cyclosporin A treatment, as well as by Bcl-2 expression, indicating that this process requires mitochondrial calcium uptake and permeability transition pore opening. Cell death occurred without caspase activation, oligonucleosomal/50-kilobase pair DNA cleavage, or release of cytochrome c or apoptosis inducer factor from mitochondria, but it required oxidative/nitrative stress. Thus, Ca^{2+} influx might triggers a distinct program of mitochondrial dysfunction leading to paraptotic cell death, which does not fulfill the criteria for either apoptosis or necrosis.

One of the mechanisms well known to induce apoptosis is the administration of ionomycin, a ionophore for Ca^{2+}, which results in strong and sustained growth of citosolic calcium, $[Ca^{2+}]_i$, e.g. in the case of thymocytes [4]. On the other hand, there are extremely few data concerning the involvement of Ca^{2+} fluxes in the apoptosis of the pro-B cells, and especially of type Ba/F3. Thus, we aimed the characterization of ionomycin-induced effects on Ba/F3 cells *in vitro*, using laser confocal microscopy. Our obtained data show that cytosolic Ca^{2+} increased in Ba/F3 cells by 1 μM ionomycin, a well known inducer of dissipation of mitochondrial membrane potential, in the presence of 1 mM Ca^{2+} for 24 hours did not induced significant effects on the mitochondrial membrane potential as compared with control cells as has been shown through the help of the JC-1 fluorescent dye. The same effects were also associated by the higher concentrations of ionomycin, e.g. 10 μM (data not shown). These results are in contrast with that induced by the treatment with staurosporine 10 μM, also a well known inducer of collapsing of mitochondrial membrane potential.

CONCLUSIONS

Ba/F3 cells, a murine early pro-B cells type, are resistant toward the collapsing of mitochondrial membrane potential induced by cytosolic Ca^{2+} overload, as shown by the use of JC-1 fluorescent dye and laser confocal microscopy.

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