FEBS Letters 522 (2002) 168–172 FEBS 26231

PUVA-induced apoptosis involves mitochondrial dysfunction caused by the opening of the permeability transition pore

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Received 29 April 2002; revised 3 June 2002; accepted 4 June 2002

First published online 11 June 2002

Edited by Vladimir Skulachev

Abstract The mechanism of cell death was investigated in Jurkat cells exposed to the combination of psoralen and UVA irradiation (PUVA). Apoptosis was by far prevailing over necrosis and involved mitochondrial dysfunction. The collapse of mitochondrial membrane potential, appears to be caused by the opening of the mitochondrial permeability transition pore since its inhibitor, cyclosporin A, prevented mitochondrial dysfunction and largely attenuated apoptosis. Apoptosis also occurred in cells treated with the photoproducts generated by irradiating psoralen in vitro with an oxygen-dependent process. Thus, the involvement of reactive oxygen species in the onset of PUVA-induced apoptosis appears mostly related to psoralen photooxidation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Psoralen; Apoptosis; Permeability transition; Mitochondria; Cyclosporin A

1. Introduction

The combination of psoralen and UVA irradiation, commonly referred to as PUVA, represents a therapeutical approach useful in the treatment of skin diseases such as psoriasis, and in some immune disorders [1]. The cellular responses to PUVA involve oxygen-independent and oxygen-dependent mechanisms. In the former case, the photosensitizer reacts directly with cellular components (i.e. nucleic acids and lipids). In the latter mechanism, the generation of radical species or singlet oxygen is likely to cause a rapid alteration of metabolic processes and cellular structures. Furthermore, irradiation of psoralen induces its photodegradation, yielding the so-called psoralen photo-oxidized products (POP) [2] that appear to be biologically active as well [3,4].

Although apoptosis has been reported to result from PUVA treatment [5], scarce information is available concerning the underlying mechanism(s). The study of mitochondrial function could provide crucial information in understanding the biochemical mechanism underlying cellular photodamage since a close relationship between mitochondria and apoptosis is widely accepted [6–8]. A possible cause of mitochondrial dysfunction might be represented by the opening of the mitochondrial permeability transition pore (PTP), a high-conductance channel located in the inner mitochondrial membrane, whose open probability is regulated by several factors including mitochondrial membrane potential ($\Delta\psi_m$), Ca^{2+}, matrix pH and cyclosporin A (CsA), a high affinity inhibitor [9].

The present study was aimed at both assessing the prevailing form of cell death and investigating mitochondrial function in Jurkat cells treated with PUVA or POP. The results show that mitochondrial dysfunction caused by PTP opening is causally related to PUVA-induced apoptosis. Preliminary accounts of these results have been published in abstract form [10].

2. Materials and methods

2.1. Cell culture

T-lymphoblastoid Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Scotland) in 5% CO_2–95% air at 37°C.

2.2. Reagents

Polyclonal antibodies anti-poly(ADP-ribose) polymerase (PARP) were purchased from Hoffmann-La Roche (Basel, Switzerland), anti-rabbit immunoglobulins were purchased from DAKO (A/S, Denmark). Tetramethylrhodamine methyl ester (TMRM) was purchased by Molecular Probes (Eugene, OR, USA). All other chemicals were purchased from Sigma.

2.3. PUVA treatment

Jurkat cells (10^6 cells/ml) were incubated with various concentrations of psoralen (10–100 μM) in serum free medium for 15 min at 37°C and then exposed to 1.8 J/cm^2 of UVA light. The fluence rate at 365 nm at the sample level was 4 mW/cm^2.

2.4. POP treatment

Psoralen in ethanol/water solution (2:1) was photo-oxidized by UVA light (6 J/cm^2) and then added to Jurkat cells in serum free medium. Parallel experiments were performed to test the effect of oxygen by bubbling the psoralen solution with N_2 for 15 min before irradiation.

2.5. Detection of PARP cleavage

Cell lysates (15 μg) were separated on a 10% SDS/polyacrylamide minigel and electrotransferred onto 0.45 μm nitrocellulose membrane (Bio-Rad), as previously described [11]. The blots were probed with anti-PARP antibodies and anti-rabbit peroxidase-conjugated secondary antibodies. Each assay was performed at least three times.

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Abbreviations: CsA, cyclosporin A; $\Delta\psi_m$, mitochondrial membrane potential; FCCP, carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; PDT, photodynamic therapy; POP, photo-oxidized products of psoralen; PTP, mitochondrial permeability transition pore; PUVA, psoralen+UVA; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester

PII: S0014-5793(02)02926-5

2.6. TMRM staining and imaging

At different time points after PUVA treatment, Jurkat cells were washed with Hanks' balanced salt solution supplemented with 10 mM HEPES, pH 7.4 (HBSS) and then incubated for 15 min at 37°C with 25 nM TMRM. Cyclosporin H (1.6 μM) was also added to inhibit multidrug-resistance pumps, which can affect TMRM loading [8]. Cellular fluorescence images were acquired with an Olympus IMT-2 inverted microscope, as previously described [12]. For detection of TMRM fluorescence, 568 ± 25 nm excitation and 585 nm longpass emission filter settings were used. Data were acquired and analyzed using Metamorph software (Universal Imaging). Mitochondria were identified as regions of interest (ROI) and at least 30 ROIs were considered for each experiment. The decrease in fluorescence intensities induced by carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP) was expressed as the difference of the values obtained before and after the addition of the uncoupler. The values of these differences obtained in the treated cells were normalized to the difference values of the untreated cells (control).

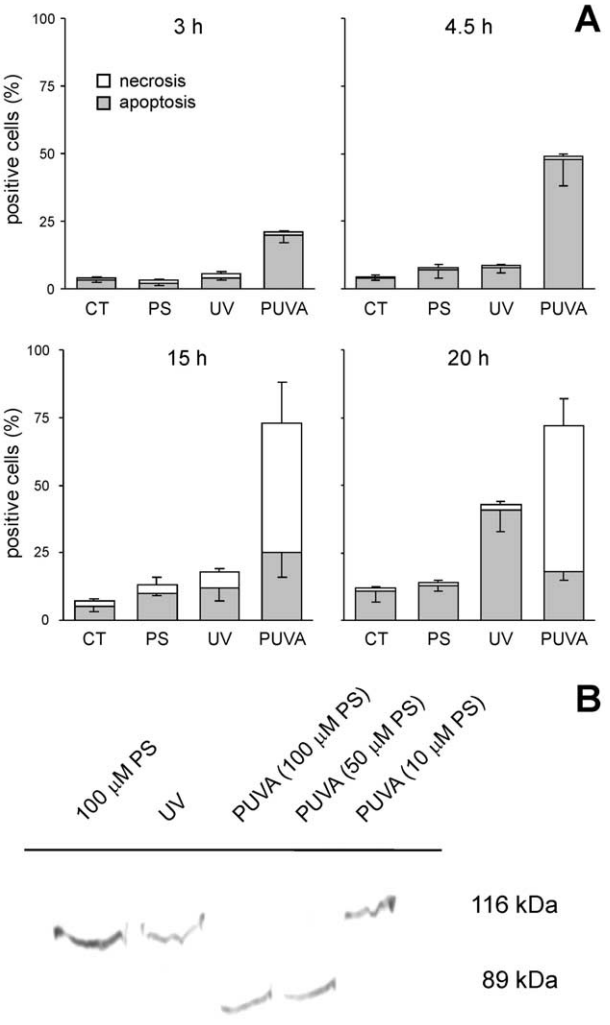

Fig. 1. Loss of cell viability and PARP fragmentation induced by Psoralen, UVA and PUVA. A: Jurkat cells (10^6/ml) were incubated for the indicated times at 37°C under the following conditions: (i) 50 μM psoralen without UVA (PS); (ii) UVA alone (1.8 J/cm^2) (UV); (iii) 50 μM psoralen followed by exposure to UVA treatment (1.8 J/cm^2) (PUVA). CT, control, i.e. incubation without PUVA. Necrosis was assessed by the staining of nuclei with PI whereas apoptosis was visualized by the appropriate changes of nuclei stained with Hoechst 33258. Values are mean ± S.D. of at least four experiments. B: Jurkat cells were incubated for 4.5 h at 37°C under the following conditions: (i) psoralen without UVA (PS); (ii) UVA alone (1.8 J/cm^2) (UV); (iii) PUVA performed with increasing concentrations of psoralen and 1.8 J/cm^2 UVA.

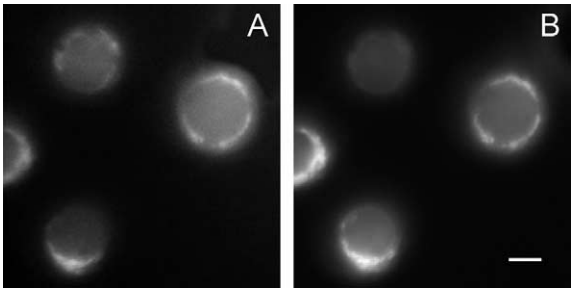

Fig. 2. Psoralen is localized to mitochondrial membrane. Jurkat cells were loaded with 50 μM psoralen (A) and 25 nM TMRM (B) and their fluorescence emission was visualized by fluorescence microscopy. The pattern of psoralen fluorescence closely matched that of TMRM. Scale bar = 5 μm.

2.7. Assessment of cell viability

Jurkat cells (10^6 cells/ml) were stained with 10 μM Hoechst 33258 and 1 μM propidium iodide (PI) for 5 min [12]. Cells were then washed with HBSS and visualized with the fluorescence microscope using excitation/emission cubes of 340/440 ± 25 nm and 568/585 ± 25 nm longpass filter for Hoechst 33258 and PI, respectively. Three randomly selected fields were acquired from each treatment. The corresponding bright field images were also acquired, and the three channels were overlaid using the appropriate function of the Metamorph software.

2.8. Statistical analysis

Data are reported as means ± S.D. The Student *t*-test was used and results were considered to be significant if $P < 0.01$.

3. Results

The initial aim of our study was to determine the prevailing form of cell death in Jurkat cells exposed to PUVA treatment, which is the exposure to UVA light after incubation with psoralen. The concentration of this photosensitizer and light fluency were chosen to obtain cell death only upon their combination. The screening for the proper conditions was performed by evaluating chromatin condensation with Hoechst 33258 as a sign of apoptosis and nuclear staining with PI as a marker of necrosis. Fig. 1A shows that psoralen alone at a concentration below 50 μM or UV irradiation alone at a dose of 1.8 J/cm^2 did not affect cell viability, except for a significant increase in the number of apoptotic cells that could be detected 20 h after UVA irradiation. Conversely, PUVA treatment caused a dramatic loss of viability represented almost exclusively by apoptosis. The apparent increase in necrosis, indicated by PI positive cells after prolonged incubations, is due to the fact that apoptotic cells eventually loose plasma membrane permeability. The occurrence of apoptosis was confirmed by PARP cleavage. As shown in Fig. 1B, immunoblot analysis showed that the typical 89 kDa fragment of PARP was detectable only when cells were treated by PUVA performed by using high psoralen concentrations. The apoptotic mode of cell death was further confirmed by fluoresceinated annexin-V staining and cytochrome *c* redistribution which were only detectable in PUVA-treated cells (not shown).

To investigate the mechanism(s) activated by PUVA and responsible for apoptosis, we focused our attention on mitochondria. In fact, not only the dysfunction of these organelles

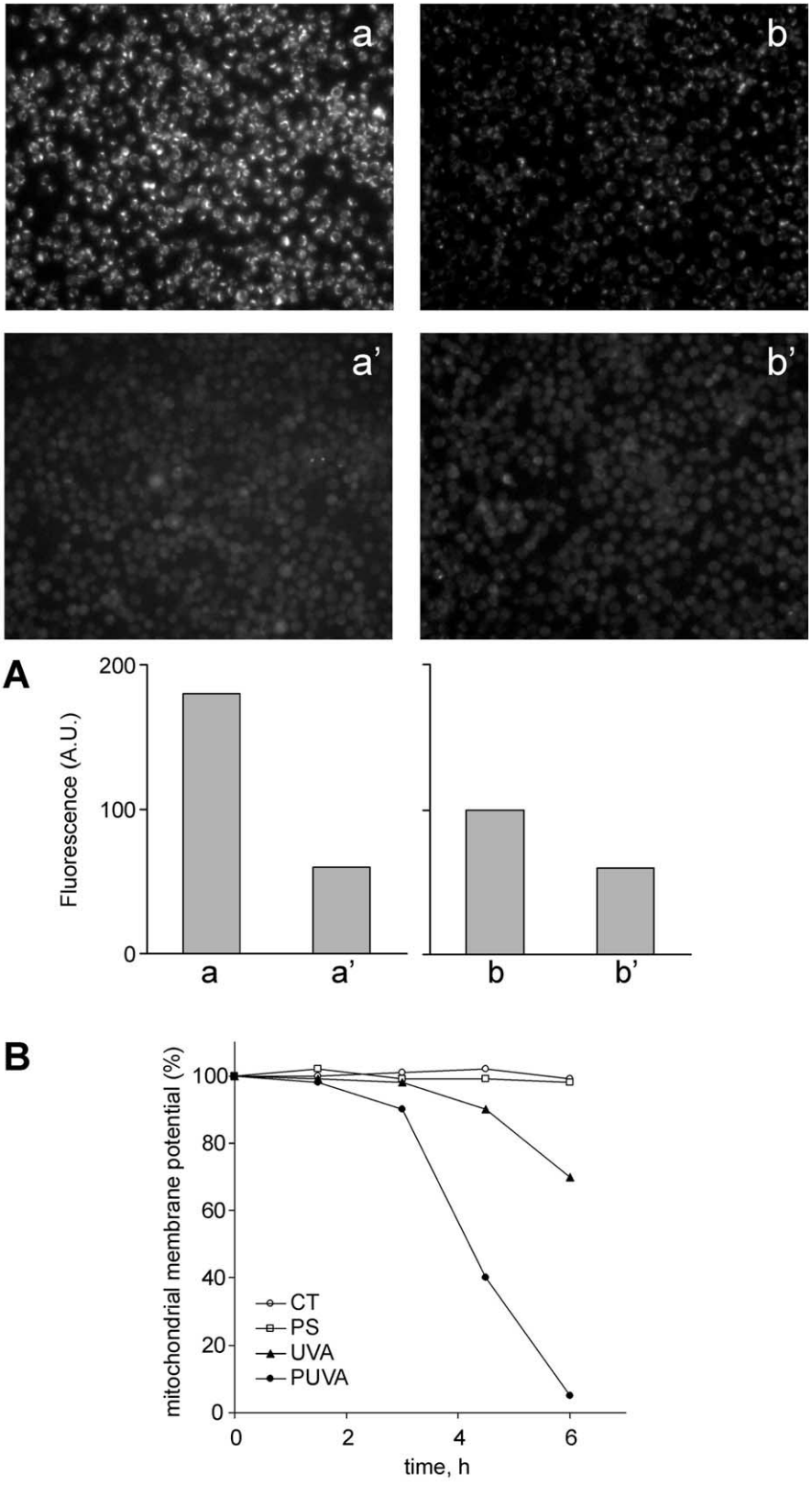

Fig. 3. $\Delta\psi_m$ decrease induced by PUVA. A: Jurkat cells were treated with UVA alone (a) or exposed to PUVA treatment (b) as described in Fig. 1. After 4.5 h of incubation cells were stained with 25 nM TMRM and 1.6 μM CsH. The images were acquired before (a, b) and after (a′, b′) the addition of 2 μM FCCP. The lower part of panel A shows the mean values of fluorescence intensities calculated on at least 30 different cells in each field. The differences between the fluorescence values of each cell before and after the uncoupler (rather than the absolute intensity before FCCP addition) reflect the actual magnitude of $\Delta\psi_m$. B: Jurkat cells were incubated under the conditions described in Fig. 1 and then stained with TMRM. $\Delta\psi_m$ was evaluated as the difference in fluorescence intensities obtained before and after FCCP addition. The different values obtained in treated cells were normalized to those obtained in untreated cells at time 0.

is considered a crucial step in the commitment of the cell to apoptosis [13], but we also found that upon psoralen addition its fluorescence was mostly localized to mitochondria and co-localized with that of TMRM (Fig. 2), a lipophilic cation commonly used for the assessment of $\Delta\psi_m$ [6,12]. The experiments performed to assess the changes in mitochondrial function are shown in Fig. 3. The $\Delta\psi_m$ was monitored by means of TMRM fluorescence. To exclude artifacts due to the different loading capacity of the various cells which can be erroneously interpreted as $\Delta\psi_m$ differences, after each treatment the cells were added with an uncoupler of oxidative phosphorylation (FCCP) which abolishes $\Delta\psi_m$. Thus, in each cell the difference of fluorescence intensities obtained before and after FCCP provided a reliable assessment of $\Delta\psi_m$. At 4.5 h after PUVA treatment Jurkat cells displayed low levels of TMRM fluorescence intensity which were slightly reduced by FCCP addition (Fig. 3A, b and b′). Conversely, at a similar interval after UVA irradiation, the fluorescence intensities were at higher levels and protonophore addition resulted in a rapid decrease of fluorescence intensity in UVA-treated cells (Fig. 3A, a and a′). Thus, $\Delta\psi_m$ reflected by the differences in TMRM fluorescence intensities before and after FCCP addition, was severely reduced by PUVA treatment. The decline in $\Delta\psi_m$ was already detectable 3 h after PUVA treatment and was maximal after 6 h, when a significant decrease of $\Delta\psi_m$ could be also assessed in cells treated with UVA alone (Fig. 3B).

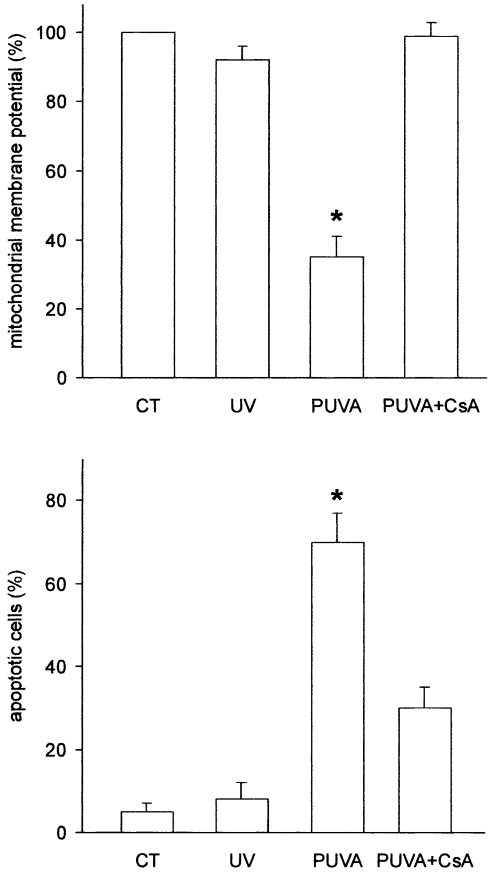

Fig. 4. CsA inhibits both mitochondrial dysfunction and apoptosis induced by PUVA. Jurkat cells were incubated for 4.5 h under the conditions described in Fig. 1. PUVA treatment was performed in the absence or presence of 1 μM CsA. Cells were loaded with Hoechst 33258 and TMRM to evaluate apoptosis and $\Delta\psi_m$, respectively. The changes in $\Delta\psi_m$ were evaluated as described in Section 2 and in Fig. 3. Values are means ± S.D. ($n = 4$). *$P < 0.01$.

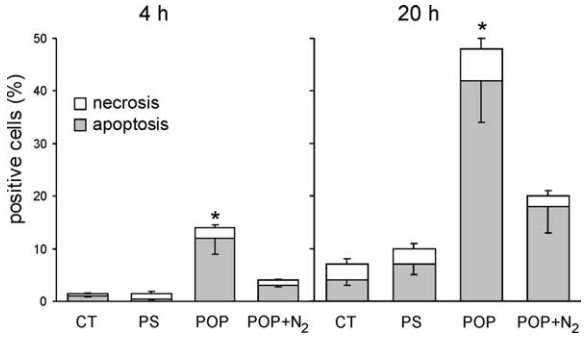

Fig. 5. Loss of cell viability induced by POP. Jurkat cells were incubated in the dark in the absence (control, CT) or the presence of 100 μM psoralen (PS) or equimolar amounts of psoralen preirradiated as described in Section 2. In particular, POP+N_2 indicates the effects elicited by a psoralen solution bubbled with N_2 before irradiation. Apoptosis and necrosis were evaluated after the indicated times by means of Hoechst 33258 and PI fluorescence, respectively. Values are means ± S.D. ($n = 4$). *$P < 0.01$.

To test the involvement of PTP in PUVA-induced mitochondrial dysfunction, Jurkat cells were added with 1 μM CsA 30 min before irradiation. This PTP inhibitor prevented both the fall in $\Delta\psi_m$ and the occurrence of apoptosis in PUVA-treated cells (Fig. 4).

Finally, we tested the hypothesis that POP might contribute to the induction of apoptosis. To this aim Jurkat cells were added with 100 μM POP generated as described in Section 2. Chromatin condensation was already detectable after 4 h in 12% of the cells and gradually increased involving 40% of the cells when the incubation time was prolonged to 20 h (Fig. 5). The apoptogenic potency of POP significantly decreased when the psoralen solution was bubbled with N_2 before irradiation.

4. Discussion

The present results demonstrate that apoptosis is the prevailing form of cell death in Jurkat cells treated with PUVA. Mitochondrial dysfunction caused by PTP opening appears to represent a crucial mechanism underlying the cytotoxic effects exerted by PUVA.

It has been previously shown that mitochondrial alterations are involved in cell death caused by photodynamic therapy (PDT; [14–16] and reviewed [17]). In particular, apoptosis was reported to result mostly as a consequence of mitochondrial photodamage, while it was not significant after selective damage to lysosomes and cell membrane induced by various photosensitizers [18]. Accordingly, besides showing the preferential localization of psoralen within mitochondria, our results illustrate for the first time the relevance of mitochondrial dysfunction in PUVA toxicity. Such a result might have been predictable based on the fact that PTP opening occurred when isolated mitochondria incubated with psoralen were irradiated with UVA [19]. However, the extrapolation of results obtained in isolated mitochondria is not always straightforward. For instance, the photosensitizer hematoporphyrin, commonly used for PDT therapy, prevents PTP opening in isolated mitochondria [20], while acting as a PTP agonist in intact cells [16]. Thus, experiments performed on intact cells are

required to clarify mechanistic issues deriving from studies performed on isolated mitochondria.

The present study clearly shows that PUVA causes the loss of $\Delta\psi_m$ which can be antagonized by CsA suggesting the involvement of PTP. A causal relationship between $\Delta\psi_m$ fall and apoptosis is not provided by the data collected in PUVA-treated cells. In fact, due to the severity of the injury, mitochondrial dysfunction occurred concomitantly with the detection of every apoptotic marker that we tested. However, when milder conditions were applied, such as UVA irradiation without psoralen, mitochondrial deenergization clearly preceded the appearance of apoptosis. In addition, since CsA administration largely prevented both the loss of $\Delta\psi_m$ and the occurrence of apoptosis, the dysfunction of mitochondria caused by PTP opening emerges as a causative factor in PUVA-induced apoptosis.

We could not assess PTP opening directly by using the calcein-cobalt procedure previously developed in our laboratory [12]. Indeed this technique proved unreliable in Jurkat cells, due to an incomplete quenching of cytosolic calcein fluorescence by Co^{2+} and minor changes of fluorescence under conditions compatible with PTP opening probably due to the paucity of mitochondria in this cell type. Thus, the measurement of $\Delta\psi_m$ remained the only way to assess mitochondrial function directly and PTP opening indirectly.

The comparison between the fluorescence intensities of membrane potential dyes, such as TMRM, measured in different moments and in different cells is a potential source of erroneous interpretations (reviewed in [6]). In fact, in comparing two different cell populations, a decrease in TMRM fluorescence does not necessarily imply a parallel reduction in $\Delta\psi_m$, since many factors, such as lipophilic interaction of the probes with cell membranes, could alter the accumulation of TMRM within mitochondria independently of $\Delta\psi_m$ modifications. Prompted by this concern, we analyzed the difference in TMRM fluorescence intensity before and after FCCP addition. Such a difference directly reflects the magnitude of $\Delta\psi_m$ independent of the ability of the cell to accumulate the fluorescent probe. Thus, while the low level of TMRM intensity in PUVA-treated cells (Fig. 3A, panel b) might result from cellular changes other than mitochondrial deenergization, the unambiguous evidence that PUVA has caused a fall in $\Delta\psi_m$ is provided by the significant reduction in the ability of FCCP to cause a decrease in TMRM fluorescence (Fig. 3A, panel b′ vs. panel b).

The results obtained with PUVA do not elucidate whether oxygen-dependent or -independent mechanisms were relevant for the induction of apoptosis. However, the observation that apoptosis was also induced by treatment with POP highlights the importance of psoralen photodegradation which generates long-lasting toxic molecules. Since the apoptogenic potency was reduced by irradiating the psoralen solution in the presence of N_2, the involvement of oxygen-dependent reactions in PUVA-induced apoptosis appears mostly related to psoralen photooxidation rather than direct damage induced by reactive oxygen species (ROS). On the other hand, photolysis products originating from oxygen-independent mechanisms [2] may also contribute to cell injury. Thus ROS, which can hardly trigger the delayed apoptosis induced by POP, likely act as amplifiers and/or accelerators of PUVA toxicity, explaining the rapid appearance of apoptosis caused by this treatment.

To reconcile the well-established damage of DNA induced by the covalent addition of psoralen upon UVA irradiation [21] with the present evidence of mitochondrial dysfunction followed by apoptosis in PUVA-treated cells, it is tempting to speculate that the induction of p53 expression or its intracellular redistribution might represent the link between the DNA damage and mitochondrial alterations leading eventually to cell death [22]. However, the involvement of p53 has been ruled out as a necessary factor in the case of PDT-induced apoptosis [17]. Further studies are necessary to verify the occurrence of such a relationship in the case of PUVA treatment.

Acknowledgements: This work was supported by Grants from the Consiglio Nazionale delle Ricerche and the Ministero per l'Università e la Ricerca Scientifica e Tecnologica to F.D.L., S.C. and F.D.A. M.C. is the recipient of a fellowship of the University of Padova. We gratefully acknowledge Dr. Valeria Petronilli for helpful discussions.

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