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Transient protection by peripheral benzodiazepine receptors during the early events of ultraviolet light-induced apoptosis

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Abstract

The peripheral benzodiazepine receptor (PBR) is a mitochondrial protein involved in the formation of mitochondrial permeability transition (PT) pores which play a critical role during the early events of apoptosis. PBRs are located in many tissues and are strongly expressed in the superficial layers of human epidermis. PBRs play a protective role against free radical damage and PBR ligands modulate apoptosis. To investigate the role of PBR during the early events of ultraviolet (UV)-mediated apoptosis we compared the effects of UVB on PBR-transfected Jurkat cells and their wild type counterparts devoid of any PBR expression. Results indicate that early after UVB exposure (up to 4 h), PBR-transfected cells were more resistant to apoptosis and exhibited a delayed mitochondrial transmembrane potential drop, a diminished superoxide anions production, and a reduced caspase-3 activation. Taken together these findings suggest that PBR may regulate early death signals leading to UV induced apoptosis. *Cell Death and Differentiation* (2001) **8,** 747–753.

Keywords: apoptosis; mitochondria; peripheral benzodiazepine receptor; ultraviolet

Abbreviations: A, annexin V; ANT, adenine nucleotide translocator; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; $\Delta\Psi m$, mitochondrial transmembrane potential; HE, dihydroethidine; MPT, mitochondrial permeability transition; PBR, peripheral benzodiazepine receptor; PBR-J, peripheral benzodiazepine receptor-transfected Jurkat cells; PT, permeability transition; PTPC, PT pore complex; PCD, programmed cell death; PI, Propidium Iodide; ROS, reactive oxygen species; UVB, ultraviolet B; VDAC, voltage-dependent anion channel; WT-J, wild type Jurkat cells

Introduction

The peripheral benzodiazepine receptor (PBR) is a 18 kDa protein that is expressed on mitochondrial outer membranes, essentially distributed in peripheral tissues, and that is distinct from central benzodiazepine receptors.[1,2] Endogenous ligands for PBRs include protoporphyrin IX and a newly described cytoplasmic protein, PRAX-1, that specifically interacts with these receptors.[3,4] PBRs are involved in steroidogenesis, heme biosynthesis, immune and stress responses, cell growth, differentiation and mitochondrial respiratory control.[5] Recent data also suggest that PBR is structurally and functionally related to the *Rhodobacter sphaeroides* tryptophan-rich sensory protein (TspO). Complementation experiments demonstrated that PBR was able to substitute for TspO taking part of a signal transduction pathway that regulates gene expression in response to oxygen.[6,7] Within the mitochondrial membrane, PBR is closely associated with voltage-dependent anion channel (VDAC) and adenine nucleotide translocator (ANT) proteins which are involved in the formation of mitochondrial permeability transition (PT) pores.[8] These multiprotein complexes play a critical role in apoptosis as opening PT pores and reduction of mitochondrial potential are the early irreversible steps of the programmed cell death (PCD) process.[9–13] Interestingly, PBR has been shown to preserve the mitochondria of hematopoietic cell lines from damage induced by oxygen radicals[14] and PBR ligands were found to modulate apoptosis.[15,16] In human skin, as PBR expression is strongly upregulated in the superficial differentiated layers of the epidermis, those receptors are thought to play a protective role against free radical damage and apoptosis generated by ultraviolet (UV) light.[17] In order to investigate the role of PBR during the early events of UV-mediated apoptosis, we analyzed the effects of UVB exposure on PBR-transfected Jurkat cells (PBR-J)[14] compared to their wild-type counterparts (WT-J) devoid of any PBR expression.

Results

We choose the Jurkat cell lines because: (i) WT-J cells are naturally devoid of any PBR expression; (ii) and are non adherent cultured cells that do not require chemical or mechanical treatments which by themselves may modify the phosphatidylserine expression. Results of all the experiments were similar between clones 12-2-F8, 31-1-B10 and 12-2-D2 and there were no difference between Jurkat cell lines transformed with vector controls (pHb Neo APR1-J) and wild type Jurkat cells (WT-J). All the experiments were repeated thrice. χ^2 and Wilcoxon tests were used for statistical analysis.

Analysis of early apoptosis induced by UVB treatment

Cells were labeled with Annexin-FITC (A) and Propidium Iodide (PI). Apoptosis was first analyzed by flow cytometric analysis with increasing UVB doses (10–500 mj/cm^2) at 1 h. A representative experiment is shown on Figure 1. Per cent of apoptotic but still viable cells (A^+PI^-) was reduced in PBR-J cells compared to WT-J cells with a dose-dependent effect. An optimal difference of apoptotic response between the two cell lines was observed with a 100 mJ/cm^2 dose. Therefore we decided to study the kinetic of apoptosis at 1, 4, 12 and 24 h by using this irradiance. A representative experiment is shown on Figure 2a. Per cent of non viable PI^+ (PI^+ A^+) cells increased after an acute UVB exposure in both cell lines. However, up to 4 h after UV treatment, the per cent of A^+PI^- cells was significantly reduced in PBR-J cells compared to WT-J cells (13% *vs* 44% at 1 h; 50% *vs* 75% at 4 h). At 12 h, most of cells have undergone apoptosis in both cell lines, the PI^+ subset being still reduced in PBR-transfected cells (46% *vs* 60%). Such differences were confirmed from multiple experiments and there was no statistically significant difference between each tested PBR-transfected clones (Figure 2b,c). Furthermore, results were similar between wild type (WT-J) and cells transfected with an empty vector (pHb Neo APR1-J). At 1 h significant changes in early apoptosis were evidenced: WT-J cells exhibited a higher percentage of A^+PI^- cells than that of PBR cells (clone 12-2-F8: 34.1$\pm$6.17% *versus* 11$\pm$1.7%, P=0.001). At 4 h, such difference was also shown with a mean per cent of A^+PI^- cells of 51.9$\pm$2.66% in PBR-J cells (clone 12-2-F8) compared to that of 70.6$\pm$5.25% in WT-J cells (P=0.001) (Figure 2b). No significant loss of viability was observed after 1 and 4 h in wild type and PBR-transfected cell lines. Twelve hours after UVB exposure, the per cent of non viable A^+PI^+ cells in WT-J was higher (clone 12-2-F8: 63.3$\pm$3.5%) than that in PBR-J cells (47.0$\pm$2.65%) (P=0.001) while the per cent of A^+PI^- PBR-J cells (36.7$\pm$5.7%) was higher than that of WT-J cells (26$\pm$6%) (Figure 2c). At 24 h, most of A^+ WT-J and PBR-J cells were PI^+ (Figure 2a). These results indicate that early after UVB exposure (up to 4 h), PBR-transfected cells were more resistant to apoptosis than WT-J cells suggesting that PBRs may exert a transient protective effect during the initial events of programmed cell death.

Mitochondrial permeability transition after UVB exposure

In a number of experimental systems, the early stage of the apoptotic process is characterized by the breakdown of the inner mitochondrial transmembrane potential ($\Delta\Psi$m).[19] As PBR is part of the protein complex involved in the formation of mitochondrial permeability transition (PT) pores,[8] we assessed the reduction of $\Delta\Psi$m that occurs after UVB exposure (100 mJ/cm^2) in WT and PBR-J cells. $DiOC_6$ is a fluorochrome which incorporates into mitochondria in strict nonlinear dependence of $\Delta\Psi$m and emits exclusively within the spectrum of green light. One representative experiment is shown in Figure 3a. One hour after UVB, 41% of WT-J cells became $DiOC_6^{low}$ PI^- whereas 54% remained PI^- $DiOC_6^{high}$.

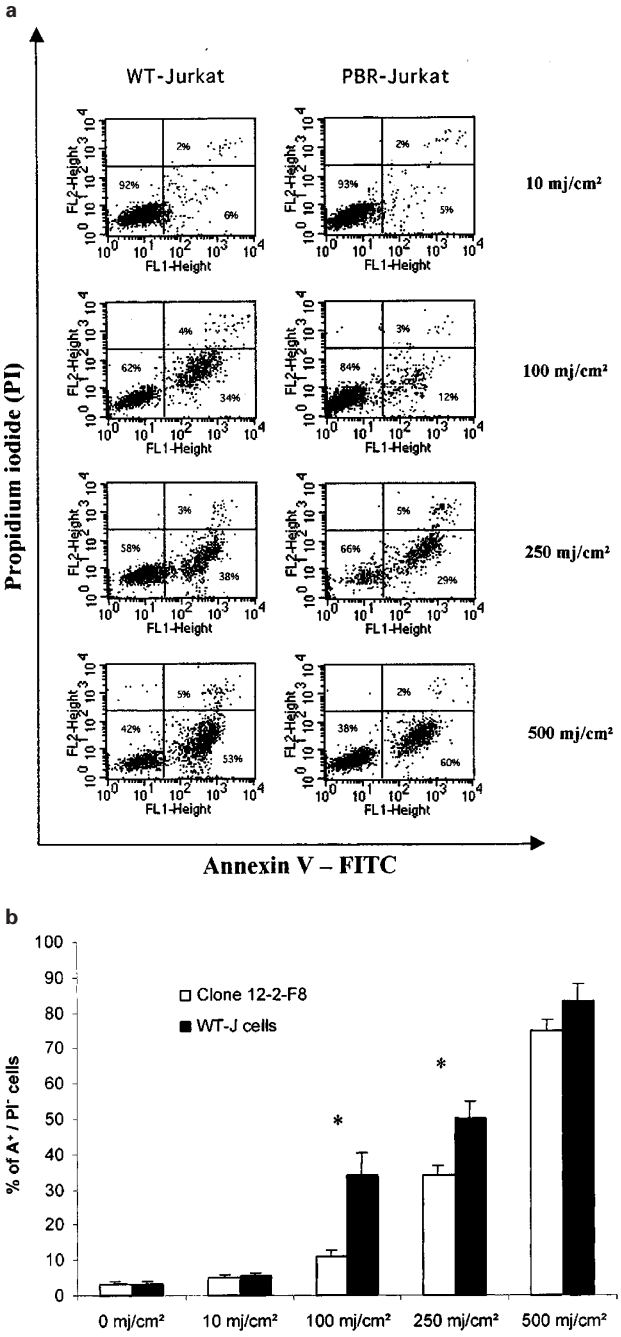

Figure 1 UVB-induced apoptosis in WT-J and PBR-J cell lines. Flow cytometric analysis of WT-J and PBR-J cells (12-2-F8 clone) labeled with Annexin V (FITC) and propidium iodide (PI) were performed at 1 h after increasing UVB doses (10 to 500 mJ/cm^2). In both cell lines per cent of A^+PI^- cells (apoptotic cells) increased in a dose-dependent manner. There were significantly less apoptotic cells (P=0.001) in PBR-J cells after 100 mJ/cm^2 and 250 mJ/cm^2 UVB exposures. The optimal difference between the apoptotic response of the two cell lines was obtained with a 100 mJ/cm^2 dose. Results are representative of three different determinations

By contrast the $DiOC_6^{low}$ PI^- cell subset was only 11% in PBR-J cells indicating that 1 h after UV, the release of $DiOC_6$ was dramatically reduced in PBR-transfected cells. Until 4 h repeated experiments confirmed those results whereas at 12

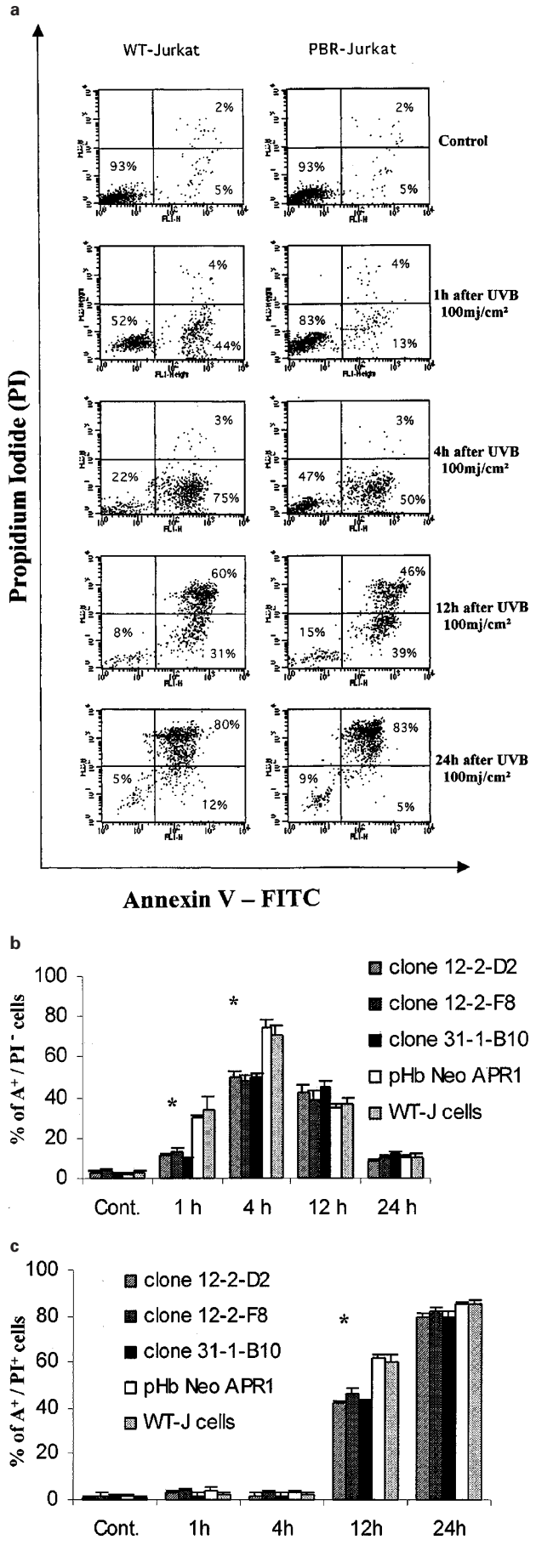

Figure 2 (**a**) UVB-induced apoptosis in WT-J and PBR-J cell lines. Flow cytometric analysis of WT-J and PBR-J cells (12-2-F8 clone) labeled with Annexin V (FITC) and propidium iodide (PI) were performed at 1, 4, 12 and 24 h after a UVB dose corresponding to 100 mJ/cm^2. Up to 4 h, A^+PI^- cellular subsets (lower right quadrants) are reduced in PBR-J cells. After 12 h, per cent of A^+PI^- cells is greater in PBR-J cells (39% *vs* 31%) but PI^+ subset is still reduced compared to WT-J cells (46% *vs* 60%). Results are representative of three independent experiments. Numbers refer to the percentage of cells contained in the indicated gate. (**b**) Kinetic study of apoptosis after UVB (100 mJ/cm^2): at 1 and 4 h, apoptotic A^+PI^- cellular subsets are significantly reduced in PBR-J cells (P=0.001). Results are representative of three different determinations. No statistically significant difference was observed between 12-2-F8, 12-2-D2 and 31-1-B10 PBR transfected clones. The kinetic study was similar between Jurkat cells transfected with the corresponding empty vector (pHb Neo APR1-J) and the WT-J cells. (**c**) Kinetic study of apoptosis after UVB (100 mJ/cm^2): at 12 h non viable A^+PI^+ cells are significantly reduced in PBR-J Jurkat cells (P=0.001). No statistically significant difference was observed between 12-2-F8, 12-2-D2 and 31-1-B10 PBR transfected clones. The kinetic study was similar between WT-J and pHb Neo APR1-J cells. Results are representative of three different determinations

and 24 h no statistically significant difference could be observed between the two cell types (Figure 3b). Taken together these results indicate that shortly following UV exposure, the $\Delta\Psi$m drop is delayed in PBR-transfected cells compared to WT Jurkat cells.

Superoxide anions production after UV exposure

Reduction in $\Delta\Psi$m and subsequent reactive oxygen species (ROS) production are observed in several *in vitro* models of programmed cell death. Early events of apoptosis that occurred before the nucleus or nuclear DNA are fragmented, included first a fall in $\Delta\Psi$m followed by mitochondrial generation of ROS.[20] To assess the production of superoxide anions, cells were labeled simultaneously with $DiOC_6$ and oxidizing hydroethidine (HE), a substance that is oxidized by superoxide anions to produce ethidium and red fluorescence emission.[21] A representative experiment is shown in Figure 4a. Flow cytometric analysis of WT and PBR-J cells (clone 12-2-F8) demonstrated that at each time point after UV exposure (1, 4 and 12 h), only $DiOC_6^{low}$ cells produced superoxide anions. One hour after UV exposure, 44% of WT-J cells displayed a reduced $\Delta\Psi$m ($DiOC_6^{low}$ population in the two left quadrants) and almost 50% of them (21% of total number of cells) were Eth^+. However, in PBR-J cells, only 8% of cells displayed a reduced $\Delta\Psi$m and one third (2% of total cells) produced superoxide anions. Repeated experiments performed at 1, 4, 12 and 24 h (Figure 4b) indicate that until 4 h the superoxide anions production is significantly reduced in PBR-transfected cells (P=0.001).

Caspase-3 activation after UV exposure

UV irradiation induces activation of caspase 3 (CPP32-like protease) in the early stages of apoptosis.[22] As mitochondria plays a critical role in the initiation of the caspase cascade,[23] we measured the activity of caspase-3 in WT-J and PBR-J cells at 1, 2, 4, 12 and 24 h after UVB exposure (100 mJ/cm^2). Shortly after irradiation, the activity of caspase-3 was increased in both WT and PBR-J cells up to 4 h, and returned to its baseline level at 24 h (Figure 5). At 2 and 4 h,

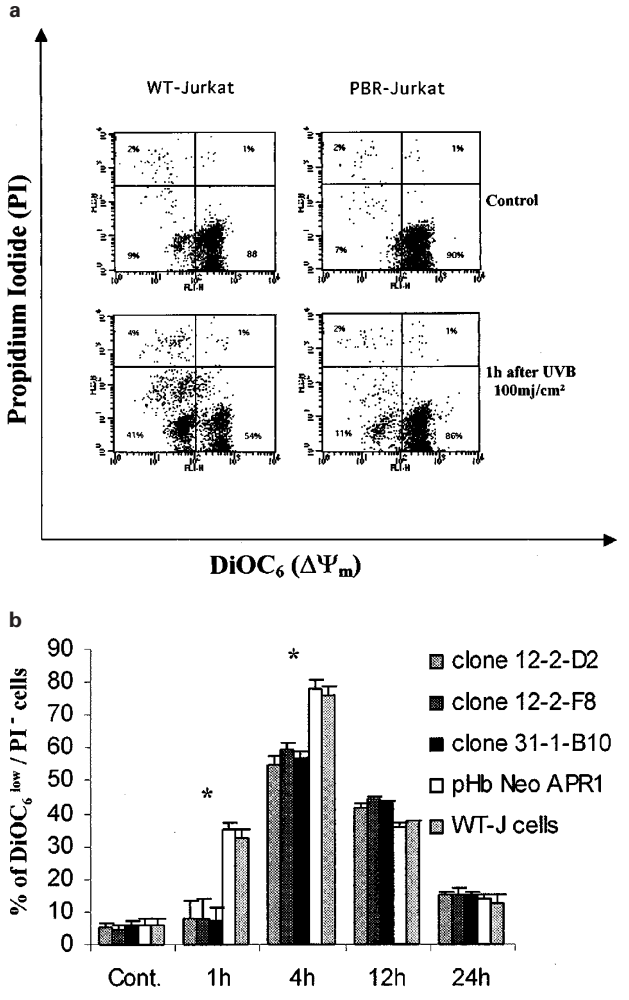

Figure 3 (**a**) UVB induced ΔΨm drop in WT-J and PBR-J cell lines. Flow cytometric analysis of WT-J and PBR-J cells (12-2-F8 clone) labeled with $DiOC_6$/PI 1 h after UVB exposure. Regarding PI expression, cells can be separated into PI^-, $PI^{+/-}$ and PI^+ populations. Gates were set on PI^+ cells (upper left quadrants). After UVB, 41% of WT-J cells were $DiOC6_{low}PI^-$ whereas only 11% of PBR-J cells dropped their ΔΨm and became $DiOC6_{low}$ PI^-. Results are representative of three independent experiments. (**b**) Kinetic study of ΔΨm drop in WT-J and PBR-J cells after UVB irradiation. $DiOC6_{low}PI^-$ cellular subsets represent cells that have released $DiOC_6$ and dropped their ΔΨm. At 1 and 4 h after UVB exposure (100 mJ/cm^2), there is a significant reduction of the $DiOC6_{low}PI^-$ subset in PBR-transfected Jurkat (PBR-J) cells (P=0.001). At 12 h, there is no statistically significant difference between the two types of cells. No statistically significant difference was observed between 12-2-F8, 12-2-D2 and 31-1-B10 PBR transfected clones. The kinetic study was similar between WT-J and pHb Neo APR1-J cells. Results are representative of three different experiments

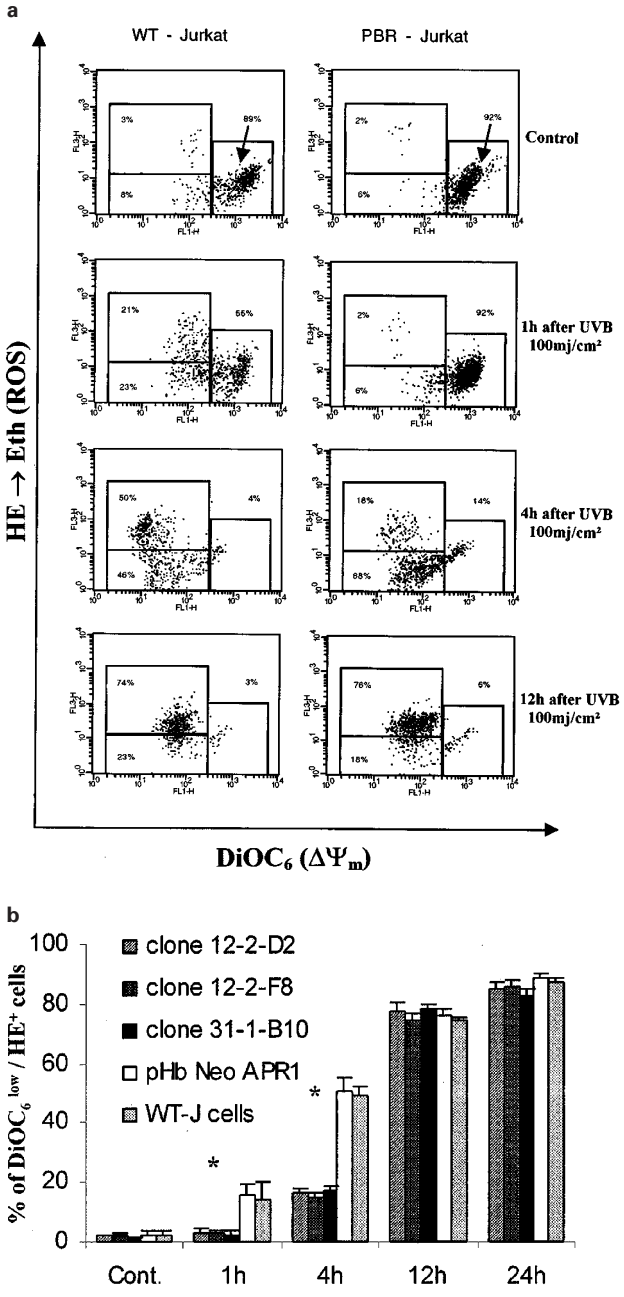

Figure 4 (**a**) UVB induced ΔΨm drop and superoxide anions production in WT-J and PBR-J (12-2-F8 clone) cell lines. Flow cytometric analysis of WT-J and PBR-J cells were performed after UVB exposure. Cells were simultaneously stained with $DiOC_6$ and HE (which allows for the determination of superoxide anion generation). Apoptotic $DiOC6_{low}Eth^+$ cellular subsets that produced ROS (upper left quadrants) are reduced in PBJ-J cells at 1 and 4 h after UV. Results are representative of three independent experiments. (**b**) Kinetic study of loss in ΔΨm and superoxide anions production in WT-J and PBR-J cells after UVB irradiation. At 1 and 4 h, superoxide anions production is significantly decreased in PBF-J cells (P=0.001). No statistically significant difference was observed between 12-2-F8, 12-2-D2 and 31-1-B10 PBR transfected clones. The kinetic study was similar between WT-J and pHb Neo APR1-J cells. Results are representative of three different determinations

caspase 3 activity was 20 – 50% higher in WT-J cells than that in PBR-J cells (P=0.01, Wilcoxon test). These results indicate that PBR expression is linked to a downregulation of caspase-3 activation that occurs in PBR-J cells shortly after UVB irradiation.

Discussion

Ultraviolet light (UV) generates singlet-oxygen damage and may trigger a PCD that does not require post-insult protein synthesis.[24] This process may involve the PT pore which is a multiprotein complex formed at the contact site between the

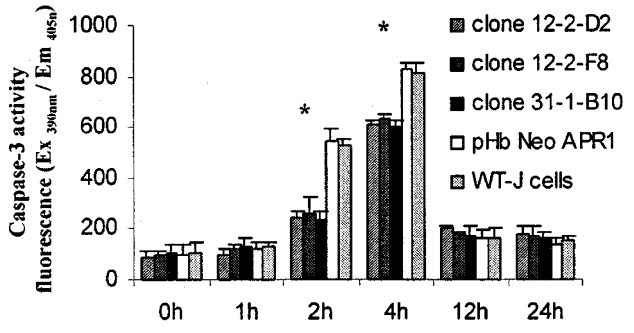

Figure 5 Kinetic study of caspase-3 activation after UVB exposure on PBR-J and WT-J cells. Jurkat cells were exposed to 100 mJ/cm^2 UVB and incubated for 24 h at 37°C. Lysates were analyzed for caspase-3 activity and fluorescence (minus fluorescence of background) was plotted versus time. Caspase-3 activity is significantly decreased in PBR-J cells at 2 and 4 h (P=0.01). No statistically significant difference was observed between 12-2-F8, 12-2-D2 and 31-1-B10 PBR transfected clones. The kinetic study was similar between WT-J and pHb Neo APR1-J cells. Results are representative of three independent experiments

mitochondrial inner and outer membranes. The exact molecular composition of the pore is still unknown although several proteins have been described involved in mitochondrial PT pore formation such as VDAC (mitochondrial porine), ANT and PBR.[8] Opening the PT pore can cause the dissipation of the inner mitochondrial transmembrane potential ($\Delta\Psi$m) that is a critical event in the process leading to apoptosis.[12,25,26] Moreover, recent evidence suggests that PT pore complex (PTPC) may constitute a crossroad of apoptosis regulation by caspases and members of the Bcl-2 family.[19,27] Indeed, Bcl-2 proteins are particularly abundant in mitochondrial membrane where they are closely associated to PBRs and other proteins of the PTPC.[10] Recent data demonstrate that proteins of the Bcl2 family bind to VDAC and regulate the mitochondrial membrane potential and the release of cytochrome *c* during apoptosis.[11] Thus, a functional interaction between Bcl-2 and other components of PTPC such as PBR cannot be excluded. Interestingly, PK11195, a specific antagonistic ligand of PBR, facilitates the induction of apoptosis by triggering $\Delta\Psi$m disruption and reverses Bcl-2 mediated cytoprotection.[15,28] Moreover, Bcl-2 protects isolated mitochondria against the opening of the PT pore induced by low doses of protoporphyrin IX, a ligand of PBR.[29] Mechanisms by which PBR and Bcl-2 exert anti-apoptotic effects are still not known and require further studies. Recent data demonstrate that a PBR agonistic ligand (Ro5-4864) is a potent anti-apoptotic compound.[16] Finally the results reported here indicate that transfection-induced hyperexpression of the PBR gene protects against early UV-induced apoptosis as evidenced by a delay in mitochondrial changes and suggest that $\Delta\Psi$m is stabilized by PBR. Altogether these data suggest a critical role for PBR in the apoptotic process.

Reactive oxygen species (ROS) are involved in many forms of PCD and in several *in vitro* models reduction of $\Delta\Psi$m is followed by enhanced production of ROS and subsequent DNA fragmentation and nuclear DNA loss.[20,30] UV-induced oxidative DNA damage is rapidly followed by the loss of $\Delta\Psi$m and the activation of caspase-3, resulting in apoptosis.[31] The demonstration of the role of PBR in the protection of hematopoietic cells against H_2O_2 suggests that PBR may prevent mitochondria from radical damage.[14] Our findings are in agreement with these results and argue that PBR may be involved in ROS production induced by UV light. Mechanisms underlying these effects are still unclear but one may hypothesize that the reduced fall in $\Delta\Psi$m due to PBR transfection results in a decreased generation of ROS that occurs shortly after UVB exposure. Indeed, PT pores participate in the generation of matrix Ca^{2+}, pH, $\Delta\Psi$m and redox-gated channel, and is implicated in cell death induced by anoxia, ROS, and calcium overload.[27] Furthermore, opening the PT pores leads to the release of cytochrome *c* from mitochondria to the cytosol, thereby triggering caspases activation and cell death by apoptosis.[32–35] Indeed, mitochondria are involved in the initiation of the caspase activation by releasing cytochrome *c* into the cytosol where it binds to the adaptator molecule Apaf-1 (apoptotic protease activating factor 1).[35] This complex activates caspase-9 which then cleaves and activates downstream caspases such as caspase-3, -6, and -7. This pathway is regulated at several steps and caspase substrates such as Bcl-2 proteins appear to promote further caspase activation as part of a positive feedback loop.[36] Caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins[37] and initiating apoptotic DNA fragmentation.[38] Recent data demonstrate that activation of caspase-3 is increased in the early stages of UVB-induced apoptosis.[22,39] Our results indicate that caspase-3 activation after UVB is delayed in PBR-transfected cells. Whether this effect is related to protein-protein interactions within the mitochondrial PT pore or results from a disruption of the feedback loop control of caspase activation remains to be elucidated.[33,40] Nevertheless, our results indicate that PBR, an important component of mitochondrial PT pore, may exert transient anti-apoptotic effects during the early events of UV-induced PCD. These findings are in agreement with pharmacological studies showing that PBR ligands may modulate apoptosis[15,16,28] of hematopoietic cells as well as epithelial or hematological malignancies.[41] Taken together, these observations provide compelling evidence indicating that PBR belongs to the protein family which may interact within the mitochondrial membrane to regulate death signals leading to apoptosis.

Materials and Methods

Materials

Stable transfectants PBJ-J (12-2-F8, 31-1-B10 and 12-2-D2 clones) and the corresponding WT-J cell lines and empty-vector transfected Jurkat cell lines (pHb Neo APR1-J) were kindly provided by Sanofi-Synthelabo (Montpellier, France). [14] Quantitation of the number of PBR sites per cell was performed by flow cytometry using no conjugated anti-PBR MoAb and the QIFI assay (QIFIKIT; Dako).[18] Clones 12-2-F8, 31-B-10 and 12-2-D2 expressed 90 000 $\pm$ 10 000, 50 000 $\pm$ 10 000 and 45 000 $\pm$ 5000 PBR sites per cell respectively. WT-J and pHb Neo APR1-J cells were devoid of any PBR

expression.[14] RPMI 1640 medium, phosphate-buffered saline (PBS), pyruvic acid, and essential and nonessential amino acids solutions were obtained from Gibco BRL (Cergy-pontoise, France). Fetal calf serum (FCS), penicillin, streptomycin sulfate and glutamine were purchased at Boehringer Mannheim (Meylan, Claix, France).

Cell cultures and UVB irradiation

WT-J and PBJ-J cell lines were routinely cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS, 4 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin sulfate at 37°C under 5% CO_2 in a humidified atmosphere. WT-J and PBJ-J cells were seeded in 35 mm Petri dishes (10^6 cells/well) in phenol red-free RPMI 1640 containing 10% FCS. Cells were exposed to UVB radiation (290–320 nm, doses ranged from 0 to 500 mJ/cm^2) by using a UV 800 Waldman device (Reichstett, France). Power density was monitored before each experiment by using a Waldmann UV-Meter (Waldmann, Schwenningen, Germany). The source to target distance was 30 cm and the cell culture temperature was kept constant at 25°C during irradiation. Sham-irradiated samples were used as controls. After irradiation, the cells were incubated at standard culture conditions for the given time periods.

Flow cytometric detection of apoptosis

The UVB induced cell death was analyzed at 1, 4, 12, 24 h after irradiation using flow cytometric detection of phosphatidylserine expression with the Annexin V-FITC kit (Bender MedSystems, Vienna, Austria). Briefly, cells (5×10^5 cells) were washed twice in PBS and resuspended in 195 μl binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and then incubated for 10 min at room temperature with 5 μl annexinV-FITC. After wash, cells were resuspended in 190 μl binding buffer and Propidium Iodide were added to a final concentration of 1 μg/ml for flow cytometric analysis. Bivariate analysis was performed on a FACSCan (Becton Dickinson, Mountain View, USA) equipped with a 488 nm argon laser. A minimum of 10 000 cells per sample was recorded and stored in list mode files. Data analysis was performed using the CELLQuest software (Becton Dickinson, St. Louis, USA).

Cytofluorometric analysis of mitochondrial transmembrane potential ($\Delta\Psi$m), and superoxide anions generation

After UVB irradiation, cells were incubated from 0 to 24 h at 37°C in the standard medium. Following published protocols[19,20] the following fluorochromes were employed to determine different apoptosis-associated change: to assess $\Delta\Psi$m and cell mortality, cells (5×10^5 cells/ml) were washed twice and incubated with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), 40 nM in PBS; Molecular Probes Inc., Eugene, OR, USA) and propidium iodide (PI, 5 μg/ml). To measure $\Delta\Psi$m and superoxide anions generation, cells (5×10^5 cells/ml) were washed twice and incubated with $DiOC_6$ and dihydroethidine (HE; 2 μM, Molecular Probes Inc., Eugene, OR, USA) for 15 min at room temperature. After incubation, cells were washed and suspended in PBS, and then analyzed on a FACScan.

Measurement of caspase-3 activity

After a 100 mJ/cm^2 UVB irradiation, cells were continuously incubated at 37°C for 1, 2, 4, 12 and 24 h in the standard medium. Caspase-3 activity was determined using a Fluorometric Immunosorbent Enzyme Assay kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells (2×10^6 cells) were washed with ice cold PBS and suspended in 200 μl of lysis buffer for 1 min on ice. Cell lysates were centrifugated at $1800 \times g$ for 15 mn and supernatants were collected. The reaction was initiated by addition of Ac-DEVD-AFC (Z-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin) to anti-caspase 3-coated microtiter containing apoptotic extract. Product formation was measured by using a Hitachi F-2000 spectrofluorometer with excitation at 400 nm and emission at 505 nm.

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