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PKC ζ counteracts UV-C signaling

PKC ζ PROTECTS AGAINST UV-C INDUCED APOPTOSIS BY INHIBITING ACID SPHINGOMYELINASE –DEPENDENT CERAMIDE PRODUCTION

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Short title: PKC ζ counteracts UV-C signaling

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SYNOPSIS

In a recent study, we described that UV-C irradiation resulted in redox-dependent activation and relocalization of acid sphingomyelinase (A-SMase) to the external surface of raft membrane microdomains, hydrolysis of sphingomyelin (SM) associated to the plasma membrane outer leaflet, ceramide (CER) generation and apoptosis. In the present study, we have investigated the influence of PKC ζ , an atypical form of PKC on this pathway. This study shows that PKC ζ overexpression resulted in the abrogation of UV-C-induced A-SMase translocation and activation into the raft microdomains, lack of CER generation, and apoptosis inhibition. Moreover, PKC ζ overexpression resulted in the reduction of UV-C induced radical oxygen species (ROS) production, which correlated with increased gene expression level of various anti-oxidant enzymes, including thioredoxin (TRx), thioredoxin reductase 1 (TR1), TR2, and peroxiredoxin 1/thioredoxin peroxidase 2 (TPx2). Importantly, enforced-TPx 2 gene expression inhibited UV-C induced A-SMase translocation. Finally,

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PKC ζ inhibition led to significant reduction in TPx 2 protein expression level. Altogether, these results suggest that PKC ζ interferes with UV-activated sphingolipid signaling pathway by regulating the thioredoxin system. These findings may have important consequences for UV-induced carcinogenesis and resistance to phototherapy.

KEYWORDS

UV-C, PKC ζ , acid sphingomyelinase, raft, antioxidant defenses, thioredoxin peroxidase.

ABBREVIATIONS

UV-C : ultra-violet-C ; A-SMase : acid sphingomyelinase ; CER : ceramide ; ROS : radical oxygen species ; TPx : thioredoxin peroxidase.

INTRODUCTION

Mammalian cells respond to UV irradiation by activating a complex signaling network which implies both serine-threonine and tyrosine kinases stimulation, resulting in activation of transcription factors which, in turn, regulate a large variety of genes involved in cell growth and apoptosis. This pathway is under the control of two main types of mediators: radical oxygen species (ROS) and lipid messengers, including diacylglycerol, arachidonic acid, phosphatidic acid, and ceramide (CER) [1-3]. Previous studies have extensively documented the role of sphingomyelin (SM)-CER pathway in apoptosis induced by both UV-B and UV-C [4]. In this signaling cascade, UV irradiation stimulates an acid sphingomyelinase (A-SMase), SM hydrolysis and the generation of CER, which in turn, induces apoptosis through a JNK-dependent pathway [5]. However, the mechanism by which A-SMase operates remained unclear until a recent study, in which we described that UV-C irradiation resulted in a multistep process consisting in redox-dependent activation and relocalization of a Zn²⁺-independent A-SMase to the external surface of raft membrane microdomains, hydrolysis of plasma membrane outer leaflet-associated SM, and accumulation of CER at the cell surface [6]. These results have been confirmed by another group using a different cellular model [7]. From these studies, one can speculate that any mechanism, which could inhibit either A-

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SMase activation or relocalization, might seriously interfere with UV-induced SM-CER pathway activation and apoptosis.

In this context, we have speculated that PKC ζ could be one of these regulators. Three lines of evidence argued for this hypothesis. First, we have previously described that this enzyme is a potent inhibitor of apoptosis induced by genotoxic agents [8, 9]. Second, our group has reported that enforced expression of PKC ζ confers a significant protection against UV-C irradiation [10]. Third, we have reported that PKC ζ contributes to the detoxication of ROS [8], the latter being an important contributor to UV-C mediated A-SMase activation and membrane externalization [6].

In this study, we show that PKC ζ does interfere with UV-induced A-SMase relocalization and activation by limiting UV-induced ROS production.

EXPERIMENTAL

Material

Silica gel 60 thin-layer chromatography plates were obtained from Merck (Darmstadt, Germany). All other drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO), Alexis Biochemicals (Paris, France), Matreya Biochemicals (Marne-La-Vallée, France), Carlo Erba (Rueil-Malmaison, France), or Prolabo (Paris, France). Peptide PKC ζ inhibitory pseudosubstrat was purchased from Millegen (Labège, France).

Cell culture

The human myeloblastic cell line U937 was obtained from the ATCC (Rockville, MD).

U937- ζ J cells were obtained from separated co-transfections with a full-length rat PKC ζ cDNA construct subcloned into the pSV₂M(2)6 vector and neomycin-resistant plasmid. U937-neo cells were obtained by transfection with the empty vector. All these cell lines were kindly provided by Dr. D.K. Ways (Lilly Corporate Center, Indianapolis, IN) [11]. These cells displayed a 3-fold increased of both PKC ζ expression and activity as we described in two previous studies [12, 13].

U937-TPx2 cells were obtained by stably transfection with thioredoxin peroxidase (TPx2/PAG) cDNA subcloned into the pEGFP-N1 vector. The plasmid encoding for PAG

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was kindly provided by Dr Goubin (Institut Curie, Paris, France). U937-TPx2 neo were obtained by transfection with the empty vector. Clone selection was done using geneticin.

All cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C in 5% CO₂. Culture medium was supplemented with 2 mM L-glutamine, 200 units/ml penicillin, and 100 µg/ml streptomycin (all from Eurobio, Les Ulis, France).

Cell irradiation

Cells were irradiated with UV-C light (254 nm) in PBS during 30 seconds corresponding to 30 joules/m², at a concentration of 1 million cells/ml.

DAPI nuclear staining assay

Changes in cellular nuclear chromatin were evaluated by fluorescence microscopy (Diaplan; Leica, Wetzlar, Germany) by 4',6'-diamidino-2-phenylindol (DAPI) staining using the following method. Briefly, cells were fixed by 4% formaldehyde (PFA) pH 7.4, for 15 minutes. After washing in PBS, cells were dried and stained by 0.5 µg/ml DAPI.

Metabolic cell labeling and quantitation of ceramide

Total cellular CER was performed by labeling 5x10⁶ cells to isotopic equilibrium with 1 µCi/ml of [9, 10-³H]palmitic acid (53.0 Ci/mmol, Amersham, Les Ulis, France) for 48 h in complete medium as previously described [14]. Cells were then washed and resuspended in serum-free medium for kinetic experiments. Lipids were extracted and resolved by thin-layer chromatography developed in chloroform/methanol/acetic acid/formic acid/water (65:30:10:4:2, vol/vol.) up to two-thirds of the plate and then in chloroform/methanol/acetic acid (94:5:5, vol/vol.). CER was scraped and quantitated by liquid scintillation counting. Lipid standards were used to identify the various metabolic products.

Isolation of membrane rafts microdomains

Raft microdomains were isolated from cells as previously described [15]. For each isolation, 100x10⁶ cells were washed twice with PBS. Cells were pelleted by centrifugation, resuspended in 1 ml of ice-cold MES-buffered saline (MBS) (150 mM NaCl, 25 mM 2-(N-morpholino) ethanesulfonic acid, pH 6.5), containing 1% (w/v) Triton X-100. After 30 minutes on ice, cells were further homogenized by 10 strokes of a Dounce homogenizer on ice. 1.5 ml of ice-cold MBS were added and 2 ml of this suspension were mixed with 2 ml of 80% (w/v) sucrose in MBS. This mixture was subsequently loaded under a linear gradient

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consisting of 8 ml 5% to 40% (w/v) sucrose in MBS. All solutions contained the following protease inhibitors: 100 μ M PMSF, 1 mM EDTA and 1 μ M each of aprotinin, leupeptin and pepstatin A. Gradients were centrifuged in a Beckman SW 41 swinging-rotor at 39000 rpm for 20 h at 4°C. Twelve fractions of 1 ml each were collected (from top to bottom), vortexed and stored at -80°C. The protein content of both fractions and the total initial cell suspension were measured using bovine serum albumin as standard [16]. GM1 was used as a marker of rafts [17, 18].

Acid sphingomyelinase activity

A-SMase activity was evaluated as we previously described [6; 19]. Briefly,

In whole cells. Cells were irradiated for different times with 30 joules/m² of UV-C. For A-SMase activity, the substrate solution consisted of [¹⁴C-methylcholine]SM (1x10⁵ dpm/assay; NEN, Paris, France) and 0.1% (w/v) Triton X-100 in 200 mM Tris-HCl buffer (pH 5) containing 10 mM DTT and 10 mM MgCl₂. After 2 h incubation at 37°C, reactions were terminated by adding 300 μ l of H₂O and 2.5 ml of chloroform/methanol (2:1, v/v). Phases were separated by centrifugation (1000 x g, 5 minutes) and the amount of released radioactive phosphocholine was determined by subjecting 700 μ l of the upper phase to scintillation counting.

The amount of radiolabeled substrate hydrolyzed during an assay never exceeded 10% of the total amount of substrate added. For calculation of the specific activities in total cell homogenates, values were corrected for protein content, reaction time and specific activity of the substrate.

In raft fractions. A-SMase activity was assayed on 250 μ l of each fraction, and reactions were started by adding 250 μ l of substrate solution as previously described [19].

A-SMase translocation

A-SMase translocation was visualized by two distinct methods as we previously described [6; 19]:

By FACS analysis: cells were irradiated or not with 30 joules/m² of UV-C, fixed for 10 minutes in 4% PFA (w/v) in PBS. Cells were then washed and further incubated for 45 minutes with rabbit polyclonal anti-A-SMase antibody (Santa Cruz Biotechnology, Le Perray-en-Yvelines, France) at 2 μ g/ml. Cells were then washed in PBS containing 1% FCS, and

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stained for 45 minutes with FITC-labeled goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA). After a final wash in PBS, cells were analyzed on a fluorescence-activated cell sorter (FacsCalibur, Becton-Dickinson, San Jose, CA).

By confocal microscopy: cells were irradiated with 30 joules/m² of UV-C, fixed for 10 minutes in 4% paraformaldehyde (w/v) in PBS and washed with PBS containing 3% BSA (w/v) and 1 mM HEPES (PBS-BSA). Cells were then incubated for 45 minutes with a rabbit polyclonal anti-A-SMase antibody (Santa Cruz Biotechnology, Le Perray-en-Yvelines, France) at 2 μ g/ml. Cells were then washed in PBS-BSA and stained for 45 minutes with 200 ng/ml Cy5-labeled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA). After a final wash in PBS, cells were mounted on glass coverslips with Dako mounting medium (Dako, Trappes, France). Control staining were performed without primary antibody. Slides were examined with a Carl Zeiss LSM confocal microscope (Carl Zeiss, Oberkochen, Germany) using a Plan-Apochromat 63 X objective (1.4oil). An argon laser at 488 nm was used to excite FITC (emission 515-540 nm).

Western-blot

1 x 10⁶ cells were washed in PBS before addition of Laemmli sample buffer (2 % SDS; 10 % glycerol; 5 % β -mercaptoethanol; 60 mM Tris pH 6.8; 0.001 % bromophenol blue). Samples were sonicated for 15-20 seconds and boiled 5 minutes at 95°C. The samples were resolved by electrophoresis in a 10% SDS-PAGE, transferred onto nitrocellulose membrane (Hybond-C, Amersham, Les Ulis, France), blocked with 10% nonfat milk in Tris-buffered saline-Tween 20 (0.1%) at 4°C for 2 h and incubated overnight at 4°C with a rabbit-anti TPx2 (PAG) (Santa Cruz, Le Perray-en-Yvelines, France) antibody, and 30 minutes with peroxidase-anti-rabbit. Bound proteins were detected by enhanced chemiluminescence detection system (Amersham, Les Ulis, France).

Determination of ROS

Production of ROS was detected using a C2938 fluorescent probe (Molecular Probes, Invitrogen, Cergy Pontoise, France). Briefly, exponentially growing cells were labeled with 0.5 μ M C2938 for 1 h and then irradiated with 30 J/m² UV-C light. The cells were washed in PBS, and cell fluorescence was determined using flow cytometry on a FACScan cytometer (BD Biosciences, Erembodegem, Belgium).

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Total RNA extraction

Total RNA was isolated by Trizol Reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's protocol and resuspended in DEPC-treated water. RNA quantification, purity and integrity were determined by spectrophotometry (A260, A280 and A320) and 1.5 % agarose gel electrophoresis.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR multiplex reaction was done by SuperscriptTM kit one-step RT-PCR with PLATINUM[®] Taq (Invitrogen, Cergy-Pontoise, France) according to the manufacturer recommendations, on 0.2 μ g of total RNA with the following cycles: reverse transcription 30 minutes at 50°C, amplification at 94°C for 5 minutes, then 30 cycles at 94°C for 1 minutes, 60°C for 1 minutes, 72°C for 1 minutes, and a final elongation at 72°C for 10 minutes. PCR amplification was carried out using the following primer pairs: TPx2 fwd: (5'-TTT GGTATCAGACCCGAAGC-3'), TPx2 rev: (5'- TCCCCATGTTTGTCAGTGAA-3'), TR1 fwd: (5'-TGTGGACTGACCAAAAAGCA-3'), TR1 rev: (5'-CTGCCAAATGTCAGCTTCAA-3'), TR2 fwd: (5'-GACCAGCAAATGTCCTCCAT-3'), TR2 rev: (5'-ATCGCTATGGGTGTCAGCTC-3'), β -actin fwd (5'-ACACTGTGCCCATCTACGA GG-3'), β -actin rev (5'-AGGGGCCGGACTCGTCATACT-3'). Primer concentrations were 1 μ M for TPx2, TR1 or TR2 and 0.1 μ M for β -actin. Distilled DEPC water was used as negative control. RT-PCR products were analyzed after electrophoresis in a 2% agarose gel stained with 0.5 μ g/ml ethidium bromide, and visualised by UV light. Results are expressed in mRNA level fold increase with U937-neo cells as 100%.

Real-time quantitative polymerase chain reaction (PCR)

Total cellular RNA was extracted with TRIzol[®]. The cDNA was synthesized using random hexamers and OligodT from 4 μ g of mRNA and performed with the SuperScriptTM First-Strand Synthesis System for RT-PCR (InvitrogenTM Life Technologies, Cergy Pontoise, France). TR1 rev (5'-TTGCAGTCTTGGCAACAGCATC-3'), TR1 fwd (5'-CAGCTGGACAGCACAATTGGAA-3'), TR2 rev (5'-CCAACGTTTTTCCAGGGGATTC-3'), TR2 fwd (5'-CAGCCGATCATCATCATTGC-3'), TPx2 rev (5'-GCACACTTCCCCATGTTTGTC-3'), TPx2 fwd (5'-CATCTCGTTCAGGGGCCTTTTT-

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3'), TRx rev (5'-GATCATTTTGCAAGGCCACAC-3'), TRx fwd (5'-TGGTGAAGCAGATCGAGAGCAA-3'). Real-time PCR was performed using an iCycler thermal cycler (Applied Biosystems 7000 Real-Time PCR System, Courtaboeuf, France) according to the manufacturer's instructions. Reactions were performed with 0.3 μ M of primers. Nucleotides, Taq DNA polymerase, and buffer were included in SYBR Green JumpStart™ Taq ReadyMix™ for quantitative PCR. cDNA amplification consisted of one cycle at 95°C for 1 minutes 30 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 minutes. The threshold cycle (C_T) values were determined by iCycler software (ABI Prism 7000) and the quantification data were analyzed following the $\Delta\Delta C_T$ method using S14 as reference. We have checked that PCR efficiency (E) of the amplification was similar whatever the primers and we calculated the relative amount (RA): $RA=(1+E)^{-\Delta\Delta C_T}$.

Statistics

The Student's t test was performed to evaluate the statistical significance.

RESULTS

Effect of PKC ζ on UV-C-induced apoptosis

The influence of PKC ζ overexpression on UV-C-induced apoptosis was evaluated in U937-neo and PKC ζ -overexpressing U937- ζ J cells. DAPI nuclear staining revealed that, 12 hours after 30 joules/m² irradiation, UV-C induced 55 \pm 11% of apoptotic cells in U937-neo, whereas only 16 \pm 10% of apoptotic cells were observed in U937- ζ J cells (dat not shown).

Effect of PKC ζ on acid sphingomyelinase redistribution

In our previous study, we showed that UV-C induced A-SMase translocation from the cytosol to the cell surface and enzyme activation [6]. Based on these results we first evaluated the effect of PKC ζ on A-SMase translocation. Flow cytometry (data not shown) and confocal (Fig. 1) analysis performed on non-permeabilized U937-neo and U937- ζ J cells with an anti-A-SMase antibody revealed that UV-C induced A-SMase accumulation at the external surface in U937-neo cells but not in U937- ζ J cells, suggesting that the enzyme did interfere with A-SMase translocation. Moreover, PKC ζ overexpression had no impact on A-SMase expression as revealed by Western-blot analysis (data not shown).

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Effect of PKC ζ on acid sphingomyelinase stimulation in intact cells

We therefore hypothesized that PKC ζ could interfere with A-SMase stimulation. In untreated cells, basal A-SMase activity was similar in U937- ζ J compared to U937-neo cells (40.8 ± 3.3 and 40 ± 2.2 pmol/h/mg protein respectively). Moreover, as expected from our previous study, UV-C induced a significant A-SMase stimulation in U937-neo cells, which was detected as soon as 5 minutes after irradiation and peaked at 10 to 15 minutes. However, no A-SMase stimulation was detectable in U937- ζ J cells following irradiation up to 20 minutes (Fig. 2A).

Then, we evaluated the influence of PKC ζ in UV-induced stimulation in raft-associated fraction of A-SMase. Cells were irradiated or not with 30 J/m^2 UV-C, then 12 minutes post-UV-C irradiation (corresponding to the peak of A-SMase stimulation in U937-neo), cells were lysed in cold Triton X-100, separated on a sucrose density gradient, and A-SMase was measured on pooled fraction. U937 rafts were previously characterized by the Triton insoluble material (fractions 4-6), the high SM content, and the raft marker ganglioside GM1 (data not shown). As shown in Fig. 2B, UV-C induced an increase in A-SMase activity exclusively in raft fractions in U937-neo but not in U937- ζ J cells.

These results suggest that PKC ζ interferes with the SM-CER pathway by inhibiting UV-induced A-SMase translocation and consequently its stimulation into raft microdomains.

Effect of PKC ζ on ceramide production

We next investigated the role of PKC ζ on UV-C induced SM-CER pathway. For this reason, U937-neo and U937- ζ J cells were prelabeled with [9, 10- ^3H] palmitic acid to equilibrium for 48 hours and then irradiated in PBS at 30 J/m^2 of UV-C. CER was quantified as described in experimental procedures. As shown in Fig. 3, UV-C induced CER accumulation in U937-neo but not in U937- ζ J cells.

Effect of PKC ζ on UV-C induced ROS generation

As we have recently described, UV-C-induced A-SMase translocation and its activation are mediated by ROS production, generated 5 minutes after irradiation [6]. In this context, we determined if PKC ζ could interfere with this UV-C-induced ROS production. As shown in Fig. 4, overexpression of PKC ζ resulted in the partial although significant inhibition of ROS accumulation in UV-treated cells.

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Altogether, these results suggest that PKC ζ acts upstream A-SMase stimulation by interfering with UV-mediated ROS production.

Effect of PKC ζ on H₂O₂ detoxication system

We have previously reported that PKC ζ overexpression resulted in an increased in H₂O₂ detoxication and that U937- ζ J cells were resistant to high level of exogenous H₂O₂ [8]. H₂O₂ may be detoxified by a variety of enzymes, including catalase, glutathione peroxidase, or the thioredoxin system. Therefore, we hypothesized that PKC ζ may influence the expression level of these enzymes. No difference on either catalase or glutathione peroxidase mRNA level was observed in U937- ζ J compared to U937-neo cells (data not shown). However, as measured by RT-PCR (Fig. 5A), real time quantitative PCR (Fig. 5B), and Western-blot (Fig. 5C), PKC ζ overexpressing cells displayed much higher mRNA and protein levels of TPx2/PAG, the prime candidate for H₂O₂ detoxication (for review see [20]). Thioredoxin (TRx) and thioredoxin reductases mRNA were also increased in PKC ζ overexpressing cells (data not shown). Moreover, treatment of both U937-neo and U937- ζ J cells with PKC ζ pseudosubstrate resulted in an impressive reduction of TPx2 protein expression level (Fig. 5D).

Effect of Thioredoxin peroxidase overexpression on acid sphingomyelinase delocalization and ceramide formation

To further confirm the role of TPx2 in the regulation of UV-induced SM-CER pathway stimulation, U937 cells were stably transfected with a plasmid encoding for TPx2/PAG. TPx2 overexpression resulted in the inhibition of both A-SMase membrane translocation (Fig. 6A) and CER generation at the cell surface (Fig. 6B) upon UV-C irradiation, thus mimicking the effect of PKC ζ . A-SMase expression level was unaffected by TPx2 overexpression (data not shown).

DISCUSSION

In this study, we describe for the first time that the atypical PKC ζ inhibits UV-C induced apoptosis. This observation may have important implications since this enzyme is regulated by a large variety of signaling pathways, including tyrosine kinase receptors, Ras

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oncogenic variants, phospholipase C and D, through various messengers such as CER, diacylglycerol, and phosphatidic acid [21-24]. Therefore, it is conceivable that each of these pathways are regulatory candidates for UV-induced apoptosis through PKC ζ . Based on previous studies it appears that classic and novel PKC isozymes are also implicated in the negative regulation of UV-induced apoptosis [25, 26]. Altogether, these observations suggest a general protective function for PKC family enzymes in the context of UV irradiation. This may have significant consequences not only for malignant transformation through survival of cells with damaged DNA, but also in the resistance to phototherapy in the treatment of malignant or inflammatory cutaneous diseases.

Several studies coming from our laboratory have described that PKC ζ may exert a protective function following other genotoxic stress, including treatment with topoisomerase II inhibitors [9, 27], nucleoside analogs [8], cisplatin, and alkylating agents [10]. These findings suggest a global function of this enzyme in cellular protection against various stress conditions. In the latter studies, we presented evidences that PKC ζ acts by regulating the level of DNA damage. As UV irradiation is concerned, we have previously reported that PKC ζ significantly enhances nucleotide excision repair capacity [10]. The present study shows that PKC ζ may also act by regulating post-damage apoptotic pathways such as the SM cycle. These observations suggest that PKC ζ , and perhaps, other PKC isozymes, may exert their cellular protective function by regulating both the level of damage and the post damage response.

We show here that PKC ζ interferes with UV-mediated ROS production, which is an important contributor for A-SMase stimulation [6]. From the present study, we propose that the anti-oxidant effect of PKC ζ is related to its capacity to regulate the expression of different anti-oxidant enzymes, and particularly, the enzymes of the thioredoxin system. Indeed, we show here for the first time that PKC ζ regulates the expression of TPx2, which, in turn, acts as a potent inhibitor of A-SMase translocation upon UV-C irradiation. However, it is possible that other anti-oxidant systems are involved although it appears that catalase and the glutathione-related enzymes are unlikely implicated in PKC ζ anti-oxidant effect [8, this study]. The mechanism by which PKC ζ regulates TPx2 remains to be elucidated. Interestingly, a previous study described phorbol ester-dependent activation of TPx2 via PKC [28] suggesting that classic or novel PKC isozymes may also regulate TPx2. Combined with

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our study, these findings suggest that PKC in general could be a critical regulator of thioredoxin system. In a previous study, we described that PKC ζ overexpression did not confer protection towards cell permeant CER [8]. Based on these findings and the present study, we can therefore assume that PKC ζ exerts its protective effect at early step of UV-C signaling by interfering with CER production.

To conclude, our study shows that PKC ζ is a potent regulator of UV-induced apoptosis. We propose that this regulatory mechanism operates through the inhibition of the UV-induced ROS production and subsequent inhibition of A-SMase activation and translocation to cell surface into raft microdomains. These results indicate that PKC ζ is an important player in the UV response.

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FIGURE LEGENDS

Figure 1. Effect of PKC ζ on UV-C-induced A-SMase localization.

U937-neo (\square) and U937- ζ J (\blacksquare) were irradiated or not with 30 joules/m² of UV-C. A-SMase translocation was observed at 10 minutes post-UV-C by confocal microscopy on non permeabilized cells using a FITC-conjugated rabbit anti-A-SMase antibody. Results are representative of three independent experiments.

Figure 2. Effect of PKC ζ on UV-C-induced A-SMase stimulation on whole cells and on raft microdomains.

(A) U937-neo (\square) and U937- ζ J (\blacksquare) were irradiated or not with 30 joules/m² UV-C. Total cellular A-SMase activity was measured at various times as described in experimental procedures. Results are mean of triplicate determinations of a representative experiment (one of three independent experiments) \pm SD, * $p < 0.05$. (B) U937-neo and U937- ζ J were irradiated with 30 joules/m² UV-C, lysed in cold Triton X-100 and fractionated on a sucrose density

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gradient. Aliquots were collected and analyzed for A-SMase activity. Peak A-SMase activities (10-15 minutes) are expressed compared against the amount of proteins in each fraction, rafts (4-6) and heavy (7-10). Results are mean of triplicate determinations of a representative experiment (one of three independent experiments) \pm SD, * $p < 0.05$.

Figure 3. Effect of PKC ζ on UV-C-induced ceramide generation.

U937-neo (\square) and U937- ζ J (\blacksquare) cells were prelabeled with [9, 10- 3 H] palmitic acid to equilibrium for 48 hours and then irradiated or not with 30 joules/m 2 UV-C. (A) CER was extracted at various times post-irradiation as described in experimental procedures, results are representative of three independent experiments. (B) Results are expressed at 12 minutes post-UV-C which represents the peak of CER generation and are the mean of three independent experiments \pm SD, * $p < 0.05$.

Figure 4. Effect of PKC ζ overexpression on UV-C-induced ROS production.

U937-neo and U937- ζ J cells were labeled with C2938, a fluorescent probe, and irradiated with 30 joules/m 2 UV-C light, and ROS production was then analyzed at different time points by flow cytometry. Results correspond to the difference of fluorescence (Δ FL1) between treated and untreated cells and are mean of triplicate determinations of a representative experiment.

Figure 5. Effect of PKC ζ overexpression on TPx2/PAG.

mRNA level of TPx2 was analyzed by RT-PCR (A) or by real-time quantitative PCR (B) in U937-neo and U937- ζ J cells as described in experimental procedures. Results are representative (A) or the mean (B) of three independent experiments. (C) TPx2 protein levels were visualized by Western-blot using specific anti-PAG antibody in U937-neo and U937- ζ J cells. (D) U937-neo and U937- ζ J cells were treated during 1 hour with PKC ζ pseudosubstrate at 50 μ M. TPx2 expression was evaluated by Western-blot using anti-PAG antibody. Actin was used as control of protein expression.

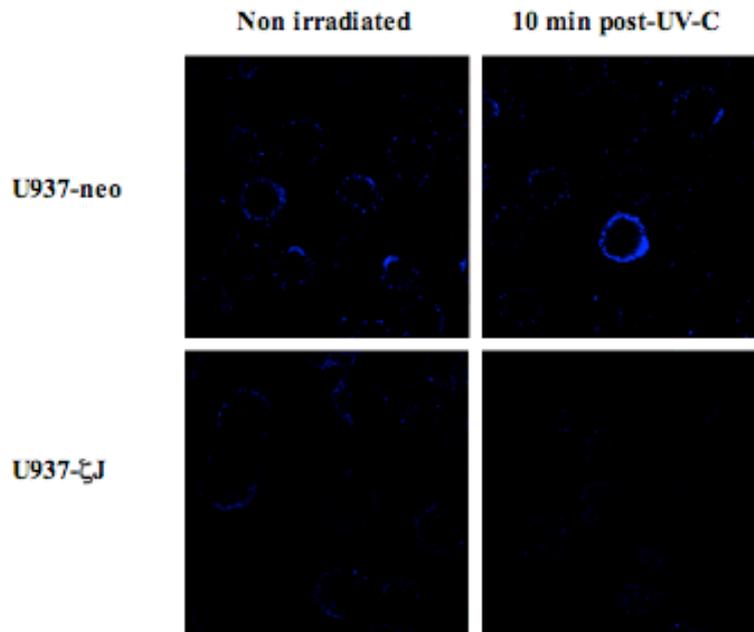
Figure 6. Effect of TPx2/PAG overexpression on A-SMase delocalization and ceramide generation.

U937-TPx2 neo and U937-TPx2 cells were irradiated at 30 J/m 2 . A-SMase delocalization (A) or CER generation (B) were performed on non permeabilized cells as described in

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experimental procedures by FACS analysis 10 minutes post-UV-C. Results correspond to the difference of fluorescence (Δ FL1) between treated and untreated cells and are mean of three independent experiments \pm SD, * $p < 0.01$. Results are representative of all clones tested.

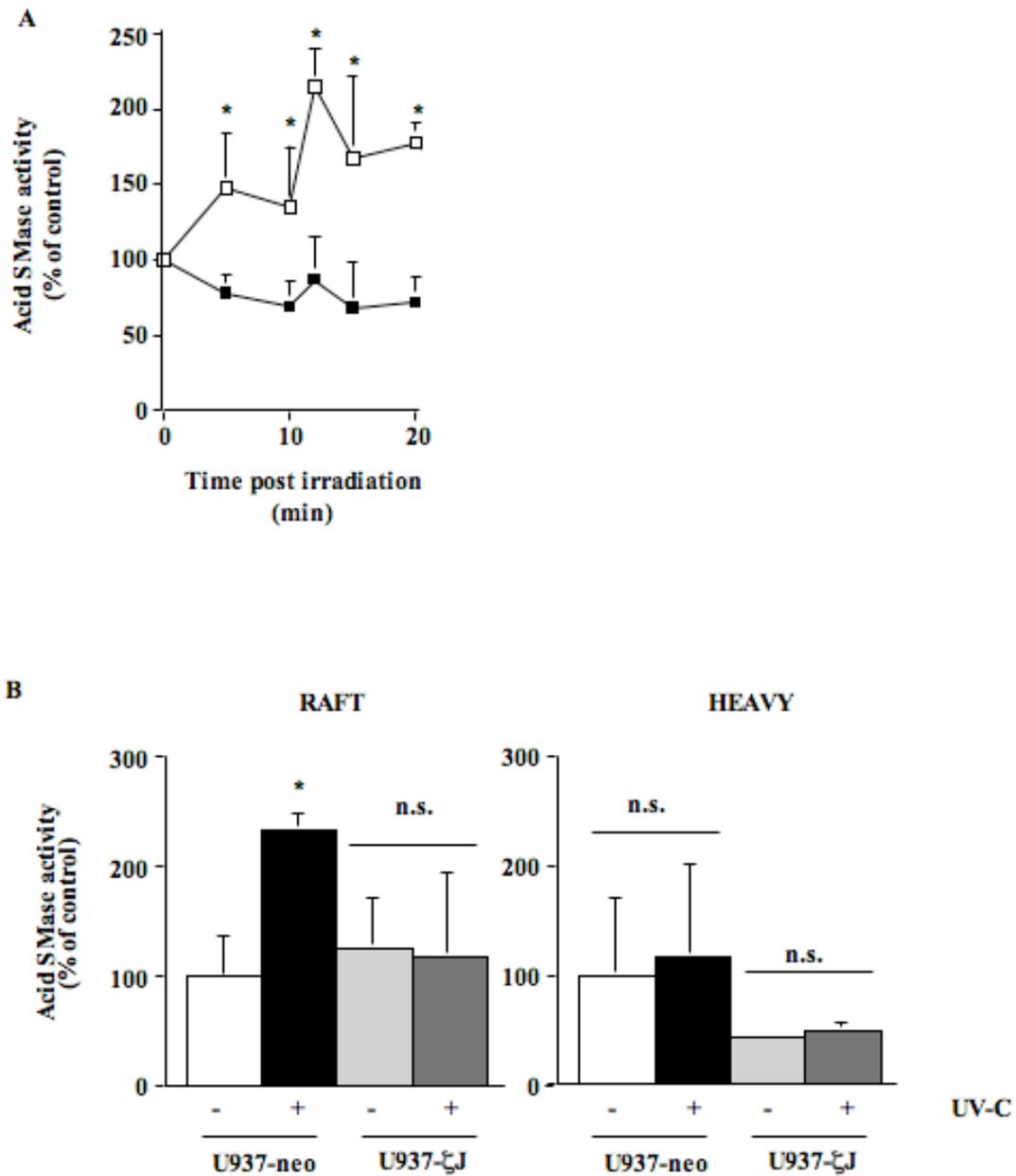
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Figure 1

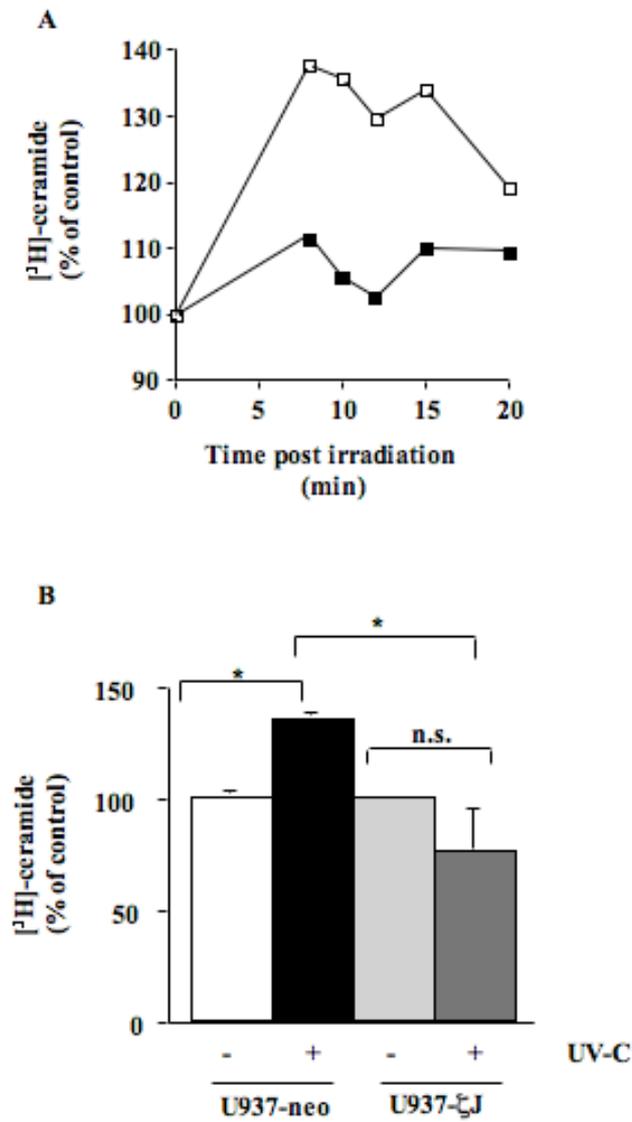
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Figure 2

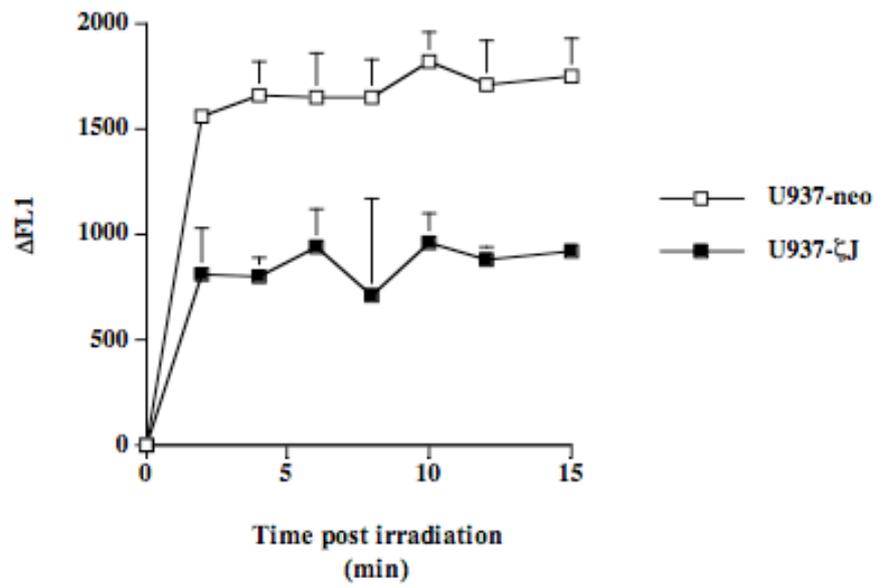
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Figure 3

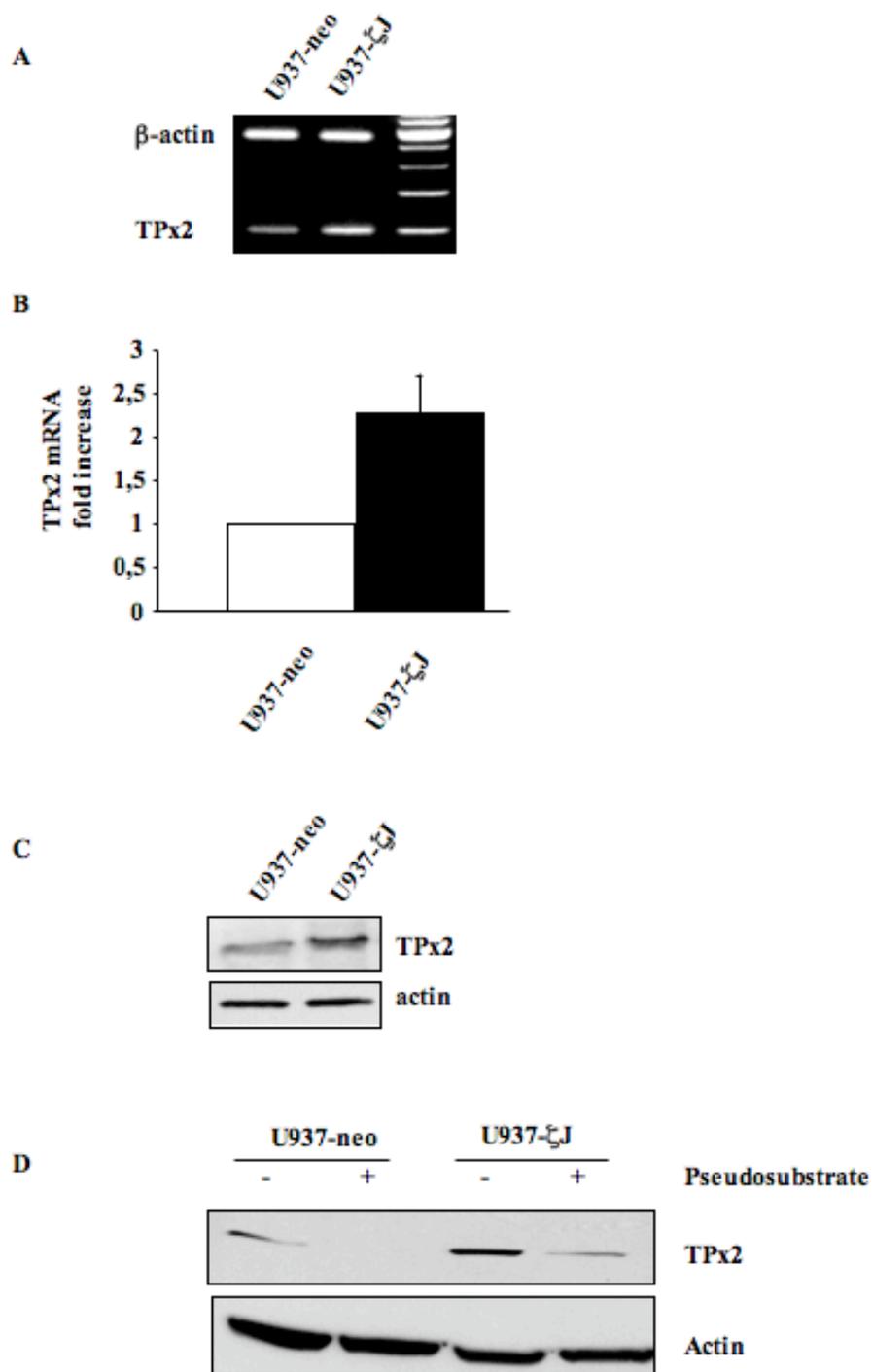
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Figure 4

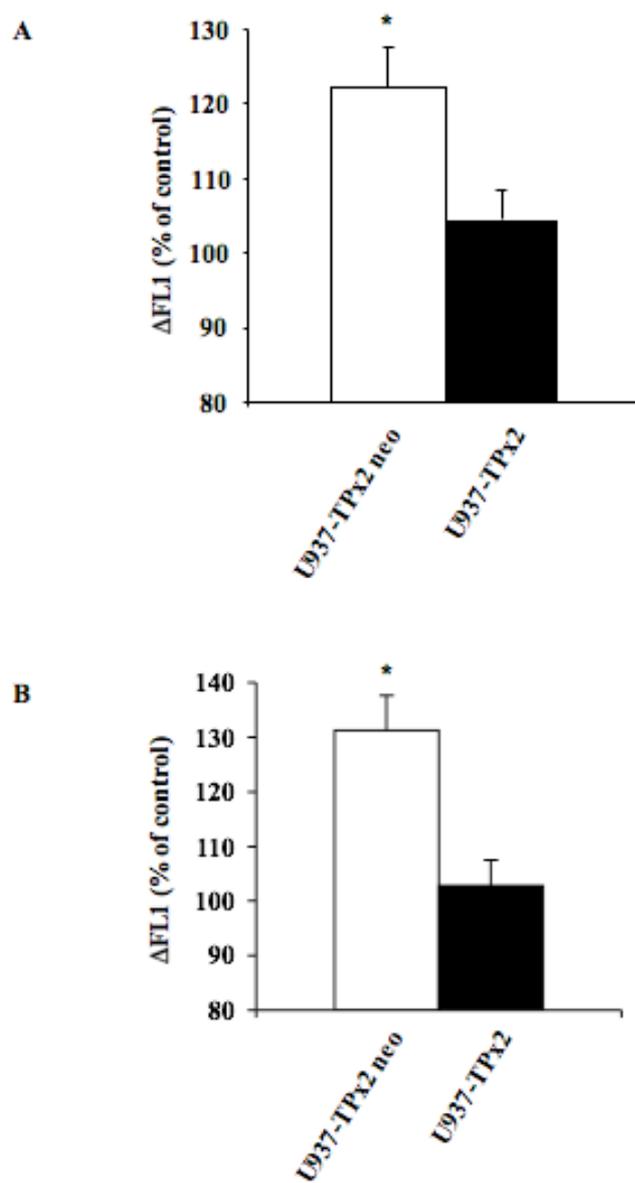
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Figure 5

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Figure 6