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UV Irradiation Increases ROS Production Via PKCδ Signaling in Primary Murine Fibroblasts

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ABSTRACT

Ultraviolet (UV) irradiation is a major environmental factor responsible for a high incidence of premature skin aging, referred to as photoaging, as well as skin cancer and melanoma. UVA irradiation represents 90% of the solar UV light reaching the earth's surface, and yet the mechanisms by which it exerts its biological effects are not clear. UVA penetrates into the skin tissue, reaching the basal layers of the active dividing cells and, therefore, the contribution of UVA to skin damage may be significant. The majority of UVA energy is absorbed by unidentified photosensitizers in the cells which are postulated to generate reactive oxygen species (ROS). It has been believed that both chronological aging and photoaging share the same molecular features and, as such, it is very common to utilize UV irradiation for induction of skin aging. To determine the involvement of protein kinase isoforms in chronological aging and photoaging, we utilized in vitro aging model systems of primary murine fibroblasts and primary fibroblasts isolated from PKC null mice. We show for the first time distinct involvement of PKC isoforms PKCδ and PKCα in photoaging versus cellular senescence. While chronological aging is accompanied by overexpression and activation of PKCα, UV irradiation and ROS production are associated with photoaging accompanied by PKCδ downregulation and nuclear translocation. J. Cell. Biochem. 105: 194–207, 2008.

KEY WORDS: ADENOVIRUS; NULL MICE; UV; ROS; PKC

Protein kinase C (PKC) plays a crucial role in the initial events of signal transduction. The PKC family of serine/threonine kinases was found to be involved in ultraviolet (UV) irradiation and the aging process. PKC activation leads to a wide range of metabolic effects including cellular proliferation, differentiation, activation of growth factor receptors, and apoptosis in different cell types [Peak et al., 1991; Chen et al., 1999; Tupet et al., 1999]. The PKC family of serine threonine kinases is composed of several isoforms characterized by their differential activation by phospholipids and Ca^{2+}. Members of the PKC family have been shown to serve as intracellular receptors for tumor-promoting phorbol diesters. The PKC family is divided into three major groups: classical PKCs: α, β1, β2, and γ, which are calcium- and diacylglycerol (DAG)-dependent isoforms; the novel PKCs: δ, ε, η, θ, which are DAG-dependent and calcium-independent isoforms; and the atypical PKCs: ζ and ι, whose cofactor requirements remain to be elucidated.

Several PKC isoforms were shown to be involved in UV-induced signal transduction pathways. PKC α, a classical PKC isoform, is irreversibly inhibited in UVA irradiated cells. PKCs from the novel class are also regulated by UV irradiation. PKCε can act as an endogenous photosensitizer that enhances UV-induced cutaneous damage and is involved in the development of squamous cell carcinomas, associated with Stat3. In addition, translocation of PKCε and PKCδ to the membrane is required for UVB-induced activation of mitogen-activated protein kinase and apoptosis [Chen et al., 1999; Wheeler et al., 2004; Aziz et al., 2007]. In contrast, overexpression of the atypical PKCs (aPKCs) results in cell protection from UV damage, which leads to apoptosis [Diaz-Meco et al., 1996; Berra et al., 1997]. Moreover, overexpression of λ/ιPKC and ζPKC inhibited UV-induced cell death [Diaz-Meco et al., 1996], whereas exposure of cells to UV irradiation leads to a dramatic reduction of ζPKC activity [Berra et al., 1997].

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Low doses of UV irradiation stimulate adhesion of fibroblasts in culture to collagen matrices through a mechanism involving activation of PKC isoforms and integrins [Tupet et al., 1999].

The ultraviolet (UV) component (290–400 nm, including UVA, UVB, and UVC) of sunlight is, by far, the most effective in triggering these responses in humans or animal models and in producing lethal and mutagenic damage in skin culture cells [Masaki et al., 1995; Jurkiewicz and Buettner, 1996; Barber et al., 1998; Brenneisen et al., 1998; Yasui and Sakurai, 2000]. In contrast to UVB and UVC, UVA irradiation is known to reach the reticular dermis, making fibroblasts an accessible target.

UV irradiation is directly associated with accelerated skin aging mediated by production of reactive oxygen species (ROS) which accumulates in the affected cells [Black, 1987; Tyrrell and Keyse, 1990; van der Leun, 1996; Gniadecka et al., 1998]. UV irradiation has been linked to membrane lipid peroxidation and formation of hydroperoxides and other secondary products such as aldehydes, which can lead to DNA damage [Peak and Peak, 1989].

Exposure of human skin to sunlight is common in Western culture and may result in short-term damage including pigmentation or erythema, or long-term damage such as carcinogenesis and skin aging [Gange and Rosen, 1986; Kligman and Kligman, 1989; Brash, 1997; Fuchs and Kern, 1998; Griffiths et al., 1998]. Little is known about the molecular and cellular mechanisms responsible for these effects.

Photoaging and chronological aging have been considered to be distinct entities. Although the typical appearance of photoaged and chronologically aged human skin can be readily distinguished, altered signal transduction pathways promote external similarities such as skin wrinkling and pigmentation.

Many research studies have shown correlation between the effects of UV irradiation, cleavage of PKCδ and apoptosis [Denning et al., 1998; Brodie and Blumberg, 2003]. Our study focused on cell damage in cells irradiated with sublethal intensity, which induced morphological changes and ROS production but did not induce cell apoptosis. It is important to emphasize that although the cells underwent major changes following UV irradiation, sublethal irradiation can also be involved in cell survival processes which were not necessarily linked to DNA damage and apoptosis, as shown in this research.

The effects of UV irradiation on skin aging processes in an in vitro model system of aging skin fibroblasts was investigated in the present work, where we established links between oxidative stress and PKC signaling following UV irradiation. As shown here, specific PKC regulation was characterized by changes associated with both skin aging and photoactivation. Furthermore, we show distinct involvement of specific PKC isoforms in the process of chronological skin aging versus photoaging.

MATERIALS AND METHODS

All animal research performed in this study was reviewed and approved by the Institutional Review Board of Bar-Ilan University.

REAGENTS AND ANTIBODIES

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) was performed with a SuperSignal West Pico Chemiluminescent Substrate Kit, purchased from Pierce. Polyclonal antibodies to PKC isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to PKC isoforms were purchased from Transduction Laboratories (Lexington, KY). Horseradish peroxidase labeled anti-rabbit and anti-mouse IgG were obtained from Bio-Rad (Hercules, CA). Leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, orthovanadate, and pepstatin were purchased from Sigma Chemicals (St. Louis, MO).

ISOLATION AND CULTURE OF MURINE FIBROBLASTS

Primary fibroblasts were isolated from newborn Balb/c mice dermis by suspension in 0.35% collagenase for 30 min followed by the addition of 3 volumes of Dulbecco's modified eagle's medium (DMEM) (Sigma, Rehovot, Israel) containing 10% serum (Biological Industries, Beit HaEmek, Israel). The suspension was put through a nylon mesh and centrifuged at 1,200 rpm for 3 min. The pellet was suspended in DMEM, centrifuged at 400 rpm for 3 min, and the isolated fibroblasts were collected from the supernatant.

PRIMARY YOUNG AND AGING FIBROBLASTS

Cells were seeded and maintained in culture until they reached 80% confluence. They were then trypsinized and passaged at a ratio of 1:10. Young cells used in experiments were obtained from passage 2, whereas cells were considered ''aged'' (aging) after passages 5 or 6. At passage 7, cells lost their replicative capacity. The same number of young and aging fibroblasts was seeded in each dish.

CELL TREATMENT AND IRRADIATION

Cells were washed in phosphate buffered saline (PBS) and irradiated with a UVA 365 nm illuminating table equipped with five TF-20L tubes (Vilbert et Lourmat, Marne la Vallee, France). Cells were irradiated from the bottom at a light intensity of 9 J/cm^2. Following irradiation, cells were washed twice with PBS and returned to the incubator for different time periods.

MEASUREMENT OF CELL APOPTOSIS

Cell apoptosis was measured using propidium iodide staining and analyzed by flow cytometry [McCloskey et al., 1994]. Detached and trypsinized adherent cells were pooled, fixed in 70% ethanol for 1 h on ice, washed with PBS, and treated for 15 min with RNase (200 μM) at room temperature. Cells were then stained with propidium iodide (100 μg/ml) and analyzed with Dickinson FACSsort Flow Cytometer.

DETERMINATION OF ROS IN CULTURED CELLS

ROS levels in fibroblast cells were assayed according to the method of Tobi et al. [2000] using the fluorescent reagent 2′,7′-dichlorofluorescin-diacetate (DCFH-DA). Fibroblasts were maintained in DMEM containing 10% fetal calf serum. At 80% confluency, cells were detached with 0.1% trypsin solution, washed with PBS, and counted. Cells were plated at 10^5/well/per 50 μl PBS in 96-well tissue culture plates. DCFH-DA solution (2.0 mg/100 ml ethanol) was

diluted 1:100 in PBS, and 25 μl was added to each well in the microplate. All plates were incubated for 1 h at 37°C and then read with a Tecan fluorometer (wavelength 485/530 nm).

TBARS ASSAY

Thiobarbituric-reactive substances were assayed according to a slightly modified procedure of Barber and Bernheim [1967]. Thiobarbituric acid (TBA) reacts with Malondialdehyde (MDA) to give bis-thio-oxonol with an optical absorption of 532 nm and with fluorescence emission at 553 nm following excitation at 510 nm. The reaction system contained a 1 ml sample (cells suspended in 50 mM phosphate buffer, pH 7.5) and 0.5 ml trichloro acetic acid (TCA) 40%. Samples were centrifuged for 10 min at 2,500*g*, and 1 ml of supernatant was added to 0.25 ml TBA 2%. The mixture was heated to 100°C for 10 min. Fluorescence was measured in cooled samples.

SOD ACTIVITY

Superoxide dismutase (SOD) activity was determined according to McCord and Fridovich [1969]. One unit of SOD is equal to 50% inhibition in cytochrome *c* reduction.

CATALASE ACTIVITY

Catalase activity was determined spectrophotometrically according to Beers and Sizer [1952].

PROTEIN DETERMINATION

Protein was determined using a DC protein assay according to the manufacturer's instructions (Bio-Rad). In lysates which did not contain Triton, protein concentration was determined by the Bradford technique [Bradford, 1976].

TOTAL CELL EXTRACTS AND WESTERN BLOTS

Whole cell lysates were prepared by scraping cells on ice into 300 μl lysis buffer containing 5% SDS, 20% 2-beta-mercaptoethanol and 50% Western upper gel buffer (0.5 M Tris, pH 6.8). Lysates were homogenized, boiled for 5 min, and centrifuged at 13,000 rpm for 20 min. Protein loading buffer was added to an equal volume of supernatants, and samples were boiled for 5 min and again spun down. Supernatants were loaded onto SDS–PAGE gel and the separated proteins were transferred to a nitrocellulose membrane (Bio-Rad). Specific protein bands were detected by immunoblotting using specific antibodies, and visualized by enhanced chemiluminescence.

PREPARATION OF CYTOSOL AND MEMBRANE FRACTIONS

For crude membrane fractions, cells were washed twice with cold PBS, resuspended in RIPA buffer without SDS, deoxycholic acid, and Triton X-100 [containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1%, 0.25 mM ethylene diamine tetra-acetic acid (EDTA) (pH 8.0), and protease and phosphatase inhibitors: 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM PMSF, 200 μM $NaVO_4$ and 5 mM NaF]. For disruption of cell membrane, cells were frozen and thawed in dry ice five times. After centrifugation at 13,000 rpm, supernatant which contained the cytosol fraction (salt soluble fraction) was removed. The pellet was resuspended in 250 μl PBS containing 1% Triton X-100 with protease and phosphatase inhibitors. Lysates were incubated on ice for 30 min at 4°C and centrifuged at 4°C for 30 min at 13,000 rpm. The supernatant contained the soluble protein fraction (membrane fraction) [Shen et al., 2001]. Protein concentrations were measured using a modified Lowry assay (Bio-Rad DC Protein Assay Kit). Western blot analysis of cellular protein fractions was performed as described [Li et al., 1996].

WESTERN BLOT ANALYSIS

Cell lysates, prepared in RIPA buffer, were prepared by mixing 0.3 ml cell lysate with 25 μl Protein A/G Sepharose (agarose beads) (Santa Cruz Biotechnology), and the suspension was rotated continuously for 30 min at 4°C. The preparation was then centrifuged at maximal speed at 4°C for 10 min. Supernatants were incubated with specific polyclonal or monoclonal antibodies to the individual PKC isoforms (diluted 1:100) overnight at 4°C, followed by incubation with Protein A/G Sepharose (agarose beads) (Santa Cruz Biotechnology) for 2 h. The suspension was then centrifuged at maximal speed for 10 min at 4°C. The pellet was washed twice with RIPA buffer, twice with cold PBS, and subjected to Western blot analysis. Immunoprecipitates were separated by SDS–PAGE, transferred to nitrocellulose membrane (0.2 μm) (Bio-Rad), and blotted with specific polyclonal or monoclonal antibodies.

Experiments were repeated several times, and blots of repeated experiments were analyzed by densitometry. Arbitrary units were normalized to a similar scale for all original figures, maintaining the differential quantitative responses. Statistical analysis (mean ± SD) was performed accordingly.

SPECIFIC RECOMBINANT ADENOVIRUS VECTORS FOR FIBROBLAST INFECTION AND PKC OVEREXPRESSION

The culture medium was aspirated and fibroblast cultures were infected with the viral supernatant containing PKCδ or DN PKCδ, recombinant adenoviruses, at a multiplicity of infection (MOI) of 12 for 24 h. The cultures were then washed twice with PBS without Ca^{2+}–Mg^{2+} and resuspended in DMEM for 24 h. β-Galactosidase (β-GAL)-encoding adenovirus was used as a negative control to exclude possible deleterious effects of the vector itself. Following infection, cells were incubated for 24 h and then left untreated or treated with UV irradiation and used for ROS assays.

MEASUREMENT OF PKC ACTIVITY

Specific PKC activity was determined in freshly prepared immunoprecipitates from fibroblast cultures following appropriate treatments. These lysates were prepared in RIPA buffer without NaF. Activity was measured using the SignaTECT Protein Kinase C Assay System (Promega, Madison, WI), according to the manufacturer's instructions. Neurogranin (AAKIQAS*FRGHMARKK) was used in these studies as the substrate for PKC activity.

IMMUNOFLUORESCENCE STAINING

Primary fibroblasts were plated on ProbeOn Plus glass slides. After 3 days of culture, fibroblasts were left untreated or treated with UV irradiation, 9 J/cm^2. After UV irradiation, cells were washed twice

with Ca^{2+}/Mg^{2+}-free PBS, maintained in DMEM and incubated for the indicated time points, then fixed in methanol for 10 min followed by permeabilization with 0.2% Triton X-100 for 5 min. The slides were then rinsed with PBS and incubated for 18 h at 4°C, with specific anti-PKC antibodies diluted in 1% BSA in PBS. After incubation, slides were washed twice with PBS for 10 min and incubated with biotinylated secondary anti-rabbit antibody for 40 min, washed twice in PBS, and incubated with strepavidin-FITC for 40 min. Following two washes in PBS and one wash with double distilled water, slides were suspended in propidium iodide for 5 min, washed twice with PBS and once with double distilled water, and then air-dried and mounted with Vectashield mounting medium (Vector). Fluorescence was examined by laser scanning confocal imaging microscopy (MRC1024, Bio-Rad, UK).

STATISTICAL ANALYSIS

The results are presented as mean ± SE. Data were analyzed using analysis of variance and a paired Student's *t*-test to determine the level of significance between the different groups.

RESULTS

MORPHOLOGICAL CHANGES OCCURRED IN YOUNG AND AGING FIBROBLASTS

In order to characterize indicative morphological characteristics of young and aging fibroblasts, we have followed two populations of aging and young fibroblasts. Aging fibroblasts, passages 5–6 (Fig. 1A), were found to be larger and their replicate capacity dramatically decreased, as expected, compared to young fibroblasts, passages 2–3 (Fig. 1A). When young and aging cells were irradiated with a high intensity of UVA (over 9 J/cm^2), cells detached from the plates and cell death was observed. Using fluorescence activated cell sorter (FACS) analysis and Annexin assay for identifying pre-apoptotic effects of UVA irradiation, an increase in apoptotic population (as detected by sub-G1 population) was observed (data not shown). Therefore, in order to study the pre-apoptotic effects of UVA irradiation, it was limited to an intensity of 9 J/cm^2 for young and aging cells, followed by up to 24 h incubation. As seen in Figure 1B, while morphological effects were observed, no cell death was detected at this UV intensity. This lack of cell apoptosis was also confirmed by using Annexin assay and FACS analysis (data not shown).

DETERMINATION OF CELL APOPTOSIS FOLLOWING UVA IRRADIATION AT 9 J/cm^2

To determine conditions of UV irradiation before apoptotic cell death, we examined cell apoptosis following different intensities of UV irradiation and found the maximal intensity in which cells did not undergo any apoptosis (sublethal intensity). We subjected young and aging fibroblast to the same conditions. Our aim was to examine whether cell death occurs during and following UV irradiation in young and aging fibroblasts at 9 J/cm^2. As seen in Figure 1B, no cell death was detected at this UV intensity in young cells. The same results were obtained in aging cells (data not shown).

UV IRRADIATION INDUCED ROS PRODUCTION IN BOTH YOUNG AND AGING FIBROBLASTS

One of the features known to be linked with UV damage and photoaging is associated with induction of ROS production and regulation of lipid peroxidation. Therefore, first we measured ROS production over time following UV irradiation (Fig. 2A) in young and aging fibroblasts. As can be seen in Figure 2, basal levels of ROS production in aging cells were elevated 5–7 times above levels produced by young fibroblasts. Both populations induced ROS production as a result of UV irradiation. However, ROS levels in young cells peaked at 30 min following UV irradiation and rapidly returned to control levels by 60 min. In aging cells, ROS production reached maximum levels 45–60 min after UV irradiation, and the return to basal levels occurred gradually. The relative change between the young and aging population was also confirmed by fluorescence microscopy, as seen in Figure 2B. As also seen in Figure 2B, no fluorescence was detected in non-irradiated cells (−UV) while strong fluorescence was detected in irradiated cells (+UV).

Furthermore, similar results were also observed when measuring the levels of thiobarbituric acid response substance (TBARS represents production of lipid peroxidation). As illustrated in Table I, TBARS and ROS were six times higher in aging cells than in young cells. However, 30 min following UV irradiation, a significant increase in TBARS and ROS levels occurred in both young and aging fibroblasts. In young cells, lipid peroxidation and ROS levels doubled following UV irradiation, while in old cells, values increased only about 40–50%.

EFFECT OF UV IRRADIATION ON SOD AND CATALASE ACTIVITY AND EXPRESSION IN YOUNG AND AGING FIBROBLASTS

One cause of ROS elevation following UV irradiation can be due to activity reduction in endogenous antioxidants such as SOD and catalase.

In this study, we evaluated SOD and catalase activity and expression following UV irradiation. As can be seen in Figure 3A, catalase activity was significantly reduced following UV irradiation both in young and aging fibroblasts, whereas activity returned to basal level 24 h following UV irradiation. No changes in catalase expression were detected under those conditions. As for SOD activity, it can be seen in Figure 3A that SOD activity was reduced in young fibroblasts following UV irradiation but no significant reduction in SOD activity was seen in aging fibroblasts; SOD levels returned to basal level 24 h following UV irradiation.

Blots clearly show that levels of SOD and catalase are significantly lower in aging cells and UV had minimal effect on these antioxidants.

No changes in SOD expression were detected following UV irradiation both in young and aging fibroblasts (Fig. 3B).

PKC ISOFORM EXPRESSION IN YOUNG AND AGING FIBROBLASTS

UV irradiation induces changes in PKC isoforms. Therefore, we next studied changes in the expression of PKC isoforms in young and aging fibroblasts following UV irradiation. As can be seen in Figure 4, we detected the expression of PKC isoforms α, β1, β2, δ, ε, and ζ in cultured skin fibroblasts. According to Western blot

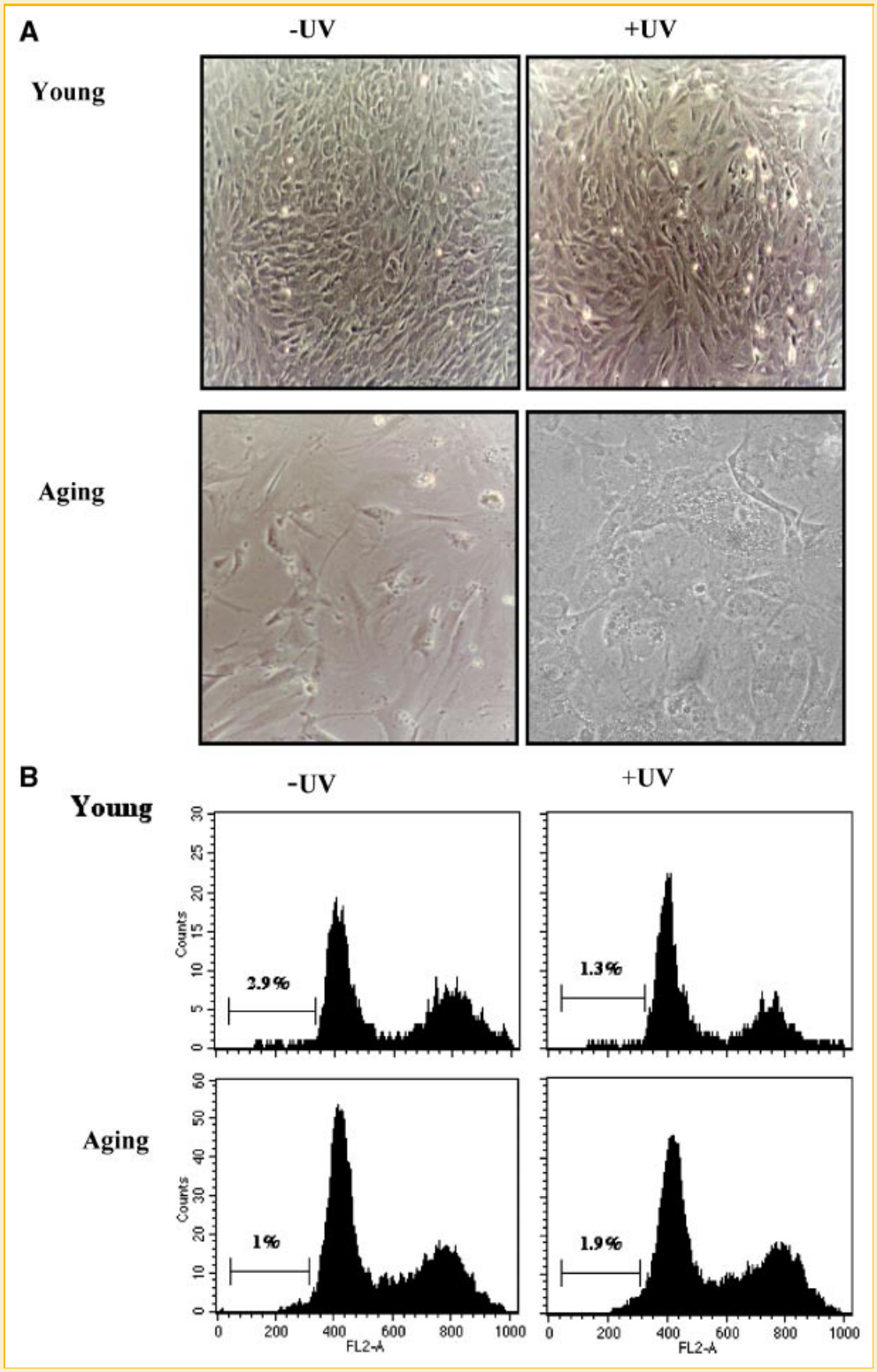

Fig. 1. A: Morphological changes occur during in vitro aging and UV irradiation in mouse primary fibroblasts. Young fibroblasts were either not irradiated or sublethally (9 J/cm^2) UV-irradiated. Aging fibroblasts were either not irradiated or sublethally (9 J/cm^2) UV-irradiated. Young fibroblasts were taken from passage 2, while aging fibroblasts were taken from passage 5. Pictures were photographed by inverted microscope, magnification 20×. B: PI staining and FACS analysis 24 h after UV irradiation at 9 J/cm^2 in young and aging fibroblasts. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

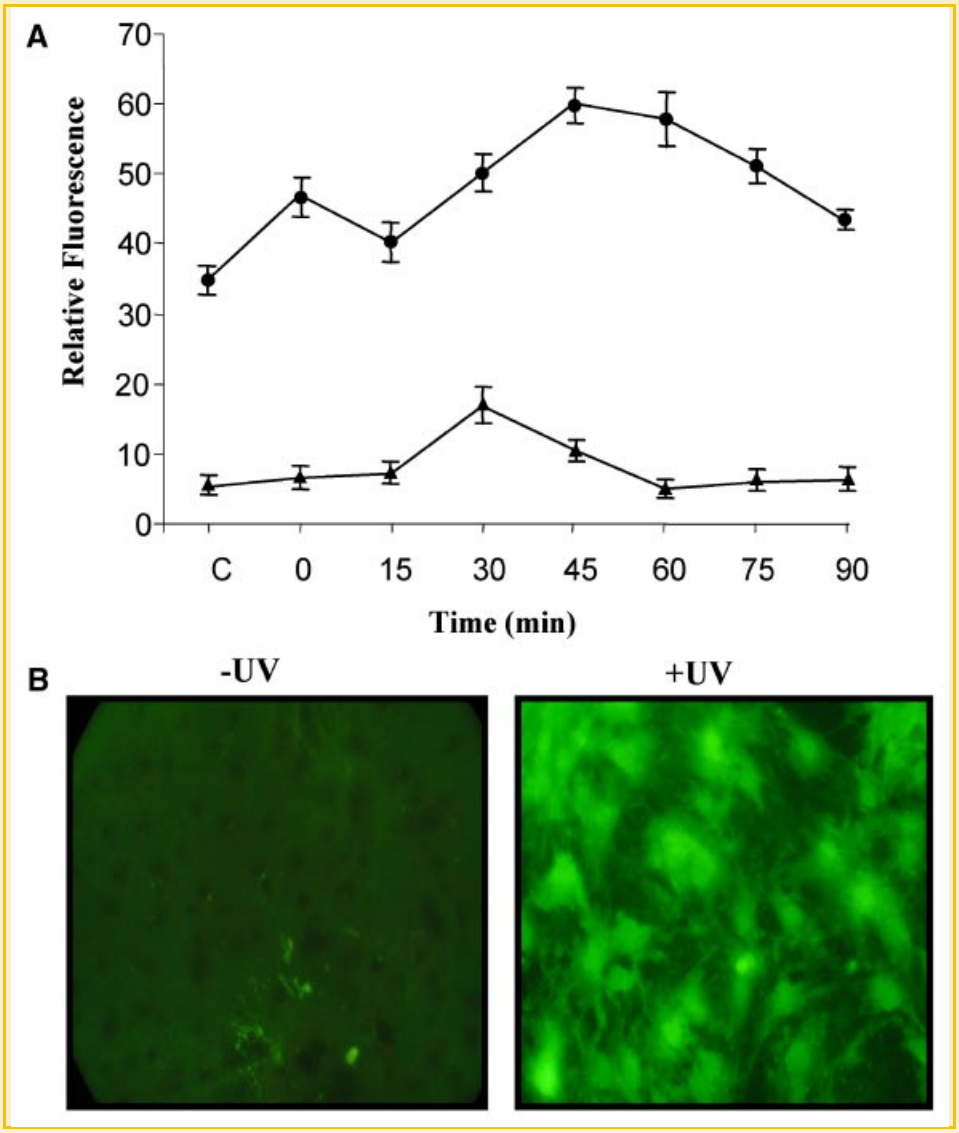

Fig. 2. ROS production at different time points following UV irradiation in young and aging fibroblasts. A: ROS production was measured in non-irradiated or irradiated young and aging fibroblasts. Following 9 J/cm^2 UV irradiation, cells were incubated for the indicated time points (0–90 min). Time (min); C, control; non-irradiated cells. ROS production was measured with DCFH-DH reagent as described in detail in Materials and Methods Section. Results are expressed as relative fluorescence (▲ young fibroblasts, ● aging fibroblasts). The results in this figure represent four experiments (n = 4). B: Illustrates young cells before UV irradiation and 30 min following UV irradiation as seen with a fluorescence microscope. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Effect of UV Irradiation on Lipid Peroxidation and ROS Production in Young and Aging Cells

	TBARS		ROS	
Treatment	Young cells	Aging cells	Young cells	Aging cells
Control	100 ± 18.5	100 ± 5.14	100 ± 24.1	100 ± 1.63
UV	188 ± 13.11	157 ± 5.9*	202 ± 17.3*	144 ± 2.9*

*$P < 0.05$. The experiment was repeated at least four times (n = 4).
Results are expressed in MDA equivalents normalized to the cell protein content. See Materials and Methods Section for further experimental details. Data are expressed as percentage of control ± SD.
ROS production was determined as described in Materials and Methods Section (basal levels of ROS in young and aging cells were 5.4 and 35.0, respectively; basal levels of lipid peroxidation in young and aging cells were 2.66 and 10.46, respectively). Values are expressed as relative fluorescence/mg protein.

analysis of total lysates, in the process of aging (young fibroblasts compared to aging fibroblasts), no significant changes were detected in expression of isoforms β2, δ, ε, and ζ. However, a dramatic elevation of PKCα expression and an increase of PKC β1 expression were determined during the process of cellular aging. In order to identify if changes in expression were also associated with changes in the activation state of these PKC isoforms, we next followed the distribution of PKC isoforms in cytosol and membrane fractions following UV irradiation.

CHANGES IN PKC ISOFORM EXPRESSION AND DISTRIBUTION IN YOUNG AND AGING FIBROBLASTS FOLLOWING UV IRRADIATION

One of the changes occurring during PKC activation state is associated with translocation of PKC isoforms from the cytosol to

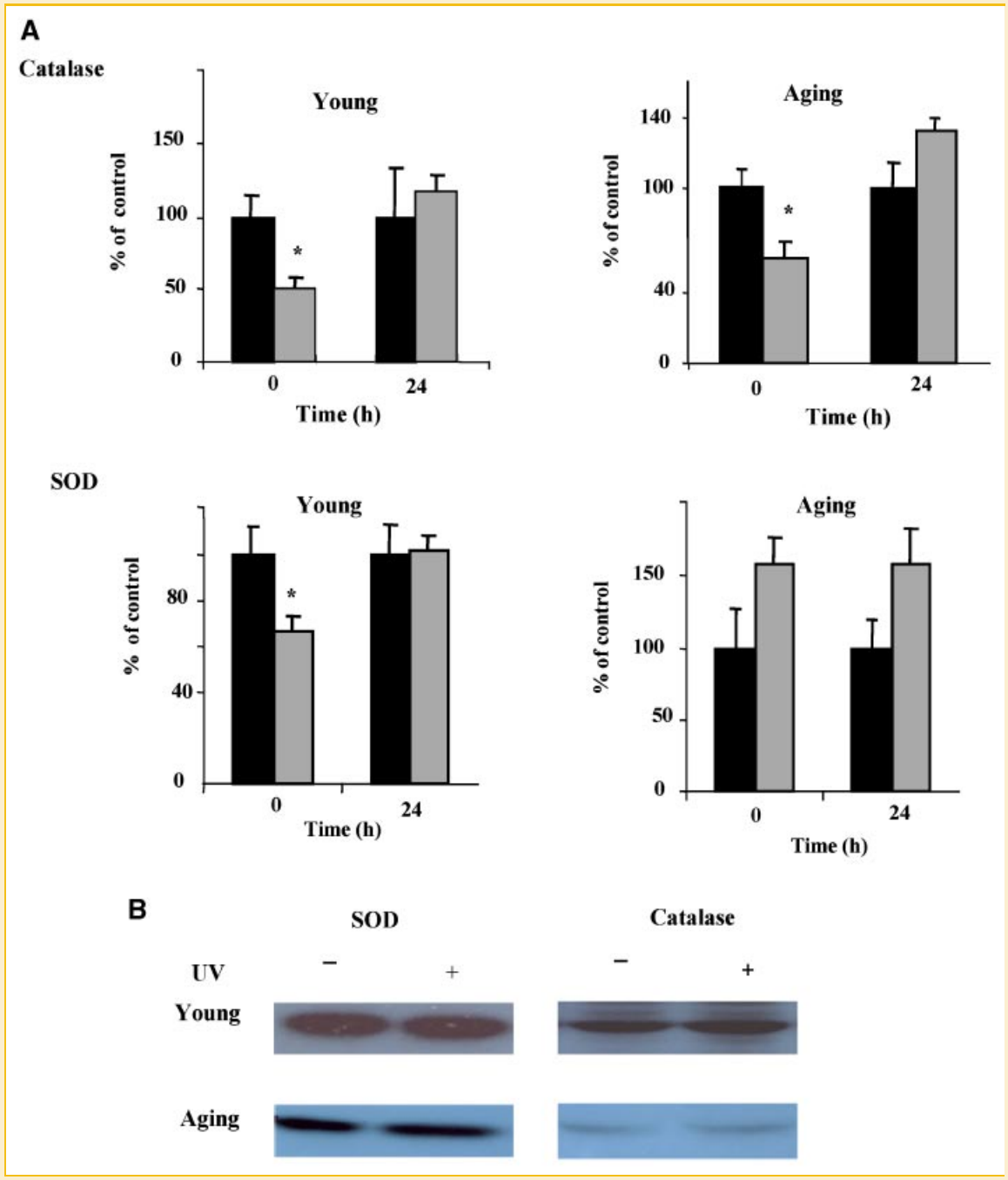

Fig. 3. SOD and catalase activity and expression in young and old fibroblasts following UV irradiation. Upper panel (A): SOD and catalase activity as described in the Methods section. Black bars indicate control, non-irradiated fibroblasts represent 100%. Grey bars indicate irradiated fibroblasts represented as percentage of control (non-irradiated cells). Results are expressed as mean ± SD of three repeated experiments ($P < 0.05$). Lower panel (B): SOD and catalase expression in young and aging fibroblasts following UV irradiation. Lysates were subjected to Western blot analysis using anti-SOD or anti-catalase antibodies. 0 h = immediately following UV irradiation; 24 h = 24 h following UV irradiation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the membrane fraction. As shown in Figure 5, when comparing young and aging fibroblasts, we found that UV induced changes in distribution of PKC isoforms PKCβ2 and PKC δ in the membrane fraction but no changes were observed in PKCα expression and distribution. In young irradiated cells, 30 min following UV irradiation, a significant transient reduction in expression of PKCδ in both cytosol and membrane fractions was observed. In aging fibroblasts, a delayed response of PKCδ was observed following UV irradiation, where PKCδ was significantly downregulated 1 h after UV irradiation. At the same time, expression of PKCα, which was elevated significantly in aging fibroblasts, was not responsive to UV irradiation, and no change in PKCα expression was observed in both cytosol and membrane fractions in young and aging cells (Fig. 5A,B). In young fibroblasts, PKCε expression decreased in

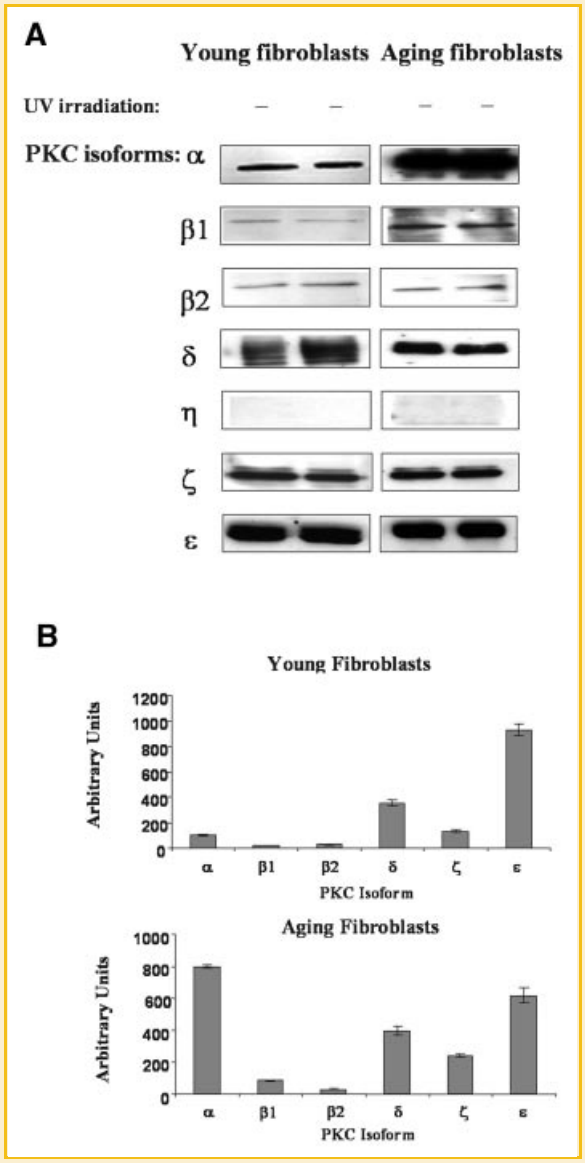

Fig. 4. PKCα isoform is overexpressed in aging fibroblasts. Upper panel: Equal amounts of protein cell lysates were obtained from young and aging fibroblasts (without UV irradiation). Lysates were subjected to Western blot analysis using anti-PKCα, anti-PKCβ1, anti-PKCβ2, anti-PKCδ, anti-PKCε, anti-PKCζ, and anti-PKCη antibodies. The figure shows one typical experiment out of four (n = 4). Lower panel: Relative optical density of the blots are presented in arbitrary units.

the cytosol fraction following UV irradiation, while in aging fibroblasts, PKCε expression was elevated in the cytosol fraction 30 min following UV irradiation. PKCξ expression decreased in both cytosol and membrane fractions following UV irradiation in both young and aging fibroblasts. Overall, we identified PKCα as the main isoform exhibiting significant changes in expression during chronological aging, while PKCδ was sensitively regulated by UV irradiation. Therefore, in our experiments that followed, we focused on these two isoforms.

PKC ACTIVITY IN YOUNG AND AGING FIBROBLASTS FOLLOWING UV IRRADIATION

We next studied the activation state of PKCα and PKCδ isoforms in young and aging fibroblasts following UV irradiation. As seen in Figure 6, a significant difference in PKC activity was detected in the different populations. In correlation with expression and translocation data, basal activity levels of PKCα increased in aging fibroblasts while PKCδ activity was reduced. Interestingly, in response to UV irradiation, specific activity of PKCα increased in young fibroblasts 1h following UV irradiation, while in aging fibroblasts, no further increase in specific activity of PKCα was observed. In correlation with reduction of PKCδ expression in the membrane fraction, PKCδ activity was dramatically decreased 30 min following UV irradiation in young fibroblasts whereas in aging fibroblasts, minor changes were detected up to 1 h after UV irradiation. These results confirmed that both PKCα and PKCδ expression and activation states were differentially regulated in young and aging fibroblasts.

UV IRRADIATION PROMOTES PKCδ TRANSLOCATION TO THE NUCLEUS IN YOUNG AND AGING FIBROBLASTS

The distribution of PKC isoforms in distinct cellular organelles and the translocation of PKC isoforms following activation and exogenous stimulation, determine the specificity of cellular signaling. We have, therefore, examined PKCα and PKCδ translocation following UV irradiation in young and aging fibroblasts. In non-irradiated cells, both PKCδ and PKCα were not distributed in the nucleus. However, 30 min following UV irradiation, PKCδ but not PKCα translocated to the nucleus of irradiated cells (Fig. 7). Similar results were obtained in aging fibroblasts (data not shown).

EFFECT OF UV IRRADIATION ON ROS PRODUCTION IN PKCα AND PKCδ KNOCKOUT (KO) MICE AND IN MICE OVEREXPRESSING SPECIFIC PKC ISOFORMS

In order to establish a link between UV-induced damage and PKC expression and activation, we studied ROS production in primary fibroblasts isolated from PKCα and PKCδ knockout mice (KOα and KOδ, respectively) (Fig. 8A). As shown in Figure 8B, fibroblasts of KOδ mice produced higher levels of ROS in the basal state as well as following UV irradiation, compared to primary fibroblasts isolated from wildtype (WT) mice. At the same time, KOα fibroblasts exhibited equal levels of ROS compared to WT control cells. In order to evaluate the importance of PKCδ to the regulation of ROS production, we further overexpressed wildtype (kinase active) PKCδ (WT PKCδ) and dominant negative (DM) (kinase inactive) PKCδ (DN PKCδ) by utilizing recombinant adenoviruses. As illustrated in Figure 8C, in fibroblasts infected by recombinant adenoviruses β-GAL, WT PKCδ, and DN PKCδ, no significant changes were observed in the basal levels of ROS prior to UV irradiation. Following UV irradiation, ROS levels increased significantly in fibroblasts overexpressing DN-PKCδ. These findings further support a link between the lack of PKCδ expression and activation, and the generation of ROS following UV irradiation.

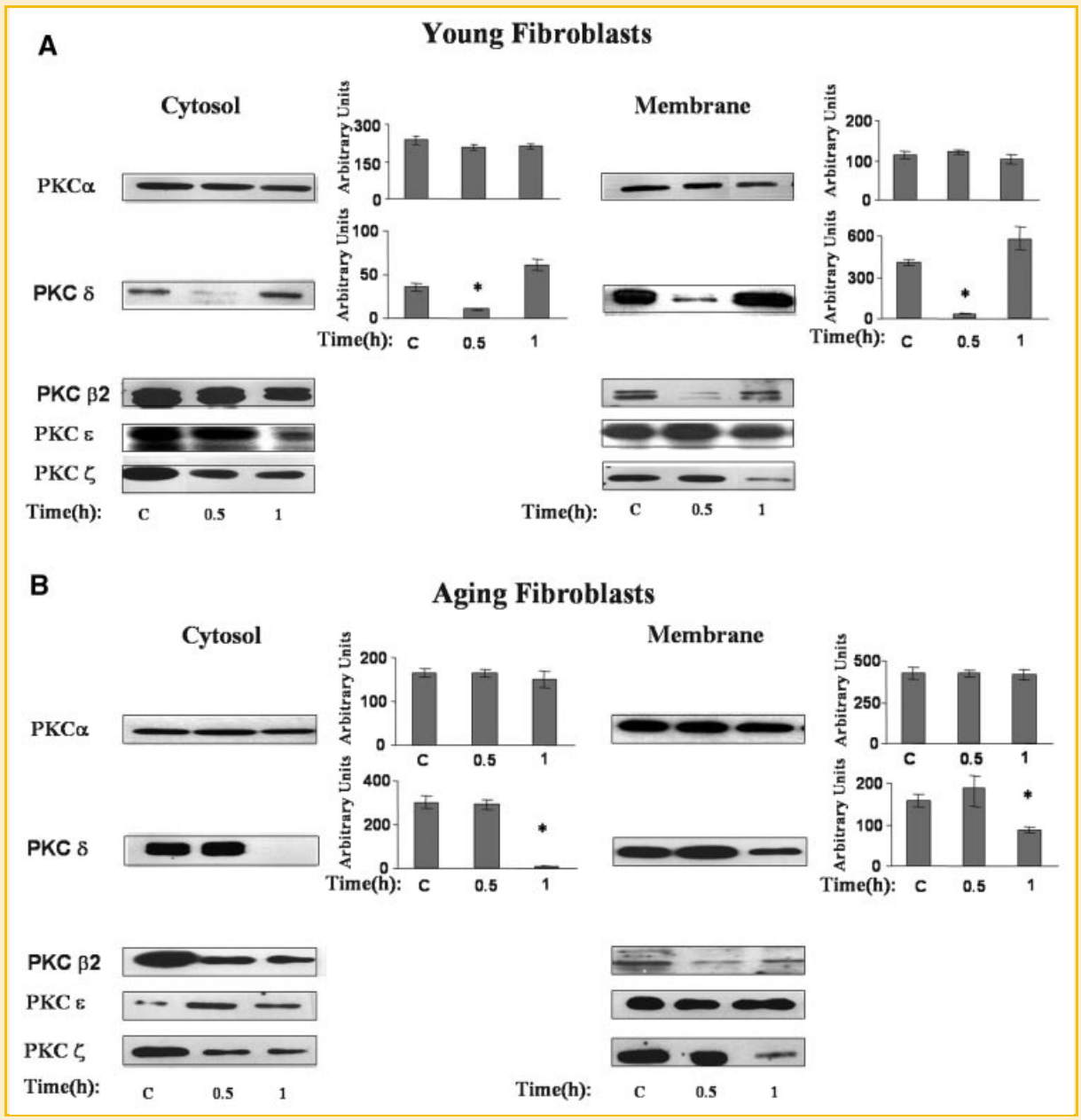

Fig. 5. Effect of UV irradiation on distribution and expression of PKC isoforms in young and aging fibroblasts. Young (A) and aging (B) fibroblasts were either non-irradiated (control—C) or UV-irradiated. Following UV irradiation, cells were incubated for the time indicated in this figure. Following incubation, cells were extracted in RIPA buffer and fractionated into cytosol and membrane fractions as detailed in Materials and Methods Section. Following fractionation, extracts were subjected to Western blot analysis using PKCα, PKCβ2, PKCδ, PKCε, and PKCζ antibodies. Experiments were repeated at least three times, and a representative experiment is shown. Relative optical densities of the blots are presented in arbitrary units. Highly significant differences were found ($P < 0.0001$) for expression level of PKCδ following UV irradiation compared with level in the respective controls.

DISCUSSION

The main goal of the present study was to shed light on the involvement of PKC isoforms in the UVA-induced signal cascades, and ROS production.

In this study, we established as a study model an in vitro aging system [Van Gansen and Van Lerberghe, 1988] consisting of young and aging fibroblasts exposed to a sublethal dose of UVA (9 J/cm^2) in order to allow the cells to initiate their protective and repair mechanisms. Under these conditions, fibroblasts did not undergo any apoptosis, as shown by cell cycle analysis (Fig. 1B) and Annexin tests (data not shown). According to recent studies, high intensity UVA causes keratinocytes to undergo detachment, leading to irreversible cell damage and apoptosis [Denning et al., 1998; Chen et al., 1999].

In the present study, we demonstrated that UVA irradiation increases ROS production and lipid peroxidation in young and aging fibroblasts (Fig. 2 and Table I). Following UV irradiation, ROS levels

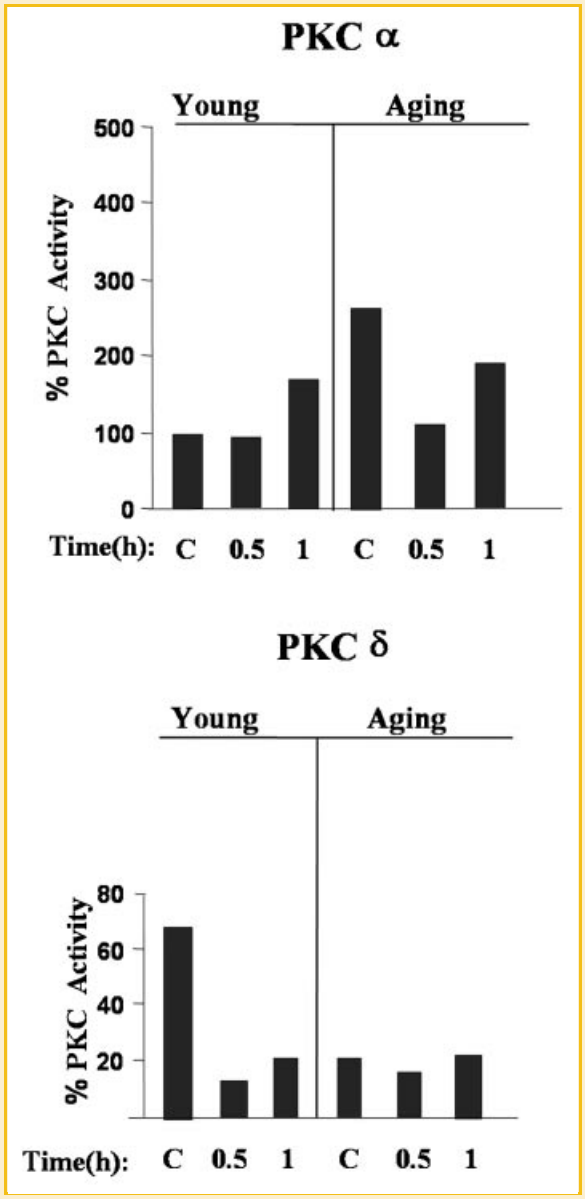

Fig. 6. UV irradiation reduces PKCδ activity in young fibroblasts. Fibroblasts were either left untreated (control—C), or treated with UVA irradiation. Following UV irradiation, cells were incubated for 30 min or 2 h. PKCα or PKCδ were immunoprecipitated from protein extracts using specific anti-PKCα and anti-PKCδ antibodies. PKCα and PKCδ immunoprecipitates were analyzed for PKC activity using an in vitro kinase assay, as described in Materials and Methods Section. Values were calculated as pico-moles of ATP per dish per min and are represented in this figure as the percentage of increase in PKC activity in relation to the level of PKCα activity in non-irradiated young fibroblasts designated as 100% (representing control). Data shown represent one out of three separate experiments (n = 3).

reached a maximum level, followed by moderate reduction until reaching basal level, in correlation with the initiation of a protective process. In aging fibroblasts, basal ROS levels were significantly higher as compared to young fibroblasts, and response to UV irradiation was delayed. The difficulty of aging cells to prevent or reduce ROS generation was probably as a result of partial endogenous antioxidant deficiency (Fig. 3) [Morliere and Santus, 1998; Jackson, 1999; Zhao et al., 2001].

UVA triggers lipid peroxidation and ROS production in young and aging fibroblasts. Increase in ROS production following UV irradiation suggests impairment of the equilibrium between oxidants and antioxidants during UV irradiation, which is important for redox maintenance in the cells. Lipid peroxidation and ROS are also detected in non-irradiated cells, since minor levels of ROS are important to maintain basal metabolic processes. In Table I, we can see that ROS levels gradually returned to basal levels 1–1.5 h after UV irradiation, probably due to endogenous antioxidants such as catalase, superoxide dismutase (SOD), glutathione, and more, that are present in the cell constitutively [Morliere and Santus, 1998; Jackson, 1999; Tobi et al., 2000; Zhao et al., 2001]. UV irradiation also reduced catalase and SOD activity in young cells (Fig. 3).

The outcome of lipid peroxidation might include structural and functional modification of membranes characterized by altered fluidity, increased permeability, and inactivation of cellular enzymes and transporters. In addition, oxyradical formation can also indirectly induce mutagenesis due to DNA damage, leading to cancer formation rather than cell death and apoptosis [Nishigori, 2006].

An important group of enzymes affected by membrane modifications is the family of PKC isoforms, whose activation state is specifically affected by the lipid environment. In addition, translocation of PKC isoforms to membranes is an important factor in the activation state of the PKC family of enzymes.

In aging fibroblasts, increased expression of PKCα was detected when compared to young cells (Fig. 4), while relatively minor changes were detected in the expression of this isoform due to UV irradiation in young and aging fibroblasts (Fig. 5). As for PKCδ, UV irradiation induced significant changes in expression and activity in both young and aging fibroblasts (Figs. 5 and 6). We detected dramatic reduction of PKCδ expression levels 30 min following UV irradiation, while protein expression level but not PKCδ activity state returned to basal level 1 h following UV irradiation.

Proteolytic cleavage and subsequent activation of protein kinase C (PKCδ) are required for apoptosis induced by a variety of genotoxic agents, including UV irradiation. In addition, overexpression of the constitutively active PKCδ catalytic fragment (PKCδ-cat) is sufficient to trigger Bax activation, cytochrome *c* release, and apoptosis [Brodie and Blumberg, 2003; D'Costa and Denning, 2005; Sitailo et al., 2006].

This is the first report to suggest a direct correlation between sublethal intensity of UVA irradiation, ROS generation, down-regulation of PKCδ, and translocation to the nucleus in both young and aging fibroblasts with no direct linkage to cell death and apoptosis [Denning et al., 1998; Chen et al., 1999].

In this study, we show that decrease in PKCδ expression and activation is associated with PKCδ translocation to the nucleus as a result of UV irradiation, in correlation with the aging process and in parallel to the elevation in ROS production (Table I and Fig. 2). These results are also supported by the induced ROS production, as seen in PKCδ knockout mice (Fig. 8B), and the ability of wildtype PKCδ

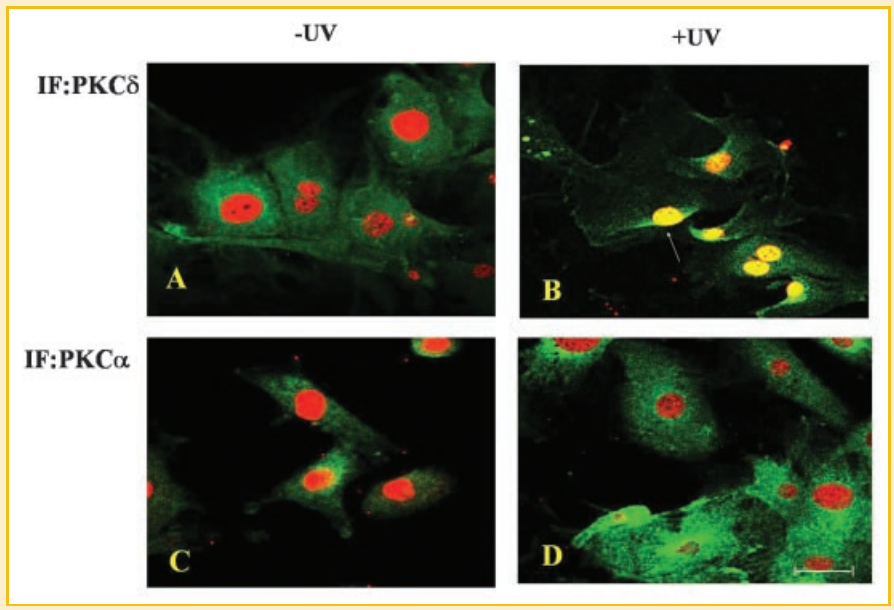

Fig. 7. Effect of UV irradiation on PKCα and PKCδ translocation in fibroblasts. Fibroblasts were seeded on glass slides. Young fibroblast cultures (3 days old) were irradiated with 9 J/cm^2, fixed in ethanol, and analyzed by immunofluorescence using anti-PKCα and PKCδ, followed by FITC-conjugated secondary antibody. Cells were viewed by confocal microscopy: (A) control non-irradiated young fibroblasts stained with anti-PKCδ; (B) fibroblasts irradiated with UVA and stained with anti-PKCδ; (C) control non-irradiated young fibroblasts stained with anti-PKCα; and (D) fibroblasts irradiated with UVA and stained with anti-PKCα. Results presented are of at least three experiments (similar results of PKCδ translocation were obtained for aging fibroblasts, data not shown). Yellow staining shows translocation of the PKC isoform to the nucleus. Pictures were photographed by confocal microscope, magnification 40×. Bar, 50 μm.

overexpression to attenuate ROS production in PKCδ overexpressing cells (Fig. 8C). Consistent with these results, ROS production was increased in cells overexpressing a dominant negative PKCδ mutant (Fig. 8C). These results indicate that cells lacking PKCδ activity generate higher levels of ROS compared to normal cells. These results suggest that PKCδ is a major factor in regulating ROS production in ROS-induced states. In young fibroblasts, PKCδ was downregulated a short time after UV irradiation, mainly in the cytosol fraction, while 24 h after UV irradiation, expression levels returned to normal (data not shown). Interestingly, in parallel, ROS production levels also returned to normal.

In aging fibroblasts, basal levels were significantly reduced and the reduction of PKCδ expression in the cytosol fraction was delayed after UV exposure. The reduced levels were maintained for up to 24 h following exposure to UV irradiation. These results support the link between loss of PKCδ expression and activation and elevation of ROS levels. Using PKCα knockout mice revealed that PKCα is not involved in this process, and there was no correlation between UV irradiation and increase of ROS production. This leads to the conclusion that PKCα, which was elevated in aging fibroblasts but not in UV-irradiated cells, is not involved in UV irradiation-induced processes. In contrast, expression of PKCδ did not change during chronological aging, but the dramatic changes in expression, distribution, and ROS production in irradiated cells suggest a link to the photoaging processes.

PKC was shown to be involved in UVA induction of signal transduction, and the different PKC isoforms might play specific roles in signal transduction [Gopalakrishna and Jaken, 2000; Huang et al., 2000; Shin et al., 2004]. PKCα was also previously linked to the aging process [Ricciarelli et al., 1999]. Activation of PKC is associated with the translocation of the enzymes from the cytosol to the particulate cell fraction. In some cases, increased activity is due to increased phosphorylation induced by oxidant-activated upstream kinases [Karin, 1998]. However, it is also known that oxidants can directly or indirectly modulate the redox status of the critical cysteine residues in the transcription factors that regulate their DNA-binding activity [Abate et al., 1990; Toledano and Leonard, 1991; Matthews et al., 1992; Anderson et al., 1994; Sun and Oberley, 1996; Flohe et al., 1997]. Thus, both the oxidant-induced increase in kinase activity and redox regulation are important mechanisms for oxidant-induced changes in gene expression that have an impact on the overall cellular response to changes in redox status.

Oxidants can directly activate PKC [Gopalakrishna and Anderson, 1989; Gopalakrishna et al., 1995]. Several signaling molecules that are regulated by redox modification are also either directly or indirectly regulated by PKC phosphorylation. It is possible that oxidation and phosphorylation represent alternative mechanisms for stimulating cellular responses relevant to the process of aging. The combined effects of PKC phosphorylation and oxidation on signaling events have not yet been described. Moreover, the possibility of cooperation and amplification of responses by the interaction of these mechanisms should be more carefully investigated.

In young and aging fibroblasts, PKCδ translocated to the nucleus as a result of UV irradiation (Fig. 7B). Decrease of this isoform in the cytoplasm could also be due to its absence from cytoplasm and membrane, while PKCα was seen in the cytoplasm (Fig. 7D). These

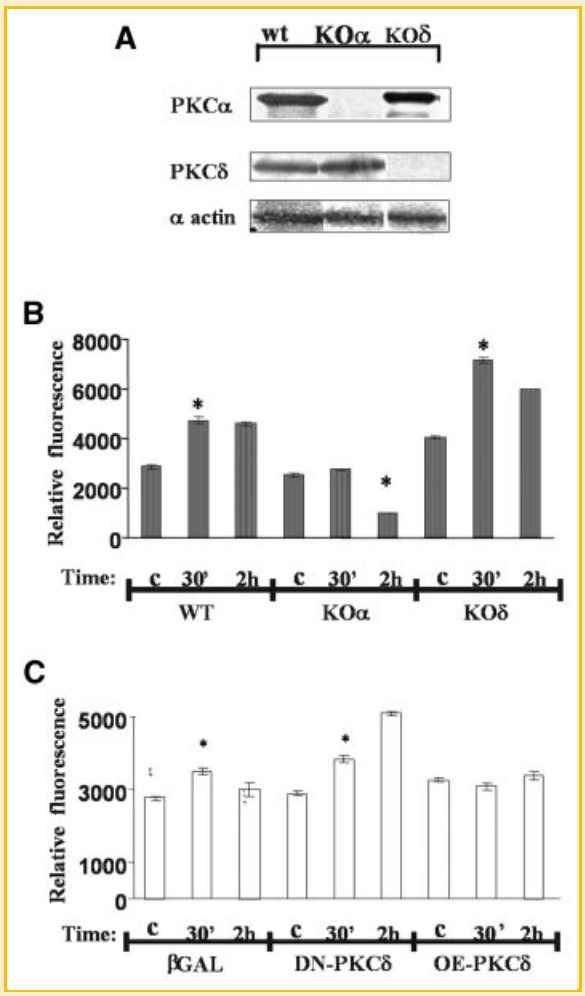

Fig. 8. DN-PKCδ and KOδ mice produce higher levels of ROS following UV irradiation. A: Primary fibroblasts were obtained from skin of PKCα and PKCδ knockout mice. Cell lysates were subjected to Western blot analysis and probed with anti-PKCα and anti-PKCδ antibodies. B: Primary fibroblasts were obtained from PKCα and PKCδ knockout mice. Fibroblasts were either not irradiated (control—C) or irradiated. Following UV irradiation, the cells were incubated for the indicated time points. Each sample (dish) was suspended in 300 μl PBS and analyzed for ROS levels. Results are expressed as relative fluorescence using the DCFH-DH. WT, primary fibroblasts isolated from wildtype mice; KOα, primary fibroblasts isolated from PKCα knockout mice; KOδ, primary fibroblasts isolated from PKCδ knockout mice. Results are expressed as mean ± SD of three repeated experiments ($P < 0.05$). C: Primary fibroblasts were infected with virus containing β-gal, wildtype (OE PKCδ), or dominant negative PKCδ (DN PKCδ) recombinant adenovirus for 24 h. After 24 h, fibroblasts were either not irradiated (control—C) or irradiated. Following UV irradiation, the cells were incubated for the indicated time points. We added 300 μl PBS to each sample (dish). DCFH-DH reagent was added to each 100 μl of sample in an ELISA microplate and incubated for 1 h at 37°C. Relative fluorescence was then measured, as described in detail in Materials and Methods Section. Results are expressed as relative fluorescence. βGAL, beta-galaclosidase (control); DN-PKCδ, primary fibroblasts infected with viral vectors encoding dominant negative PKCδ; OE, primary fibroblasts infected with viral vectors encoding wildtype PKCδ. Results are expressed as mean ± SD of three repeated experiments (n = 3) (*$P < 0.05$ in relation to control).

data also support the fact that PKCα and PKCδ play distinct roles concerning chronological aging and photoaging. In this study, we demonstrate the translocation of PKCδ (but not PKCα) to the nucleus as a result of UV irradiation in young and aging fibroblasts. Translocation of PKCδ to the nucleus can regulate activation of a number of transcription factors such as AP-1, NF-κB, Gadd53/CHOP, and STAT3, some of which are also involved in cancer development [Abate et al., 1990; Toledano and Leonard, 1991; Matthews et al., 1992; Anderson et al., 1994; Sun and Oberley, 1996; Flohe et al., 1997; Karin, 1998; Carballo et al., 1999; Zhang et al., 1999; Kim et al., 2005].

In summary, the differences between young and aging fibroblasts related to PKCδ and PKCα signaling following UV irradiation are: (1) PKCδ is important in regulating/downregulating ROS levels following UV irradiation, since in the absence of PKCδ, ROS levels were higher; and (2) The expression level of PKCα is dramatically elevated in aging cells but does not change following UV irradiation; therefore, its possible crucial role in chronological aging needs further investigation.

These findings help to distinguish, for the first time, the role of PKCα during chronological aging and PKCδ during the photoaging process. These findings also contribute to the understanding of downstream signaling of PKCα and PKCδ isoforms since they are involved in cell proliferation and tissue remodeling.

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