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Docosahexaenoic acid reverts resistance to UV-induced apoptosis in human keratinocytes: involvement of COX-2 and HuR☆

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Abstract

The dramatic increase in the incidence of nonmelanoma skin cancer over the last decades has been related to the augmented exposure to ultraviolet (UV) radiation (UVR). It is known that apoptosis is induced as a protective mechanism after the acute irradiation of keratinocytes, whereas apoptotic resistance and carcinogenesis may follow the chronic exposure to UVR. We found that not all the human keratinocytes lines studied underwent apoptosis following acute exposure to UVR (10–60 mJ/cm^2). Whereas UVR induced apoptosis in the HaCaT cells, NCTC 2544 and nr-HaCaT cells showed apoptosis resistance. The cytokeratin pattern of the apoptosis-resistant cells indicated that they possessed a degree of differentiation lower than that of HaCaT cells. They also showed an enhanced expression of cyclooxygenase-2 (COX-2), an early marker of carcinogenesis in various tissues, including skin. n-3 polyunsaturated fatty acids have drawn increasing interest as nutritional factors with the potential to reduce UVR carcinogenesis, and since they are apoptosis inducers and COX-2 inhibitors in cancer cells, we investigated the ability of n-3 polyunsaturated fatty acids to influence the resistance to UVR-induced apoptosis in keratinocytes. We observed that docosahexaenoic acid (DHA) reverted the resistance of nr-HaCaT cells to UVR-induced apoptosis, increasing the Bax/Bcl-2 ratio and caspase-3 activity, and reduced COX-2 levels by inhibiting the expression of the human antigen R (HuR), a known COX-2 mRNA stabilizer in keratinocytes. The transfection of nr-HaCaT cells with HuR siRNA mimicked the proapoptotic effect of DHA. Overall, our findings further support the role of DHA as a suitable anticarcinogenic factor against nonmelanoma skin cancers.

Keywords: Apoptosis; COX-2; DHA; HuR; Keratinocytes; UVR

1. Introduction

Ultraviolet (UV) radiation (UVR) is considered as the major environmental risk factor responsible for the development of nonmelanoma skin cancers [1]. The incidence of these types of cancer has dramatically increased over the last decades mainly in relation to the augmented UVR exposure [2]. For a long time, the UV-B were considered as the most powerful carcinogenic components of the UV solar radiation, even though their levels were much lower than those of UV-A. However, more recently, it has been recognized that UV-A also may exert strong procarcinogenic action [3]. It has been hypothesized that the dysregulation of apoptosis may play a crucial role in the carcinogenic effects of both these types of UV radiations [4]. It has been observed that, after an acute exposure to UVR, apoptosis is induced as a protective mechanism aimed at removing damaged premalignant cells [5]. On the other hand, it is known that chronic exposure may induce apoptotic resistance and carcinogenic effects in skin cells [6]. Moreover, it has been observed that UVR induces the expression of cyclooxygenase-2 (COX-2), a usually undetectable protein in most normal epithelial tissues. Its overexpression exerts an established role in carcinogenesis [7] and has been related to the malignancy of a series of cancers, including skin cancers [8,9]. In addition, COX-2 overexpression is believed to confer resistance to apoptosis [10], thus providing cancer cells with an apoptotic escape mechanism. Recently, using COX-2 knockout mice, it was shown that COX-2 expression was necessary for the development

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of UVR-induced skin cancer [6]. In addition, COX-2 deficiency in etherozygote COX-2+/- mice increases the percentage of apoptotic keratinocytes after treatment with UVR [11]. Recently, it has been suggested that UVR induced COX-2 overexpression through the increase of COX-2 mRNA stability in keratinocytes [12]. It was hypothesized that UVR can elicit this effect by inducing the cytoplasmic expression of the human antigen R (HuR), a known COX-2 mRNA stabilizer [13].

In our and other laboratories, it has been demonstrated that n-3 polyunsaturated fatty acids (PUFAs) reduce the overexpression of COX-2 in different kinds of cancer cells [14–18]. This effect has been put into relation with the growth inhibitory and proapoptotic effects exhibited by these fatty acids in various strains of cancer cells cultured in vitro or growing in animals [19–22]. Moreover, n-3 PUFAs have recently drawn increasing interest as possible protective agents against UVR-induced skin carcinogenesis [23–25], since it has become clear that the main portion of UVR exposure takes place not during special occasions as vacations but in everyday life [26]. Thus, the increased daily dietary intake of n-3 PUFAs may help to reduce UVR risk at any time [23].

Even though it is generally reported that, after the acute irradiation of keratinocytes, apoptosis is induced as a protective mechanism [27,28], in the present work, we observed that not all the human immortalized keratinocyte lines studied underwent apoptosis after acute exposure to UVR and that two cell lines, overexpressing COX-2, were resistant to UVR-induced apoptosis. On this basis, we examined whether docosahexaenoic acid (DHA, 22:6 n-3), which has often been shown to exert the maximal growth inhibitory and proapoptotic effect among the major n-3 PUFAs in vitro and in vivo [29–34], might revert the resistance to UVR-induced apoptosis exhibited by these keratinocytes. We investigated also the effect of DHA on the expression of COX-2 and HuR, a powerful stabilizer of COX-2 mRNA in many different cells, including keratinocytes [35].

2. Materials and methods

2.1. Cell lines and treatments

HaCaT human normal immortalized keratinocytes were purchased from ATCC (Rockville, MD, USA). NCTC 2544 human normal immortalized keratinocytes were kindly gifted by Dr. R. De Bellis (University of Urbino, Italy). The HaCaT cell line nonresponsive to UVR-induced apoptosis [nonresponsive HaCaT (nr-HaCaT)] was obtained by serially sub-culturing the parental HaCaT cells. Cells were grown in DMEM medium containing 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and supplemented with 10% foetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell lines were serially subcultured by treatment with trypsin-EDTA and seeded twice a week at a density of 3×10^5 cells/ml.

DHA, eicosapentaenoic acid (EPA, 20:4 n-3), linoleic acid (LA, 18:2 n-6), oleic acid (OA, 18:1 n-9) and the COX-2 specific inhibitor NS 398 were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA).

Fatty acids were added from an absolute ethanol stock solution, and the control cells were treated with the same amount of vehicle alone. The final ethanol concentration never exceeded 0.5% (v/v).

2.2. Ultraviolet (UV) irradiation of cells

A Philips TL20W/12RS lamp was used as UV source for irradiation of cells. In order to establish the amount of radiation emitted in the UV-A (320–400 nm), UV-B (290–320 nm) and UV-C (100–290 nm) wavelength ranges, the spectral distribution of radiation emitted by the lamp was measured by using an Ocean Optics USB2000 spectrometer (Supplement 1). Lamp emission consists in a broad UV emission extending from UV-C to UV-A, superimposed to several narrow peaks, the latter being present even in the visible range. We intended to perform our experiments by irradiating the cells with a mixture of both UV-A and UV-B, avoiding UV-C, being the solar UV radiation reaching the earth surface composed essentially by UV-B and UV-A. Moreover, we considered essential for our study the irradiation of keratinocytes with both UV-A and UV-B since they are both recognized as powerful pro-carcinogenic agents for skin [36]. For this reason, we performed some preliminary experiments in order to evaluate the ability of a glass filter (consisting of a microscope slide, Marienfeld, Germany) and a Kodacell K6808 filter (Eastman Kodak, Rochester, NY, USA; kindly gifted by S.M. Fischer, University of Texas) to absorb the UV-C. We observed (Supplement 1) that all UV-C and almost all UV-B emissions were filtered out by the Kodacell K6808 filter, leaving a transmitted radiation consisting of 4% UV-B and 96% UV-A and visible ranges. Also, using the glass filter, the UV-C emission was filtered out, and the residual UV-B emission represented 40% of transmitted intensity, while the remaining 60% consisted of UV-A and visible radiation. Therefore, since we intended to efficiently irradiate the cells also with the UV-B component, we used for our experiments the glass filter.

A Lutron radiometer was calibrated to give correct value of irradiance in the UV range. TL20W/12RS lamp-to-cells distance was optimized to have an irradiation difference ≤1% within the exposed area containing cell culture dishes. Exposure times were adjusted to obtain the desired UVR doses in the cells.

2.3. Apoptosis detection

Apoptosis was measured by acridine orange-ethidium bromide method [37] and analyzed by fluorescence microscopy. Apoptosis was also assessed using M30-cytodeath monoclonal primary antibody, which is directed against a specific epitope of cytokeratin 18 (CK18) that is formed by early caspase cleavage in the apoptotic cells [38]. Keratinocytes were plated in four-well chamber slides (Nunc, Rochester, NY, USA), fixed with ice-cold pure methanol at −20°C for 30 min, washed twice in 0.01 M phosphate-buffered saline (PBS) pH 7.4 and incubated with primary mouse monoclonal M30-cytodeath antibody (Alexis Biochem., San Diego, CA, USA) diluted 1:100 in PBS containing 1% bovine serum albumin (BSA) for 60 min at room temperature (RT). After two washings in PBS for 10 min, the anti-mouse IgG Cy2 conjugated antibody (Jackson Immunosearch Lab., West Grove, PA, USA) diluted 1:200 in PBS was added for 60 min at RT. Cells were then washed in PBS and mounted in Vectashield with Dapi mounting medium (Vector, Burlingame, CA, USA). Cells were observed using a Zeiss Axiophot (Germany) fluorescence microscope (10×). For quantitative analysis, M30-positive cells that fell into six randomly selected 50 μm squares for each slide were counted using a computerized system. The percentage of immunoreactivity of positive vs. total number of counted cells in each field was determined. For this purpose, images from immunostained sections were captured using a Zeiss AxioCam MRc camera (Zeiss, Germany) coupled to a Windows XP professional computer (Microsoft). The number of cells was then evaluated on the captured images using the Axiovision Release 4.4 software (Carl Zeiss Vision).

The activity of caspase-3 was measured by a fluorimetric assay as previously described [39]. Briefly, cells were incubated for the indicated times and then harvested. Cells (2×10^6) were lysed in 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 mM EDTA, 0.5% IGEPAL, and 150 mM NaCl. Cell lysates were incubated with 50 μM caspase-3 fluorogenic substrate, Ac-DEVD-AMC (Alexis Biochemicals, Lausen, Switzerland), in a reaction buffer (10 mM HEPES, pH 7.5, containing 50 mM NaCl and 2.5 mM DTT) for 120 min at 37°C. The release of AMC was measured with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer.

2.4. Western blot analysis

Cell extracts were prepared by lysing the cells (1×10^7) in ice-cold lysis buffer (1 mM $MgCl_2$, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM $Na_4P_2O_4$, 1mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 20% glycerol, 1% NP-40), as previously described [40]. The protein content was determined by Bradford method using the Biorad assay (Hercules, CA, USA) [41]. Equal amounts of proteins (50 μg) were separated on a 10% sodium dodecyl sulfate polyacrylamide gel and electroblotted on a nitrocellulose membrane. The membrane was blocked overnight at 4°C in 5% dried milk (w/v) in PBS plus 0.05% Tween 20 and then incubated with specific antibodies to COX-2 (clone 29, catalog # sc-19999), Bcl-2 (clone C-2, catalog # sc-7382), Bax (clone P-19, catalog # sc-526), HuR (clone 3A2, catalog # sc-5261), Cytokeratin 5 (clone H-40, catalog # sc-66856), Cytokeratin 10 (clone SPM262, catalog # sc-56518) and Cytokeratin 13 (clone 1C7, catalog # sc-58721) (Santa Cruz Laboratories, Santa Cruz, CA, USA).

As loading controls, the blots were reprobed with an anti-actin antibody (clone C-2, catalog # sc-8432) or anti-α-actinin antibody (clone B-12, catalog # sc-166524, Santa Cruz Laboratories) at a 1:1000 dilution. Following incubation with secondary mouse (COX-2, Bcl-2, HuR, cytokeratin 10, cytokeratin 13, actin, α-actinin) or rabbit (Bax, cytokeratin 5) antibodies (Amersham, Pharmacia Biotech Italia, Milan, Italy), the immunocomplexes were visualized using the enhanced chemiluminescence detection system (Amersham) and quantitated by densitometric scanning.

2.5. mRNA extraction and analysis

Total RNA was extracted from tissue samples using Trizol according to manufacturer's protocols (Invitrogen Life Technologies, Paisley, UK). The RNA was eluted in diethylpyrocarbonate (DEPC)-treated water (0.01% DEPC) and stored at −80°C until reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Nucleic acid concentrations were measured by spectrophotometry (Hewlett-Packard HP UV/VIS spectrophotometer 8450). RT-PCR assay was performed using the two-step method. For the first-step of reverse transcription, we used QuantiTect Reverse transcription kit (Qiagen, Hilden, Germany) with 500 ng of total RNA as template RNA, following the manufacturer's procedure. For the second-step PCR reactions, we employed QuantiTect SYBR Green Kits (Qiagen) and QuantiTect Primer Assays (Qiagen) for human β-actin

and human COX-2, according to manufacturer's protocol described for the LightCycler Real-Time PCR System (Roche, Penzberg, Germany). PCR data obtained by the LightCycler software were automatically analysed by the Relative Quantification Software (Roche) and expressed as target/reference ratio. Our approach was based on the calibrator-normalized relative quantification including correction for PCR efficiency.

2.6. Small interfering RNA (siRNA) transfection for knockdown of HuR

siRNA duplex oligonucleotides were purchased from Qiagen (Milan, Italy). The transfection was performed as indicated by the manufacturer. Briefly, nr-HaCaT cells were seeded at a density of 3×10^6/plate in 100 mm plates for Western blot analysis and at 3×10^5/well in six-well plates for apoptosis detection. On the same day, cells were transfected with two different HuR-specific siRNAs or with a fluorescently labeled siRNA as transfection efficiency control using the HiPerfect Transfection reagent (Qiagen). The concentration of siRNA in the cell culture medium was 10 nM. The target sequences were 5′-AAGTAGCAGGACACAGCTTGG-3′ (Hs-ELAVL1_1 siRNA) and 5′-ACCAGTTTCAATGGTCATAAA-3′ (Hs-ELAVL1_11 siRNA). After 12 h, the efficiency of transfection was verified by fluorescence microscopy. After 48 h from transfection (maximum HuR silencing time), cells were exposed to UVR for the times indicated, culture medium was replaced with fresh medium containing or not DHA and cells were incubated for different periods of time in order to evaluate the expression of HuR, COX-2, Bax, Bcl-2 and apoptosis induction. The two siRNAs used achieved the same extent of reduction in HuR expression, so the first one (Hs-ELAVL1_1) was used in the reported experiments. The effect of siRNA knockdown on cell viability was determined by trypan blue exclusion method.

2.7. Immunocytochemical analysis of intracellular HuR localization

nr-HaCaT keratinocytes were seeded in Nunc multiwell chamber slides at the density of 1×10^5 cells/well. After treatment, cells were fixed with 4% paraformaldehyde for 8 min, then permeabilized with 0.2% BSA and 0.1% Triton X-100 in PBS for 8 min and washed with PBS. Subsequently, cells were labeled with HuR mouse monoclonal antibody (clone 3A2, sc-5261, Santa Cruz Biotechnology) diluted 1:300 in Normal Antibody Diluent (phosphate buffered). Following incubation, cells were washed several times with PBS. Binding was visualized using biotinylated secondary antibody and the strepdavidin-biotin peroxidase complex developed with diaminobenzidine. The intensity of cytoplasmic immunocytochemical staining [integrated density (ID)] was evaluated in digitalized images, after thresholding and removal of cell nuclei, by "The image processing toolkit" (version 3.0, 1998, CRC Press, Boca Raton, FL, USA), as previously reported [42].

2.8. Statistical analysis

The results were expressed as the means±S.E. One-way analysis of variance (ANOVA) was used to determine significant differences among groups in Figs. 2, 3, 5, 6, 7 and 8. Multifactorial two-way ANOVA was adopted to assess any differences among the treatments and the times in Figs. 1, 4 and 9. When significant values were found ($P<.05$), post hoc comparisons of means were made using Tukey's honestly significant differences test.

3. Results

Fig. 1A shows that the acute exposure of two different strains of immortalized keratinocytes, HaCaT and NCTC 2544, to 10–60 mJ/cm^2 UVR produces opposite effects. Whereas HaCaT cells undergo apoptosis in a dose-dependent manner and increase the percentage of apoptotic cells by 3.2-fold upon exposure to 60 mJ/cm^2 UVR, NCTC 2544 cells do not respond to the proapoptotic effect of UVR. On the contrary, they appear to lose their ability to undergo apoptosis proportionally to the dose of UVR administered. By serially sub-culturing the HaCaT cells, we also obtained a cell line that, similar to NCTC 2544 cells, was unable to undergo UVR-induced apoptosis, and for this reason, we called them "nr-HaCaT." Apoptosis was evaluated by analyzing morphologically the cells stained with the fluorescent dye acridine-orange, but analogous results were obtained by immunocytochemical staining with M30 monoclonal antibody (Fig. 1B), a specific tool to investigate early apoptotic stages, as it is directed against the caspase-cleaved cytokeratin18 formed during early apoptosis in epithelial cells [38]. Using this method it was observed that while the percentage of HaCaT cells undergoing apoptosis increased 3.8 folds upon exposure to 60 mJ/cm^2 UVR (from 1.9% to 7.2%), the percentage of nr-HaCaT apoptotic cells decreased by 2.7-fold (from 1.6% to 0.6%) in the same conditions. Also the activation of caspase-3, which is considered a hallmark of the apoptotic process induced by UVR [43], was investigated in the two HaCaT cell lines after exposure to UVR. In the parental HaCaT cell line a significant increase in caspase-3 activity took place, in agreement to the proapoptotic

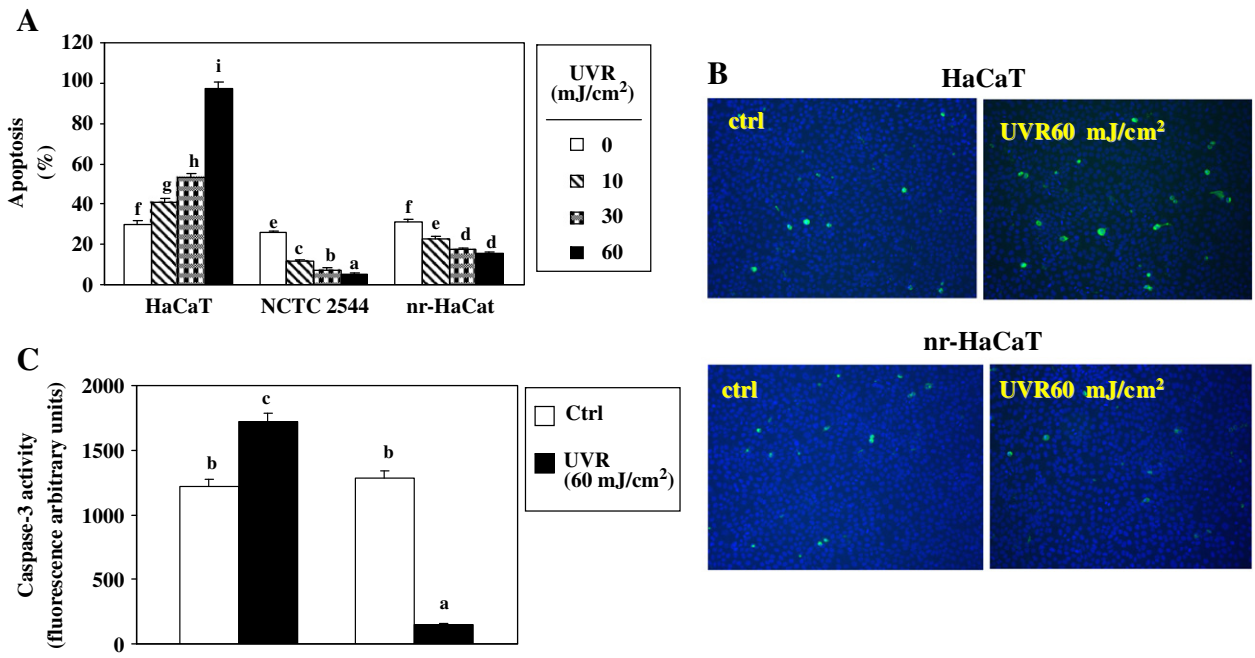

Fig. 1. Effect of an acute exposure to UVR on apoptosis in different keratinocyte cell lines. Apoptosis was evaluated 24 h after the exposure. (A) Apoptosis was evaluated by fluorescence microscopy on acridine orange-ethidium bromide stained HaCaT, NCTC 2544 and nr-HaCaT cells irradiated with increasing doses of UVR. (B) Representative images of fluorescence microscopy analysis of parental HaCaT and nr-HaCaT keratinocytes stained with the M30 cytodeath antibody. (C) Fluorimetrical analysis of caspase-3 activation in HaCaT and nr-HaCaT cells exposed to acute UV irradiation (60 mJ/cm^2, 24 h). (A and C) Data are the means±S.E. of four different experiments. Values not sharing the same superscript are significantly different ($P<.05$, multifactorial two-way ANOVA, followed by Tukey's test).

effect of UVR observed in these cells (Fig. 1C). On the contrary, a dramatic decrease in caspase-3 activation was obtained following UV irradiation in nr-HaCaT cells, and that was consistent with the inhibition of apoptosis observed in the same conditions.

Since it was reported the involvement of Bcl-2 family members in UVR-induced apoptotic cell death in HaCaT keratinocytes [44,45] and, in particular, the increased expression of the proapoptotic protein Bax and the decreased expression of Bcl-2, we evaluated how the levels of Bcl-2 and Bax varied in nr-HaCaT cells exposed to UVR (Fig. 2). Consistent with the resistance of nr-HaCaT cells to undergo UVR-induced apoptosis, we observed a marked decrease in the levels of the proapoptotic protein Bax in irradiated nr-HaCaT cells (51% decrease with 60 mJ/cm^2 UVR) (Fig. 2A), along with an even more striking increase of the anti-apoptotic protein Bcl-2 (7800% increase with 60 mJ/cm^2 UVR) (Fig. 2B). These alterations were dependent on the dose of UVR and led to the increase of the antiapoptotic/proapoptotic (Bcl-2/Bax) ratio from the initial value of 0.003 to the value of 0.52 with the maximal dose of UVR (60 mJ/cm^2). These findings confirmed that an alteration in the apoptotic machinery involving Bcl-2 proteins was taking place in these cells following UV irradiation.

It has been reported that whereas HaCaT keratinocytes possess a high degree of differentiation, NCTC 2544 cells are scarcely differentiated [46]. For this reason, we next investigated whether the different responses in terms of ability to undergo apoptosis following an UVR treatment could be in some way related to their different degree of differentiation, and to this aim, we examined the cellular levels of different cytokeratins (CKs) (Fig. 3). Some of them, such as CK10 and CK13, are indicative of a differentiate phenotype typical of the suprabasal layers of epidermis, while CK5 is a marker restricted to the undifferentiated cells of the basal layer in the epidermis [47]. We observed that CK5 was basally expressed at higher levels in NCTC 2544 (3-fold more) and nr-HaCaT cell lines (2.3-fold more) than in the HaCaT parental cell line (Fig. 3A). On the other hand, CK10 (Fig. 3B) and CK13 (Fig. 3C) were expressed at significantly lower levels in NCTC 2544 cells than in both the HaCaT cell lines, where their levels of expression did not differ significantly. Altogether, these findings suggest that the parental HaCaT cell line is the most differentiated, followed by its derived cell line nr-HaCaT and finally by the NCTC 2544 cell line, and their degree of differentiation is inversely related to their ability to respond to the UVR insult by undergoing apoptosis. These findings suggest that the newly derived nr-HaCaT cell line has lost some differentiation features present in the parental cell line and has acquired a phenotype more similar to undifferentiated cells.

UVR-induced apoptosis is considered to be a defense mechanism displayed by the cells of surface tissues acutely exposed to UVR and suffering DNA damage [48,49]. Therefore, the loss of this ability to undergo apoptosis after an acute UVR exposure exhibited by nr-HaCaT and NCTC 2544 cells may indicate that these scarcely differentiated cells have also a higher sensitivity to the carcinogenic action of UVR. A variety of studies have shown that the resistance to apoptosis may be conferred to the cells by the increased expression of the protein COX-2 [10], widely considered a marker of carcinogenesis in several tissues, including skin [8]. For this reason, we investigated the expression of this protein in NCTC 2544 and nr-HaCaT cells and compared it to that of the parental HaCaT cell line. We observed (Fig. 3D) that the expression of COX-2 protein was inversely related to the ability of the cells to undergo UVR-induced apoptosis and directly to their degree of differentiation, with the nr-HaCaT and NCTC 2544 cells showing basal levels of COX-2 respectively 3.9- and 4.3-fold higher than those of the parental HaCaT cell line. Moreover, we observed that in the nr-HaCaT cells the levels of COX-2 grew higher as the dose of UVR increased (Fig. 4). The effect was maximal at 16 h and became less evident at 24 h.

In previous works performed in our and other laboratories, it was demonstrated that n-3 PUFAs may act as proapoptotic agents in different cells by inhibiting the expression of COX-2 [14–16,18,34,50]. For this reason, we investigated whether also in our conditions DHA and EPA could induce apoptosis in nr-HaCaT cells. We used DHA and EPA in the 10–50 μM range and avoided the higher concentrations tested (75-100 μM), since they significantly induced necrosis in the cells (data not shown). After 24 h of treatment, DHA (at 30 and 50 μM) induced apoptosis in a dose-dependent manner in nr-HaCaT cells in basal conditions (86.4% increase in the percentage of apoptotic cells at 50 μM DHA) (Fig. 5A) and even more markedly, upon irradiation with 60 mJ/cm^2 UVR (490.9% increase in the percentage of apoptotic cells at 50 μM DHA), thus reverting the resistance to UVR-induced apoptosis showed by these cells. On the contrary, EPA reduced apoptosis in basal conditions and did not change the percentage of apoptotic cells observed after UV irradiation (Fig. 5B). Effects similar to those of EPA were observed in the presence of other fatty acids, the monounsaturated OA (Fig. 5C) and the n-6 PUFA LA (Fig. 5D) in

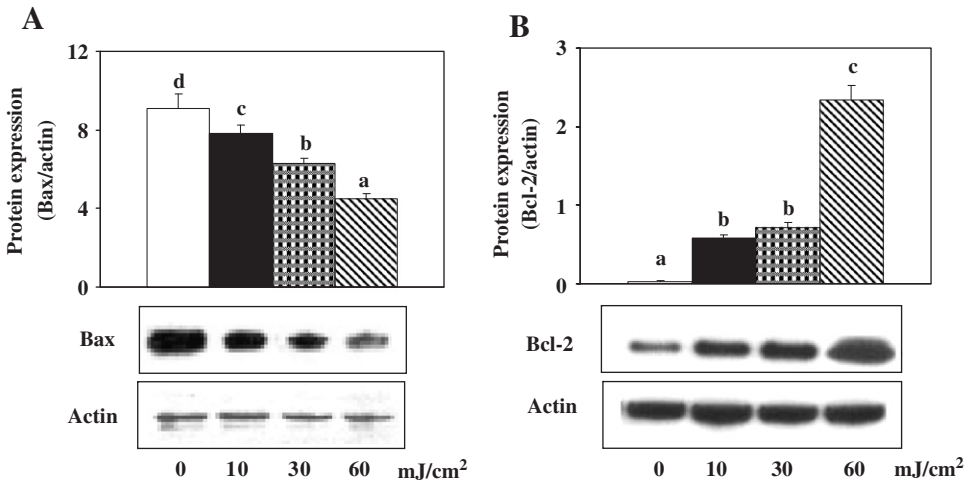

Fig. 2. Bax and Bcl-2 protein expression in nr-HaCaT cells exposed to increasing doses of UVR. The expression has been evaluated 24 h after the irradiation. (A and B, top) Data represent the means±S.E. of three different experiments. Values not sharing the same superscript are significantly different (P<.05, one-way ANOVA). (A and B, bottom) One representative of three similar Western blot analyses is shown for each panel.

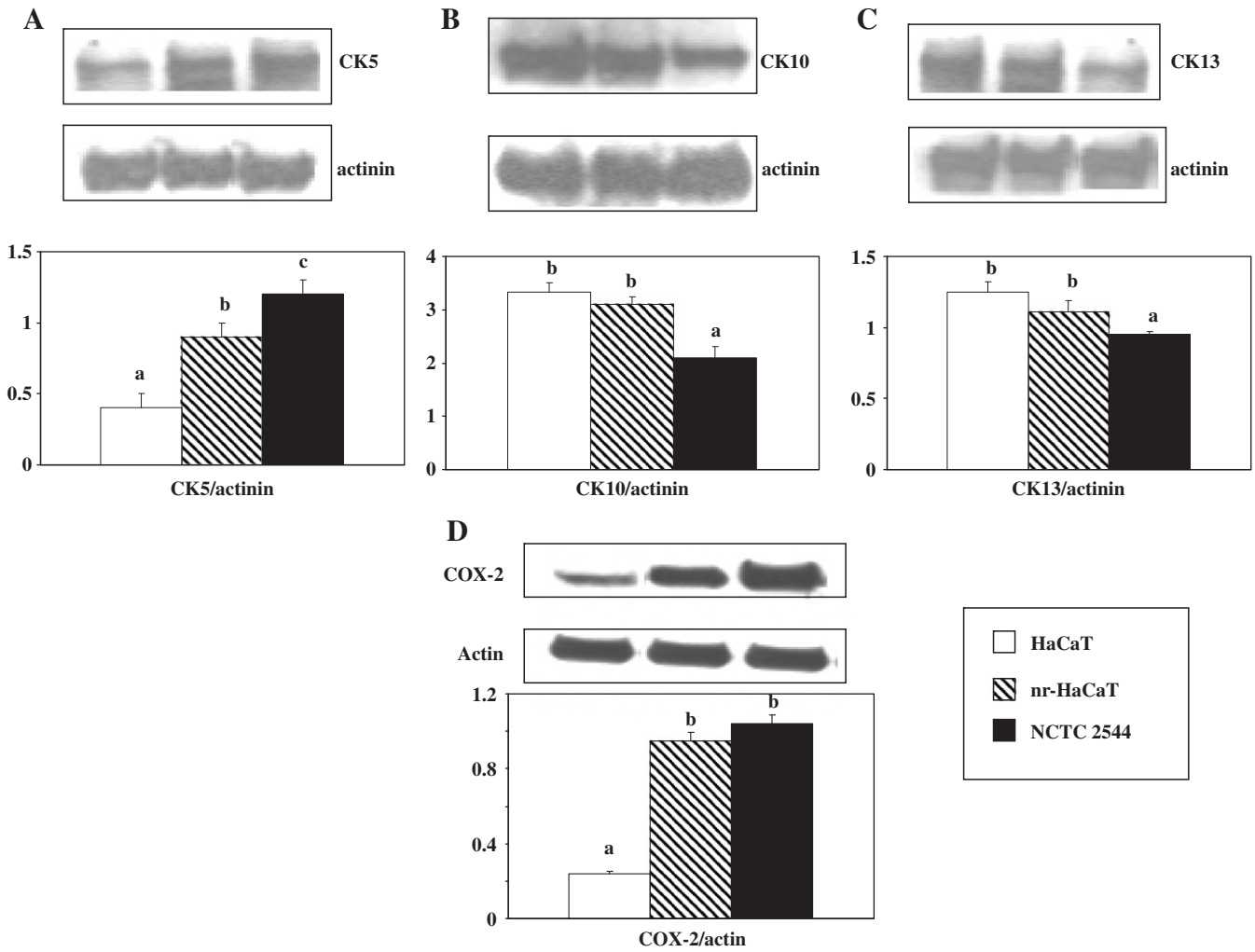

Fig. 3. Basal expression of cytokeratins (CK 5, 10 and 13) and COX-2 in HaCaT, nr-HaCaT, NCTC 2544 cells. (A–D, top) For each panel, one representative of three similar Western blot analyses is shown. (A-D, bottom) The data are the means±S.E. of three different experiments. Values not sharing the same superscript are significantly different ($P<.05$, one-way ANOVA).

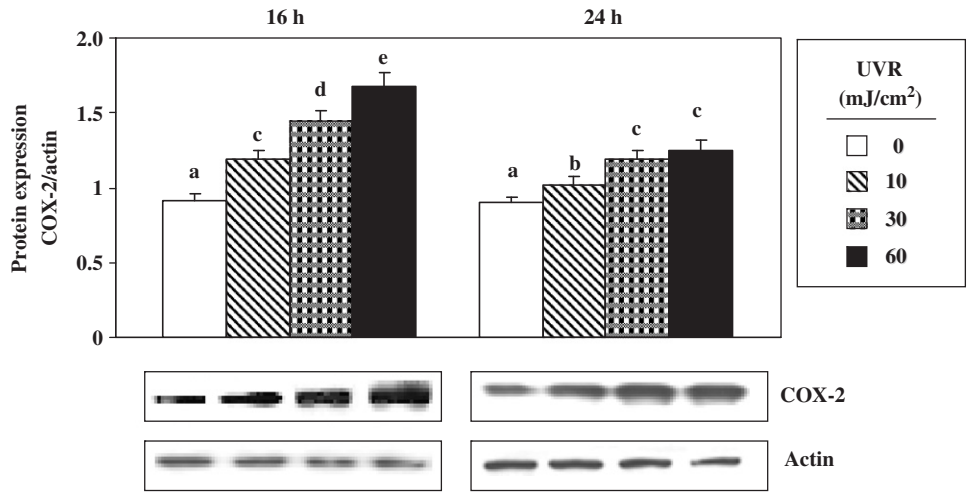

Fig. 4. UVR induction of COX-2 expression in nr-HaCaT keratinocytes. The cells were exposed to increasing doses of UVR for 16 or 24 h. (Top) Data are the means±S.E. of three different experiments. Values not sharing the same superscript are significantly different ($P<.05$, multifactorial two-way ANOVA, followed by Tukey's test). (Bottom) One representative of three similar Western blot analyses is shown for each panel.

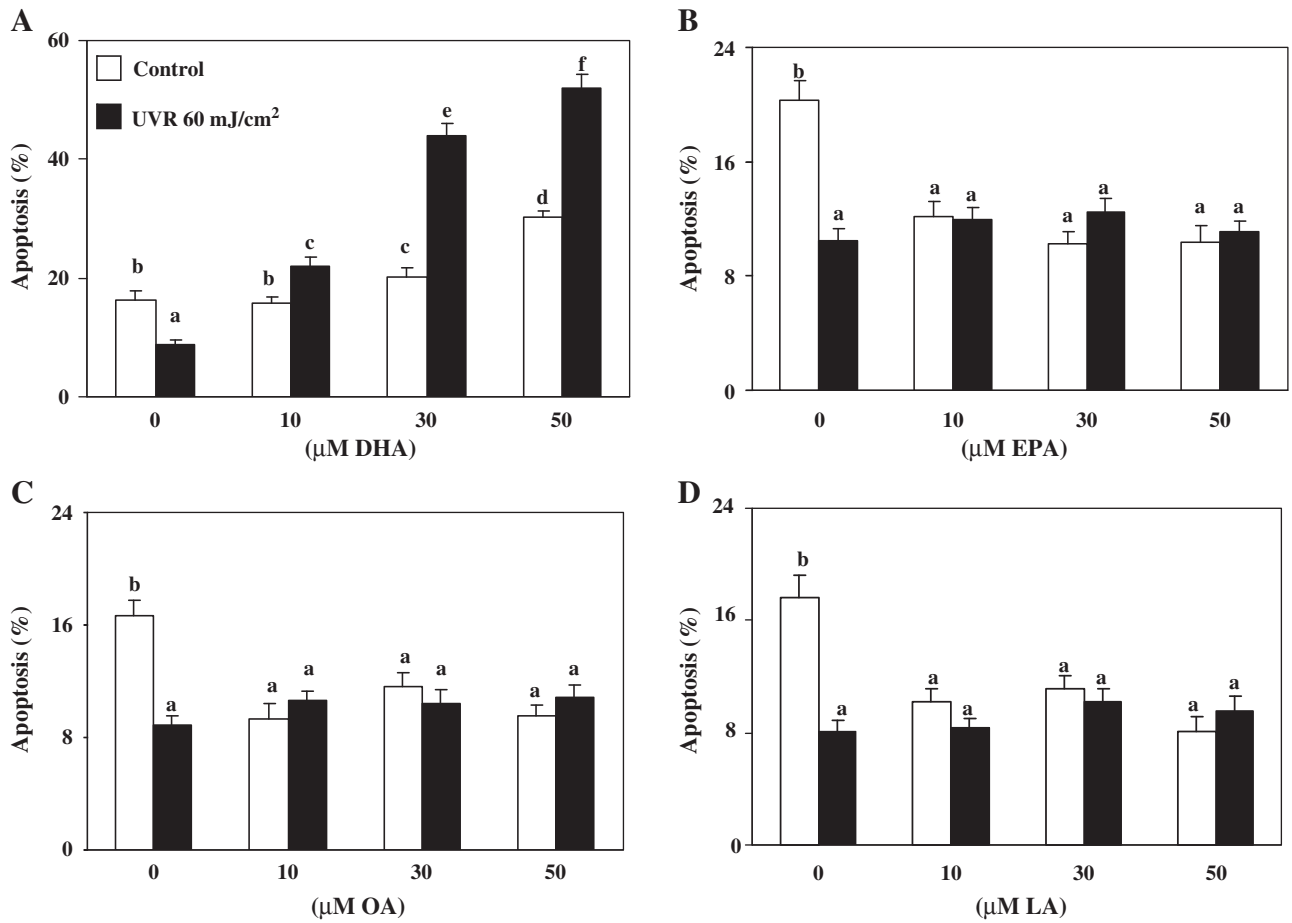

Fig. 5. Effect of DHA (A), EPA (B), OA (C) and LA (D) on apoptosis in nonirradiated and UVR-irradiated (60 mJ/cm^2) nr-HaCaT cells. The cells, treated for 24 h with increasing concentrations (0–50 μM) of fatty acids were stained with acridine orange-ethidium bromide and analyzed by fluorescence microscopy. Data are the means±S.E. of three different experiments. Values not sharing the same superscript are significantly different ($P<.05$, multifactorial two-way ANOVA, followed by Tukey's test).

nr-HaCaT cells. The proapoptotic effect of DHA was also confirmed by the finding that in the presence of this fatty acid at the highest concentration (50 μM), the levels of the proapoptotic protein Bax, which dramatically decreased after exposure to UVR, were reverted to values significantly higher than those observed in basal conditions (26.4% increase, Fig. 6A). In agreement, DHA was also able to considerably reduce the nr-HaCaT cell levels of Bcl-2 (Fig. 6B), both in basal conditions (21% decrease) and after UVR exposure (43.9% decrease). Correspondently, DHA treatment reduced markedly the high basal levels of COX-2 protein in nr-HaCaT cells and was also able to revert the UVR-induced overexpression of COX-2 protein (observed after 16 h, Fig. 7A) and mRNA (observed after 12 h, Supplement 2). We observed that EPA slightly increased the levels of COX-2 protein in basal conditions, in agreement to what previously observed by Chene et al. [51] in HaCaT cells, whereas, after the UVR exposure, EPA decreased the high level of COX-2 (Fig. 7B) of the irradiated nr-HaCaT cells, even though at a much lower degree than DHA (73.5% and 26.8% decrease for DHA and EPA, respectively), suggesting that the small EPA-induced COX-2 change could not be sufficient to induce apoptosis. We also observed that the addition of the n-6 PUFA LA, similarly to EPA slightly increased the levels of COX-2 protein in basal conditions but did not modify COX-2 expression following UVR exposure (Fig. 7C).

Zhang and Bowden [35] recently found that the COX-2 overexpression induced by UVR was related to the increased stability of its mRNA, which was shown to be induced by HuR, a powerful stabilizer of COX-2 mRNA in many different cells, including keratinocytes [12,13,52–56]. These authors [35] observed that the UV-B treatment of HaCaT cells induced the protein abundance of HuR and its localization in cytoplasm, where it exerts its stabilization activity. Accordingly, they found that the overexpression of HuR augmented the mRNA stability of COX-2. We observed that the basal levels of HuR were increased in nr-HaCaT cells as compared to the parental cell line and were similar to those observed in NCTC 2544 cells (Fig. 8A). Moreover, the levels of HuR were further increased in nr-HaCaT cells after the exposure to UVR (Fig. 8B). The increase was observed as early as 2 h after the UVR exposure, becoming maximal after 6 h, and DHA was able to revert this effect (Fig. 8C). Furthermore, the immunocytochemical analysis confirmed that UVR exposure caused an increased expression of cytoplasmic HuR in nr-HaCaT cells and that DHA inhibited this effect (Fig. 8D). In particular, the quantitative immunocytochemistry revealed that cytoplasmic HuR increased from 1.2 ID in untreated cells to 6.9 ID in UVR-exposed ones. The addition of 50 μM DHA to the irradiated cells instead decreased the HuR ID to 2.0 (71% decrease).

The *HuR* gene silencing by the specific siRNA in nr-HaCaT cells was performed to substantiate the hypothesis that DHA may act in these cells by inhibiting HuR expression (Fig. 9). Firstly, we evaluated the siRNA knockdown efficiency (Fig. 9A) by measuring the change in the levels of the cytosolic HuR protein and observed that it was maximally reduced 48 h after the transfection. Similar to what was obtained in the nr-HaCaT cells treated with DHA, and different from the nontransfected cells, in the siRNA-transfected

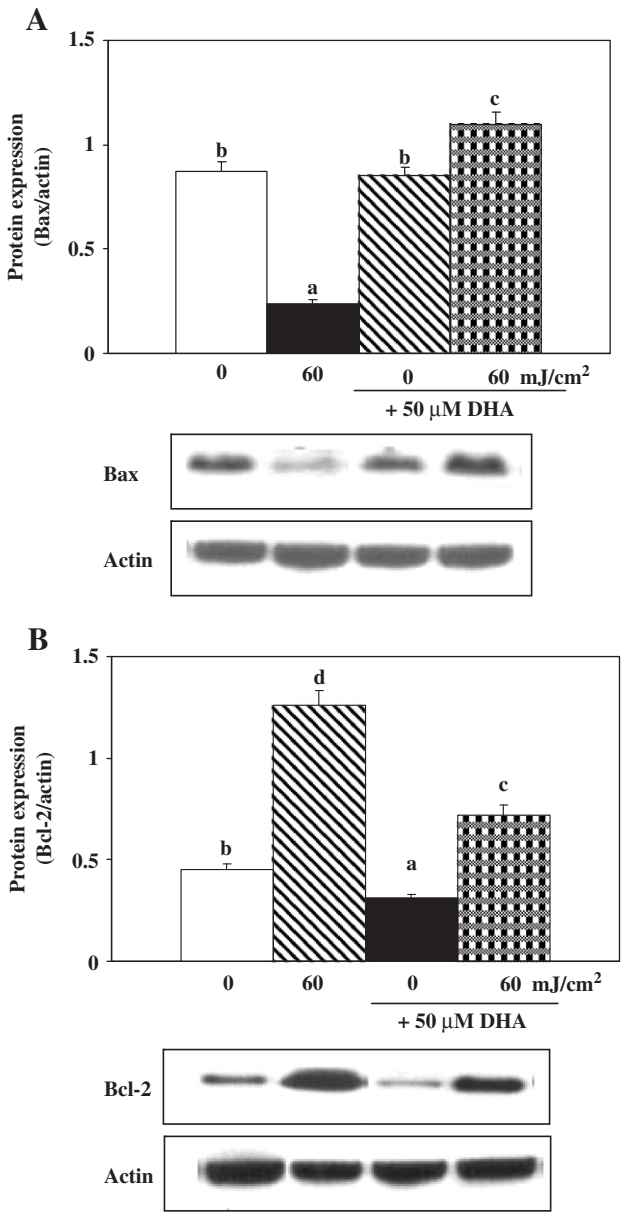

Fig. 6. Bax and Bcl-2 protein expression in nr-HaCaT keratinocytes irradiated with UVR (60 mJ/cm^2) and treated with DHA. The cells were treated for 24 h with 50 μM DHA. (A and B, top) Data are the means±S.E. of three different experiments. Values not sharing the same superscript are significantly different ($P<.05$, one-way ANOVA). (A and B, bottom) One representative of three similar Western blot analyses is shown for each panel.

nr-HaCaT cells, the exposure to UVR was able to induce apoptosis (Fig. 9B). In these cells, the levels of COX-2 protein were drastically decreased and not further modified by UV irradiation (Fig. 9C). These results demonstrated that the apoptosis resistance of nr-HaCaT cells could be overcome by reducing the expression of COX-2 and that this reduction could be due primarily to the change in HuR expression and activity, either caused by *HuR* silencing or by DHA addition. Moreover, we showed that the inhibition of COX-2 activity, obtained by the use of the specific COX-2 inhibitor NS 398 (Fig. 9D), also allowed the nr-HaCaT cells to overcome the resistance to UVR-induced apoptosis. This finding further strengthens the hypothesis that the alteration in the levels and activity of COX-2 is crucial to determine the apoptosis-resistant phenotype in human keratinocytes.

4. Discussion

In the present study, we demonstrate that three lines of human immortalized keratinocytes respond differently to acute irradiation with UV, undergoing or not apoptosis, depending on their basal COX-2 levels. We observed that the resistance to the UVR-induced apoptosis was conferred by high levels of COX-2 expression and could be reverted by the treatment with DHA. We related the effect of DHA to its ability to inhibit the expression of COX-2 through the reduced expression of HuR, a known stabilizer of COX-2 mRNA in many cells, including keratinocytes [13,35].

There is consensus that an acute treatment of normal keratinocytes with UVR both in vitro and in vivo induces apoptosis as a defense mechanism against the UVR-induced damage to DNA and carcinogenesis [4]. On the contrary, it has been observed that a chronic treatment with UVR may induce apoptosis resistance [57]. However, in the present study we observed that the apoptotic response induced by a single dose of UVR (range 10–60 mJ/cm^2) was markedly different between two immortalized keratinocyte lines (HaCaT and NCTC 2544) known to exhibit different degree of differentiation [46]. We observed that HaCaT cells exhibited higher expression of CK10 and 13, typically found in most differentiated cells of the outer layer of epidermis [47], and lower expression of CK5, typically found in the undifferentiated basal cells of epidermis, thus confirming their high differentiation degree. On the contrary, the lower expression of CK10 and CK13 as well as the higher levels CK5 in NCTC 2544 cells suggested the resemblance of these cells to the undifferentiated cells of the basal layer of epidermis [47]. Whereas HaCaT cells exposed to UVR underwent apoptosis in a dose-dependent manner, confirming what was previously observed by Chaturvedy et al. [58], the NCTC 2544 cells were more resistant to apoptosis and their ability to undergo apoptosis decreased as the UVR dose increased. This could appear in contrast with what previously reported by Leccia et al. [59], who showed that NCTC 2544 cells exposed to a single dose of UV-A1 were more sensitive to UVR-induced cytotoxicity than HaCaT cells. However, there exist many differences between their experimental model and ours: we used a mixture of both UV-B and UV-A; the UVR dose used by them (13 J/cm^2) was much higher than the doses used in the present work (10–60 mJ/cm^2). We chose these doses in order to avoid the necrotic damage and, thus, better investigate the proapoptotic effect elicited by UVR. Moreover, Leccia et al. [59] did not evaluate apoptosis, but cytotoxicity, either by counting surviving cells or using the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method.

By serially subculturing the HaCaT cells, we obtained a cell line which, similar to NCTC 2544 cells, was resistant to apoptosis, and for this reason, we named it “nr-HaCaT.” Its acquired resistance to UVR-induced apoptosis was demonstrated also by the fact that these cells completely lost the ability of the parental cell line to activate caspase-3 following the UV irradiation. In agreement, in these cells, the ratio between the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax (Bcl-2/Bax) was markedly increased by the UVR. Interestingly, these cells, similar to NCTC 2544 cells, exhibited lower levels of CK5 than the parental cells, even though they retained the high levels of CK10 and CK13 of the parental HaCaT cell line. This partial modification in the pattern of cytokeratins, together with the lower susceptibility to UVR-induced apoptosis of the nr-HaCaT cell line as compared to the parental cell line, suggested that a step towards carcinogenesis could have taken place in the newly derived cells. This hypothesis was further supported by the observation that the nr-HaCaT cells exhibited basal levels of the protein COX-2 much higher compared to the parental cells. This alteration is particularly noteworthy, since the overexpression of COX-2 has been considered a marker of carcinogenesis in several tissues [60–62], including skin [63]. Remarkable is the observation that the basal COX-2 levels of

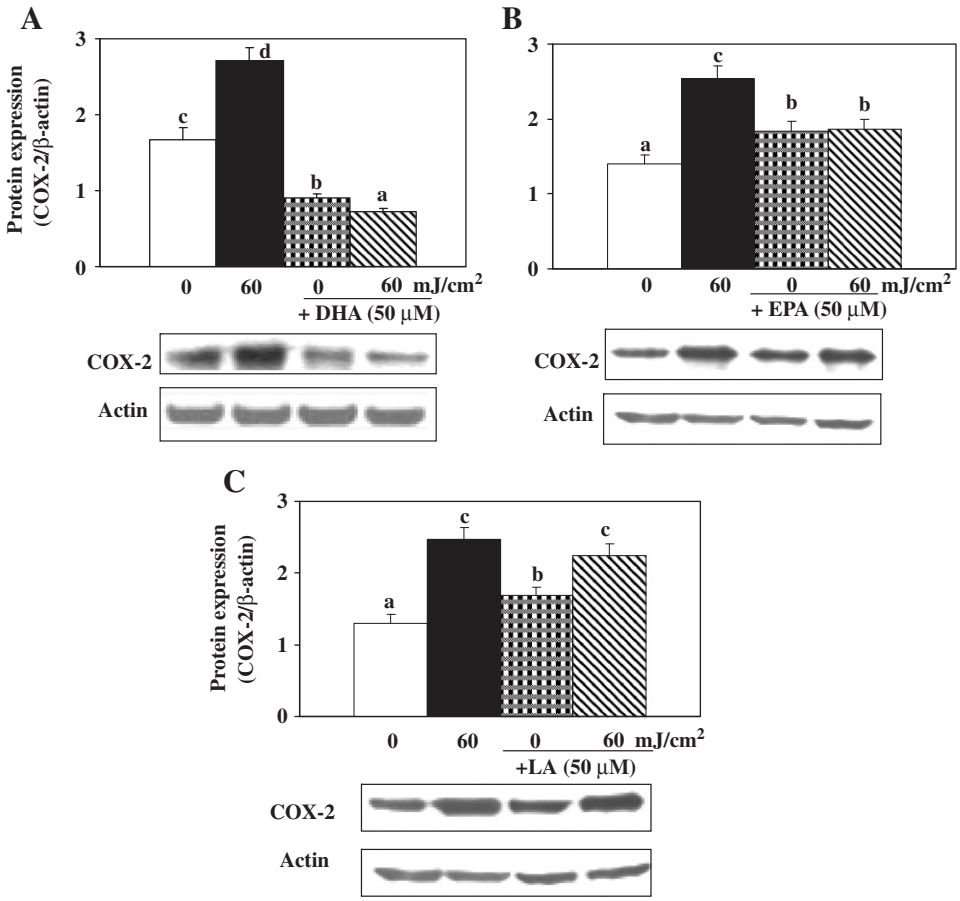

Fig. 7. Effect of DHA (A), EPA (B) and LA (C) on COX-2 protein expression in nr-HaCaT keratinocytes exposed to UVR. The cells were exposed to UVR and treated with DHA for 16 h. Values are the means±SE of three different experiments. (A–C) COX-2 protein expression. (A-C, top) Values not sharing the same superscript are significantly different ($P<.05$, one-way ANOVA). (A–C, bottom) One representative of three similar Western blot analyses is shown for each panel.

nr-HaCaT cells were comparable to those observed in NCTC 2544 cells. Since it is believed that the augmented expression of COX-2 may confer apoptosis resistance to cells [64–66], we hypothesized that the inability to undergo UVR-induced apoptosis could be related to the high basal level of COX-2 expression exhibited by both these cell lines as compared to the HaCaT parental cell line. As a confirmation of this hypothesis, we demonstrated that the inhibition of COX-2 activity with its specific inhibitor NS 398 reverted the resistance to UVR-induced apoptosis in nr-HaCaT cells. Moreover, we observed that COX-2 expression, evaluated both at mRNA and protein level, was further increased by UV irradiation in these cells and that the increase became more pronounced as the dose of UVR increased, paralleling the progressive decrease in the percentage of apoptotic cells observed as the UVR dosage increased. The observed inverse relationship between the expression of COX-2 in keratinocytes and their ability to undergo UVR-induced apoptosis confirms the recent finding that keratinocytes of transgenic COX-2-deficient (−/−) mice show increased ability to undergo UVR-induced apoptosis [11]. Moreover, the heterozygous COX-2 deficiency (COX-2+/-) reduced skin tumor formation in UVR-sensitive SKH-1 mice, as compared to wild mice when exposed chronically to UV-B [6], thus demonstrating the role exerted by COX-2 in UVR-induced skin carcinogenesis.

In agreement with the hypothesized role of COX-2 overexpression in UVR-induced carcinogenesis, there exists ample evidence that COX-2 selective inhibitors can reduce the incidence of UVR-induced skin tumors following chronic exposure in mice [67–71]. Apoptosis induction is one of the main mechanisms invoked to explain the antitumor activity of COX inhibitors [4,72–76]. However, their possible use as chemopreventive agents for skin cancer has been discouraged by the fact that some COX-2 inhibitors can cause photosensitization of skin during UVR exposure [77] and by the major adverse side-effects, which can arise from a prolonged treatment with them [78,79]. Some studies performed in our and other laboratories have previously indicated that the inhibition of COX-2 overexpression may be obtained in different kinds of cancer cells also through the treatment with safer nutritional compounds such as n-3 PUFAs [14–18]. For instance, we demonstrated that the inhibitory effect of n-3 PUFAs on COX-2 expression in colon cancer cells was related to the proapoptotic and antiangiogenic activity of these fatty acids [14]. As far as skin pathologies are concerned, there exists wide evidence in the literature showing the possible protective action of diets rich in fish oil against UVR-induced photocarcinogenesis [80]. In particular, using a mouse model in vivo, it was observed that, whereas a fish oil rich in n-3 PUFAs exerted protective activity against skin carcinogenesis, a procarcinogenic action was exhibited by dietary corn oil rich in n-6 PUFAs [81]. These findings were confirmed by an epidemiological case-control study reporting the significant association of a high n-3 PUFA/n-6 PUFA ratio in the diet with a low risk of squamous cell carcinoma [82]. Moreover, recently, a double-blind randomized trial on healthy humans evidenced an effective protection of EPA against a range of early genotoxic carcinogenic markers, including the induction of p53 protein in skin and the increased formation of DNA strand breaks in peripheral blood cells [24]. On these bases, we became interested in investigating

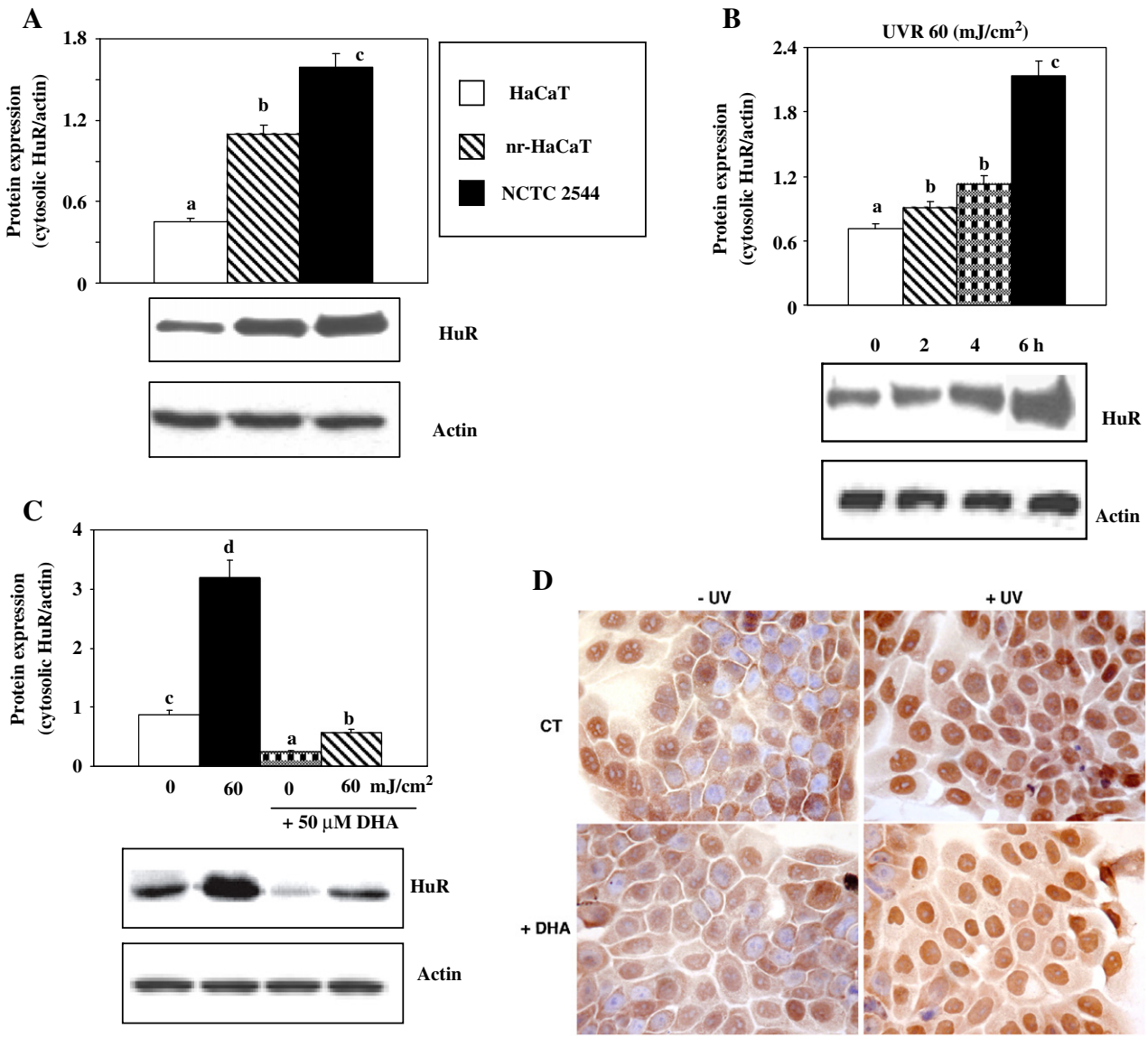

Fig. 8. Expression of cytosolic HuR protein in basal condition and after DHA treatment in keratinocytes. (A) Basal cytosolic expression of HuR in HaCaT, nr-HaCaT and NCTC 2544 keratinocytes. (B) Cytosolic expression of HuR in nr-HaCaT keratinocytes irradiated with 60 mJ/cm^2 UVR for 2-6 h. (C) Cytosolic expression of HuR in nr-HaCaT keratinocytes irradiated with 60 mJ/cm^2 UVR for 2–6 h and exposed to 50 μM DHA for 6 h. (D) Intracellular localization of HuR protein in nr-HaCaT cells irradiated with 60 mJ/cm^2 UVR in the presence and in the absence of 50 μM DHA, evaluated by immunocytochemistry. (A–C, top) The data are the means±S.E. of three different experiments. Values not sharing the same superscript are significantly different (P<.05, one-way ANOVA). (A–C, bottom) One representative of three similar Western blot analyses is shown in each panel.

whether n-3 PUFAs could be able to induce apoptosis also in nr-HaCaT cells in spite of their resistance to UVR-induced apoptosis and increased basal levels of COX-2. We observed that, in the range of concentrations used (30–50 μM), DHA was able to significantly induce apoptosis in irradiated nr-HaCaT cells. Correspondently, DHA was able to revert the UVR-induced COX-2 expression (observed both as mRNA and protein) to levels lower than those observed in the untreated nr-HaCaT cells. These results suggest that the DHA-induced down-regulation of COX-2 may render the nr-HaCaT cells similar to their parental cells as far as the lower COX-2 expression and the ability to undergo apoptosis after receiving an UVR acute treatment are concerned. The ability of DHA to revert the resistance to UVR-induced apoptosis was also demonstrated by the marked decrease induced by DHA in the Bcl-2/Bax ratio, which was instead substantially increased by the UVR in these cells in basal conditions. On the contrary, EPA was not as efficient as DHA. At the concentrations used, it did not revert the nr-HaCaT cell resistance to UVR-induced apoptosis, even though it caused a slight decrease in the COX-2 expression of irradiated cells, which, however, could not be sufficient for the promotion of apoptosis. Still, this does not mean that EPA cannot function as a protective agent against the carcinogenic action of UVR, since it could act not by inducing apoptosis like DHA but preferentially by inhibiting cell proliferation [33] or UVR-induced inflammation [83]. Nevertheless, it could also be possible that higher concentrations of EPA could be needed to obtain the same proapoptotic effect of DHA. In fact, DHA has been often reported to have greater cell growth inhibiting and proapoptotic activities than EPA, and higher concentrations of EPA were sometimes needed to obtain the same effects showed by DHA [29–32,34]. However, in our cellular model, it was not possible to investigate EPA at higher concentrations, since, as already mentioned, they induced remarkable necrotic effects. Moreover, we observed that the proapoptotic action of DHA, as well as its ability to decrease COX-2 expression in nr-HaCaT cells, was specific, as indicated by the finding that other fatty acids,

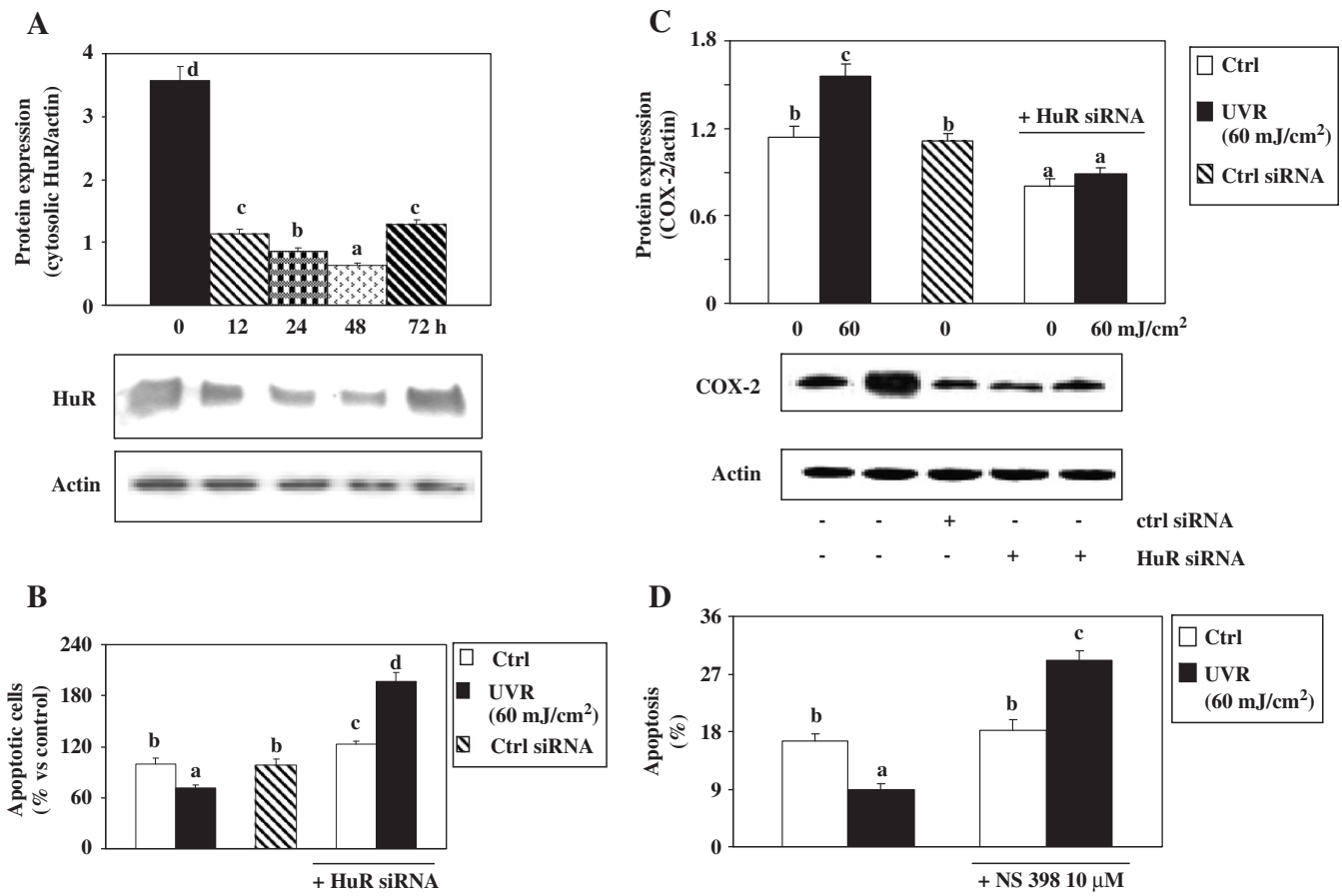

Fig. 9. Effect on COX-2 expression and apoptosis in nr-HaCaT keratinocytes transfected with the HuR specific siRNA or treated with the COX-2 specific inhibitor NS 398. (A) The cytosolic expression of HuR was evaluated at different intervals following transfection. (B) The effect of HuR silencing on COX-2 expression was evaluated 24 h after the maximal silencing effect (48 h). (C and D) The effect of HuR silencing or NS 398 on the apoptosis induced by UVR (at 24 h from UVR treatment) was evaluated by fluorescence microscopy on cells stained with the acridine orange-ethidium bromide. (A and C, top) The data represent the means±S.E. of four different experiments. Values not sharing the same superscript are significantly different ($P<.05$, two-way multifactorial ANOVA, followed by Tukey's test). (A and C, bottom) One representative of three similar Western blot analyses is shown in each panel.

both monounsaturated, such as OA, and polyunsaturated of the n-6 class, such as LA, were not able either to induce apoptosis nor to inhibit COX-2 expression in the irradiated cells.

In many different cells, including keratinocytes [12,13,52–56], it has been observed that the mRNA of COX-2 may be stabilized by HuR, thus leading to the increased expression of COX-2 protein. In particular, it has been recently found [35] that the overexpression of COX-2 protein induced by UVR in HaCaT cells was related to the increased stability of its mRNA. The authors found that the UV-B treatment induced both the abundance of the protein HuR and its localization in the cytosolic compartment of HaCaT cells, where it performed its stabilizing mRNA action. We observed that the basal levels of HuR were increased in nr-HaCaT cells as compared to those of the parental cell line, suggesting that the augmented basal expression of COX-2 in these cells was related to the basal high expression of its mRNA stabilizer HuR. In agreement, the NCTC 2544 cells, which had basal levels of COX-2 similar to those found in nr-HaCaT cells and much higher than the parental HaCaT cells, showed also HuR basal levels that were higher than those of parental HaCaT cells and, conversely, comparable to those found in nr-HaCaT cells.

The UV irradiation caused a further increase of HuR levels in these cells, which was observed very early (6 h) after the irradiation. Remarkably, DHA was able to revert both the UVR-induced increase of HuR and to impede the preferential cytosolic location of HuR induced by UVR in these cells.

To substantiate the hypothesis that the capacity of DHA to reduce HuR expression and consequently decrease the COX-2 levels was strictly related to its ability to overcome the resistance to UVR-induced apoptosis, we used the transfection with the specific siRNA to inhibit the expression of HuR. We observed that, in the transfected cells, the basal COX-2 level decreased as compared to the nontransfected cells, and that the UVR treatment caused an even lower decrease of COX-2. These modifications are perfectly comparable to those observed in these cells after the DHA treatment. In agreement to the results obtained with DHA, the silencing of HuR also allowed the nr-HaCaT cells to change their apoptotic response to UVR and become more susceptible to apoptosis, thus reverting the resistance to UVR-induced apoptosis observed in basal conditions.

Overall, these results show that a treatment with DHA may prevent the apoptosis resistance to UVR that may arise in skin keratinocytes and may represent a step towards carcinogenesis. Moreover, they suggest that this effect may be related to the inhibitory action of DHA on the expression of COX-2 and of its mRNA stabilizer HuR. Further studies are in progress to have a more complete view of the potential efficacy of DHA against the resistance to UVR-induced apoptosis, by evaluating the incorporation of DHA into the immortalized keratinocytes, by comparing the fatty acid profiles of the immortalized cultured cells in vitro and the keratinocytes directly derived from the human skin as well as by

analyzing the tumorigenic potential of the different keratinocyte lines implanted in athymic mice in vivo.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.08.004.

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