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Regulation of the redox-sensitive transcription factors NF-kB and AP-1 by ultraviolet light-B in human melanocytes

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Abstract. *Background.* Dietary micronutrients such as α-tocopherol, ascorbic acid, and β-carotene are important secondary antioxidants that protect cells against damage from reactive oxygen species (ROS), including singlet oxygen and free radical molecules. The skin is an organ rich in dietary antioxidants that exists in an oxygen-rich environment and is subject to the constant generation of ROS by ultraviolet light (UVL) exposure. A variety of mechanisms have been proposed by which antioxidants protect against ROS, and the recent recognition of ROS as cellular messengers operating through redox-sensitive transcription factors provides a common pathway by which their actions may be modulated.

Methods. The content of RNA transcripts in normal and β-carotene (BC) treated human neonatal melanocytes (NHM) was measured before and after UVL-B irradiation using Northern blot and gel shift analysis.

Results. UVL-B markedly stimulated c-fos and c-jun expression, but AP-1 binding was largely unaffected. BC decreased c-jun expression at early time points. In contrast, UVL-B had little effect on DNA-binding in control cells, but in BC-treated cells, NF-kB binding was markedly decreased. This process seemed to be PKA but not PKC dependent.

Discussion. Redox transcriptional factors respond to UVL-B irradiation in NHM and their response can be modified by BC.

Key words: melanocytes, NF-kB, redox, ultraviolet light.

Introduction

For the past decade our research has involved the human melanocyte, a neuroectodermally derived cell resident on the basement membrane at the epidermal-dermal junction of the skin. The melanocyte is responsible for the major expression of cutaneous pigmentation and interacts prominently with UVL both directly and indirectly in the generation of melanin pigment via a complex biochemical pathway of which the enzyme tyrosinase is a major regulatory component and during which H_2O_2 is generated at some steps [1,2]. The end-product, melanin, is responsive to UVL by the generation of semiquinone radicals that interact with ground-state oxygen to produce ROS [3]. We have studied certain aspects of signal transduction in depth in the human melanocyte system and have identified abnormalities in gene expression acquired during the process of carcinogenesis to melanoma cells; most prominently,

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defects in the expression of protein kinase C-β_{II} and retinoic acid receptor-β as well as dysregulation of the AP-1 transcription regulatory pathway occur [4–6]. Our attention in the past 5 years has turned to understanding the mechanistic role of UVL-B in melanocyte transformation and a considerable amount of effort has been devoted to setting up an appropriate in vitro system (review [3]).

Classically, ROS have been viewed as compounds that damage the cell in a multiplicity of ways. The recognition of ROS as cellular messengers [7] and the identification of redox-sensitive transcription factors in bacterial and eukaryotic cells [8] provides a common pathway by which to attempt to understand the effect of dietary antioxidants in cellular systems. In eukaryotes the transcription factors AP-1 and NF-kB have prominent roles in responding to oxidant stress [9,10]. AP-1 is a transcription factor most commonly comprised of heterodimers from the c-jun and c-fos families which binds to recognition sites on genes and activates their expression [11]. We have studied their role in melanocyte carcinogenesis and found an increase in c-fos RNA and a decrease in c-jun RNA expression in melanoma cells compared to normal melanocytes [4]. NF-kB responds to a wide range of insults including drugs and ionizing and nonionizing radiation [9,12]. The transcription factor NF-kB resides in the cytoplasm bound to an inhibitory unit IkB, which, when phosphorylated, is released from NF-kB and promptly degraded. The released and activated NF-kB subunit then moves rapidly to the nucleus, recognizes various transcription sites on DNA, and affects the expression of a wide variety of genes.

Dietary antioxidants play an important role in controlling cellular and tissue damage from free radicals and are one of the three major arms that comprise the antioxidant system. Secondary antioxidants that trap free radicals or interrupt oxidizing processes (and thereby prevent damaging chain reactions in biomolecules) include major dietary substances such as α-tocopherol (vitamin E), ascorbic acid (vitamin C), and β-carotene (BC).

Our preliminary results suggest that NF-kB may be particularly important as a redox-sensitive molecule in melanocytes and sensitive to BC, and we therefore have made this transcription factor a major focus. A schematic diagram of the system under study is shown in Fig. 1 and provides a useful pictorial and conceptual overview of the redox/transcriptional factor system overall.

Materials and Methods

Cell culture

Human neonatal foreskins were placed in 0.25% trypsin at 4°C overnight. Following this incubation, the tissue was scraped to recover the melanocytes. The melanocytes were cultured in MCDB 153 (Sigma) medium containing 2% fetal calf serum, 0.3% bovine pituitary extract (Clonetics Corp.), 10 ng/ml TPA, 2 mM $CaCl_2$, 5 µg/ml insulin and 0.1 mM IBMX (Sigma).

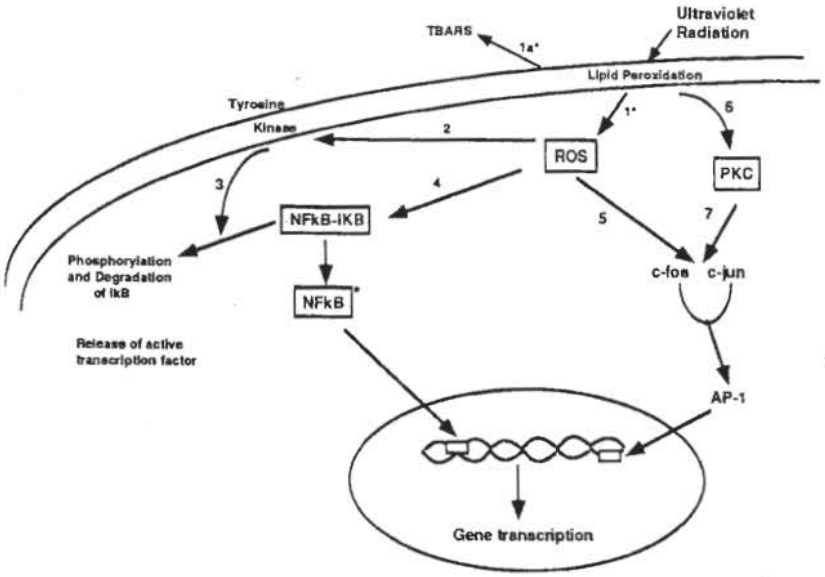

Fig. 1. Broad conceptual framework of redox transcriptional factor regulation in human melanocytes. UVL generates ROS which regulates NF-kB and AP-1. Up- and down-regulation can be modulated by dietary antioxidants at various points (1–7).

Micronutrients

The BC and soybean oil emulsion were provided by Lance Schlipalius of Betatene Limited (Australia). The BC was isolated from the alga *Dunaliella salina* in which it represents 85–90% of the total carotenoids, with half of the balance consisting of oxycarotenoids (lutein and zeaxanthin) and the remaining half of BC as characterized by HPLC. The BC was emulsified in a soybean oil carrier. The soybean oil emulsion was used at a final concentration of 0.05%. A fresh vial of BC was used for each experiment and protected from direct light throughout all procedures. D-α-tocopherol acid succinate and L-ascorbic acid were from Sigma.

Thymidine incorporation/cell viability

DNA synthesis was measured by labeling with (methyl-^{3}H)-thymidine (2.5 mCi/ml, 20 Ci/mmol, NEN) added to the medium during the last 4 h of incubation. Cells were harvested using a Ph.D. cell harvestor (Cambridge Research Inc.). Amount of radioactivity incorporated was determined by liquid scintillation counting with an efficiency of 68% (Beckman). Cells were harvested with 0.25% trypsin, counted by hemacytometer and viability determined by trypan blue (0.4%) exclusion.

UVL irradiation

UVL-B irradiation of the melanocytes was performed with a Stratalinker Crosslinker (Stratagene) equipped with five UVP bulbs which exhibit an emission maximum centered at 302 nm as measured by a UVX-31 radiometer (UPV Inc.). The spectrum distribution has been characterized. Wavelengths shorter than 280 nm were cut off by keeping plastic lids on tissue culture dishes.

RNA isolation, Northern analysis and cDNA probes

Total RNA was isolated by detergent lysis followed by phenol-chloroform extraction and ethanol precipitation. Ten micrograms of RNA was size-fractionated on denaturing formaldehyde agarose gels and transferred to nylon filters by capillary blotting. Blots were exposed to ^{32}P-labelled cDNA probes and hybridized at 42°C for 24 h in 50% formamide, 2X SSC, 5X Denhardts, 0.1% SDS, 10% dextran sulfate and 100 µg/ml salmon sperm DNA. After hybridization, filters were washed to a stringency of 0.5–0.1X SSC at 60°C and autoradiographed at –80°C for 3 to 10 days using Fuji film. Autoradiographs were quantitated by densitometry using the Molecular Analyst software (Bio-Rad). The c-jun probe was a 1.8 kb Xho I/EcoRI fragment from the plasmid BK 28. The 18S probe was isolated by an EcoRI digest and isolation of the 5.6 kb fragment from the plasmid pB. The NF-kB1 RNA expression levels were detected using a 3.8 kb EcoRI insert from the plasmid clone 9 (ATCC, Rockville, MD, USA).

Nuclear protein extracts

Cells were grown to approximately 50–60% confluency and treated as described in the experimental protocol. Briefly, cells were allowed to swell in hypotonic buffer (10 mM HEPES, pH 7.8, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT and 1 mM PMSF (Sigma)) for 15 min on ice. Cytosolic protein fraction was obtained after the addition of Nonidet P-40 (NP-40) (USB) to a final concentration of 0.5% and separation from the nuclei by centrifugation (500 × *g*, 5 min). Nuclear proteins were extracted from the remaining pellet by dialysis with hypertonic buffer (20 mM HEPES, pH 7.9, 350 mM NaCl, 1 mM DTT, 1 mM PMSF) for 2 h at 4°C. Isolated nuclear protein was frozen and stored at –80°C. Quantitation was calculated from optical density readings of 230, 260 and 320 wavelengths.

Gel mobility shift assays

Five microgram samples of each extract were incubated with 10,000 cpm of a ^{32}P-labeled consensus oligonucleotide of NF-kB (Promega). Following a 1-h incubation, samples were electrophoresed in a low ionic strength polyacrylamide gel. Quantitation of protein:DNA complexes was accomplished by densitometry using the Molecular Analyst software (Bio-Rad).

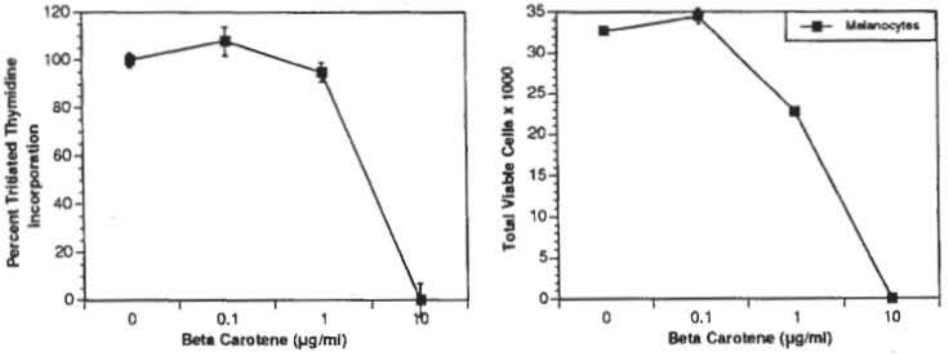

Fig. 2. Effect of BC (72-h incubation) on DNA synthesis and total viable melanocytes.

Results

Effect of BC (72-h incubation) on DNA synthesis and total viable cells in NHM (Fig. 2).

No effect on either parameter was noted at lower doses (<0.1 µg/ml). There was little effect on DNA synthesis even at higher doses (1 µg/ml), although the total viable cell count seems to be affected slightly. Based on these results, a dose of BC of 1 µg/ml was chosen for subsequent experiments.

Effect of BC on c-fos and c-jun expression in UVL-B perturbed NHM (Fig. 3).

Melanocytes were treated for 24 h with or without BC (1 µg/ml) with the appropriate

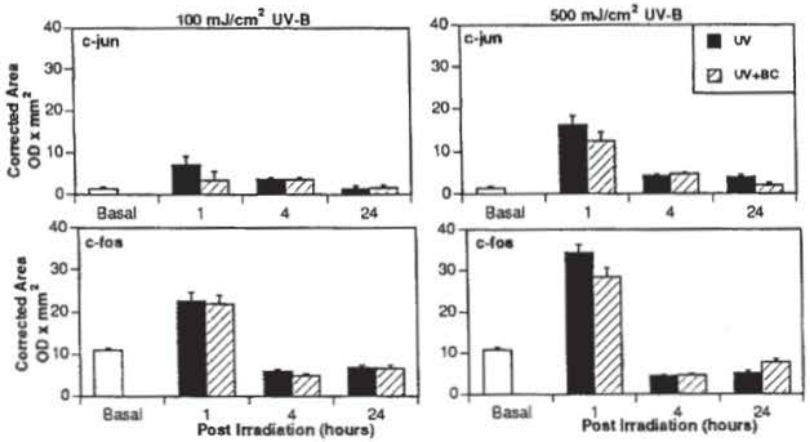

Fig. 3. Effect of BC on c-fos and c-jun expression in UVL-B perturbed melanocytes. Melanocytes were treated for 24 h with or without BC (1 µg/ml) with the appropriate controls. Media were then removed and cells were exposed to 100 or 500 mJ/cm² of UV-B. Fresh media were then added and cells were incubated for an additional 1, 4 and 24 h and total RNA isolated. This histogram is representative of four independent Northerns (corrected for loading) from four normal Caucasian melanocyte lines.

controls. Media were removed and cells were exposed to 100 or 500 mJ/cm^2 of UVL-B. Fresh media were added and cells were incubated for an additional 1, 4, or 24 h and total RNA isolated. The histogram is representative of four independent Northerns (corrected for loading) from four Caucasian NHM lines. Both c-jun and c-fos were induced at the 1-h time point, and c-jun induction was significantly inhibited by pretreatment with BC. No effect of BC was evident at later time points.

DNA binding activity of AP-1 in NHM following BC and UVL-B treatment (Fig. 4).

Gel mobility shift assay using an AP-1 consensus sequence was performed to determine DNA binding activity of nuclear extracts from melanocytes. NHM were pretreated with 1.0 μg/ml of BC for 24 h followed by 100 or 500 mJ/cm^2 of UVL-B, and assayed at 1, 4, and 24 h following irradiation. Histograms and autoradiograph are representative of four separate experiments with standard error shown. No change in AP-1 binding at any time point was noted after a dose of 100 mJ/cm^2, and the BC had no effect. At 500 mJ/cm^2 AP-1 binding was induced, but BC had no further effect.

DNA binding activity of NF-kB in NHM following BC and UVL-B treatment (Fig. 5).

Gel mobility shift assay using an NF-kB consensus sequence was performed to determine DNA binding activity of nuclear extracts from melanocytes. NHM were pretreated with 1.0 μg/ml of BC for 24 h followed by 100 or 500 mJ/cm^2 of UVL-B and assayed at 1, 4, and 24 h following irradiation. Histograms and autoradiograph representative of four separate experiments with standard error are shown. At both the 100 and 500 mJ/cm^2 dose, no increase in DNA binding of NF-kB occurred in the

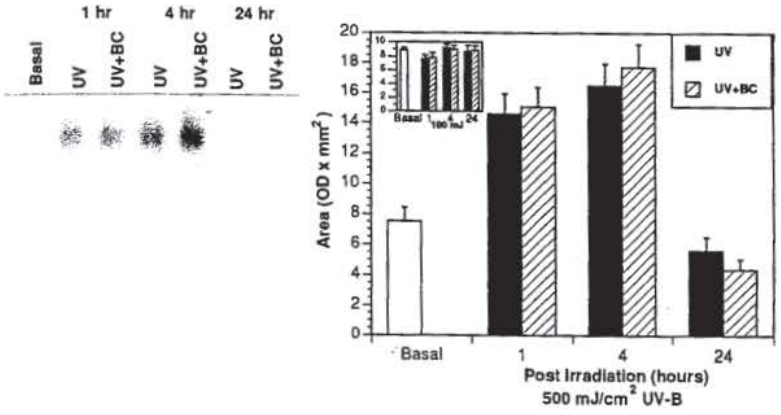

Fig. 4. DNA-binding activity of AP-1 in melanocytes following BC and UVL-B treatment. Gel mobility shift assay using an AP-1 consensus sequence was performed to determine DNA binding activity of nuclear extracts from melanocytes. Cells were pretreated with 1.0 μg/ml of BC for 24 h followed by 100 or 500 mJ/cm^2 of UV-B and assayed at 1, 4 and 24 h following irradiation. Histograms and autoradiograph are representative of four separate experiments with standard error shown.

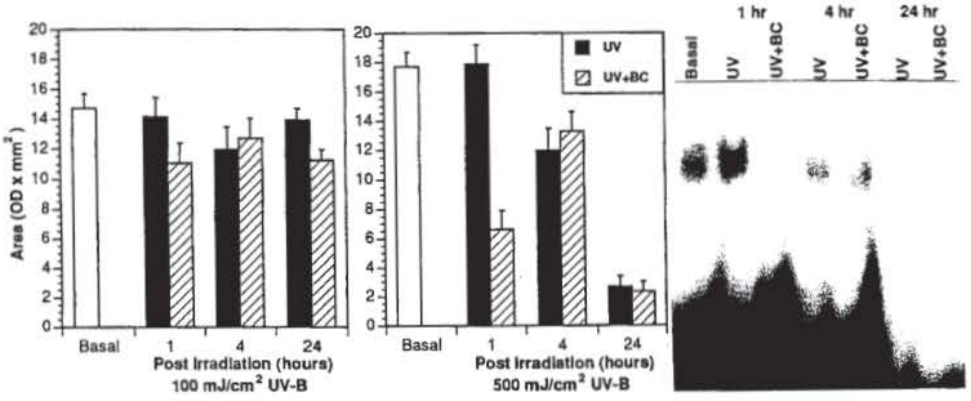

Fig. 5. DNA-binding activity of NF-kB in melanocytes following BC and UVL-B treatment. Gel mobility shift assay using an NF-kB consensus sequence was performed to determine DNA binding activity of nuclear extracts from melanocytes. Cells were pretreated with 1.0 μg/ml of BC for 24 h followed by 100 or 500 mJ/cm^2 of UV-B and assayed at 1, 4 and 24 h following irradiation. Histograms and autoradiograph are representative of four separate experiments with standard error shown.

melanocytes that were not pretreated with BC. However, in melanocytes which were pretreated with BC, NF-kB binding was decreased after UVL-B treatment in a dose response fashion. This response is consistent with BC acting as an antioxidant with increased effect under the greater oxidant stress (i.e., 500 mJ/cm^2). Additional studies (data not shown) have shown that NF-kB binding is increased at earlier time points (before 1 h) after UVL.

Effect of TPA and IBMX withdrawal on AP-1 DNA binding (Fig. 6)

Gel mobility shift assay using an AP-1 consensus sequence was performed to determine DNA binding activity of nuclear extracts from two NHM lines. NHM were pretreated with or without TPA (10 ng/ml) and IBMX (0.1 nM) for 24 h followed by 100 mJ/cm^2 of UVL-B. Fresh media were added with or without TPA or IBMX and cells were incubated for 1 h following irradiation.

DNA binding of AP-1 was decreased 60% when TPA was removed from the medium and the response was similar in control and UVL-irradiated cells. In contrast, AP-1 binding was little affected by IBMX withdrawal in control cells, but was markedly decreased in UVL-irradiated cells.

Effect of TPA and IBMX withdrawal on NF-kB DNA binding (Fig. 7)

Gel mobility shift assay using an NF-kB consensus sequence was performed to determine DNA binding activity of nuclear extracts. NHM were pretreated with or without TPA (10 ng/ml) and IBMX (0.1 mM) for 24 h, then irradiated in PBS (100 mJ/cm^2 UVL-B). Following irradiation, fresh media were added with or without TPA

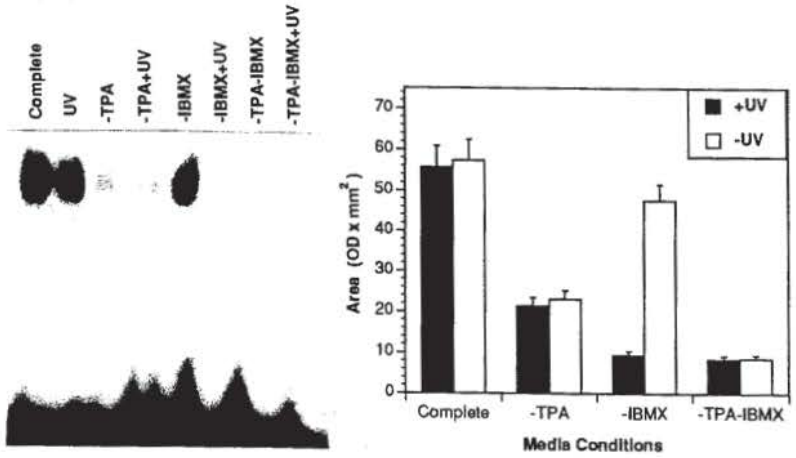

Fig. 6. DNA-binding activity of AP-1 in unperturbed and UVL-perturbed melanocytes after TPA or IBMX withdrawal. Gel mobility shift assay using an AP-1 consensus sequence was performed to determine DNA binding activity of nuclear extracts from two normal Caucasian melanocyte (NCM) lines. NCM were pretreated with or without TPA (10 ng/ml) and IBMX (0.1 mM) for 24 h followed by 100 mJ/cm^2 of UV-B. Fresh media were then added with or without TPA or IBMX and cells were incubated for 1 h following irradiation.

or IBMX and cells were incubated for 1 h. Nuclear extracts were then isolated and analyzed.

DNA binding of NF-kB was decreased nearly 90% in control and UVL-irradiated

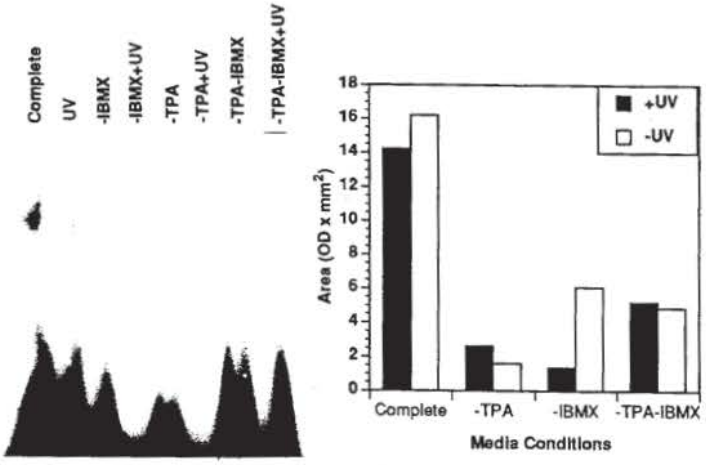

Fig. 7. DNA-binding activity of NF-kB in unperturbed and UVL-perturbed melanocytes after TPA or IBMX withdrawal. Gel mobility shift assay using an NF-kB consensus sequence was performed to determine DNA binding activity of nuclear extracts. NHM were pretreated with or without TPA (10 ng/ml) and IBMX (0.1 mM) for 24 h then irradiated in PBS (100 mJ/cm^2 UV-B). Following irradiation, fresh media was added with or without TPA or IBMX and cells were incubated for 1 h. Nuclear extracts were then isolated and analyzed.

Table 1. Summary of effects of β-carotene (BC) on UVL-B-induced early response gene expression in melanocytes[a].

	No UVL	100 mJ/cm^2		500 mJ/cm^2	
		Control	BC	Control	BC
c-jun	× 1	× 4	× 2	× 15	× 10
c-fos	× 1	× 2	× 2	× 3.5	× 3.0
AP-1 binding	× 1	× 1	× 1	× 2	× 2
AP-1 binding, no TPA	0.37	0.37 (1.0)[b]			
AP-1 binding, no IBMX	0.85	0.16 (0.17)			
NF-kB binding	× 1	× 1.0	× 0.8	× 1.0	× 0.3
NF-kB binding, no TPA	0.10	0.17 (1.7)			
NF-kB binding, no IBMX	0.37	0.08 (0.21)			

[a]Fold-change as measured by laser densitometry of Northern blot (c-jun, c-fos) or gel shift blot (AP-1 binding, NF-kB binding).
[b]Represents ratio of UV irradiated/true control

cells when IBMX was withdrawn. In cells in which TPA was withdrawn, NF-kB binding decreased 35% in control cells and was further decreased in UVL-irradiated cells (10% of control).

Discussion

The overall data of the current experiments are summarized in Table 1. UVL-B affected the expression of c-fos and c-jun, causing an increase in transcriptional activity early on that was dose dependent. This was reflected in an increased binding of AP-1, particularly at the higher UVL doses.

BC had little effect on c-fos or c-jun levels or AP-1 or NF-kB binding in the control unirradiated melanocytes. In contrast, NF-kB binding was unaffected by BC, but markedly decreased after UVL (20% decrease, 100 mJ/cm^2; 70% decrease, 500 mJ/cm^2).

The requirement for PKC and PKA activation for melanocyte growth has been evident for some time. The differential effect of UVL-B on DNA binding in IBMX-starved cells is striking and suggests that a PKA-activated process is required for UVL effect. The effect of BC on this parameter in this setting has not been answered.

These preliminary experiments suggest that redox-regulated transcriptional factors play a role in UVL perturbation of cellular processes. Additionally, BC seems to affect NF-kB and this process may be selectively modulated by the PKA pathway. No selective role for PKC mediation was evident. To further clarify these issues will require: 1) use of lower UVL doses, both single and cumulative; and 2) determination of BC effects in IBMX-depleted media.

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