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ORIGINAL RESEARCH COMMUNICATION

The Involvement of Smac/DIABLO, p53, NF-kB, and MAPK Pathways in Apoptosis of Keratinocytes from Perilesional Vitiligo Skin: Protective Effects of Curcumin and Capsaicin

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Abstract

Oxidative stress has been suggested as the initial pathogenetic event in melanocyte degeneration in vitiligo. Our previous results indicate that keratinocytes from perilesional skin show the features of damaged cells. In the present study, biopsies were taken from the perilesional skin of 12 patients suffering from nonsegmental vitiligo. The intracellular pathways involved in keratinocyte damage and apoptosis and the antioxidant protection of curcumin and capsaicin in these cells were investigated. In keratinocytes from perilesional vitiligo skin, we observed high levels of activated p38, NF-kB p65 subunit, p53, and Smac/DIABLO proteins. In contrast, low levels of ERK phosphorylation were present. To investigate the relationship between these pathways, we used specific inhibitors and evaluated the alteration of each pathway. For the first time, our study demonstrates the pivotal role of p38 MAP kinase as an upstream signal of perilesional keratinocyte damage, and the important contribution of p38 and NF-kB on p53 accumulation. Curcumin and capsaicin also increase ERK phosphorylation, thus inhibiting apoptosis. Moreover, pretreatment with such natural antioxidants inhibited caspase activation, increased total antioxidant capacity, repressed intracellular ROS generation and lipid peroxidation, and improved mitochondrial activity. These results suggest that antioxidants might represent an alternative approach to protect against vitiligo progression. *Antioxid. Redox Signal.* 13, 1309–1321.

Introduction

Vitiligo is a depigmenting disorder characterized by the development of white patches with a varying distribution in the body. The appearance of skin lesions is due to the loss of functional melanocytes from the epidermis. Different forms of vitiligo have been described, according to the distribution and the extent of the achromic lesions: in segmental and focal vitiligo, the disease maintains a local character, whereas generalized vitiligo typically shows a symmetrical distribution of multiple scattered lesions. The cellular mechanisms and biochemical changes that are responsible for the development of achromic lesions are still uncertain. Several hypotheses have been proposed to explain the pathogenesis of vitiligo: one of these assigns a primary role to oxidative stress in the development of skin damage (24). Whatever hypothesis is considered, however, the pivotal role of the melanocyte in the pathogenesis of the disease is still under debate. Recent experimental data underline the complex interactions that exist between melanocytes and other typical skin cells, keratinocytes in particular (28). Many studies have shown that patients display varying extents of cellular vacuolation and cellular debris in epidermal keratinocytes (2, 26). It was shown that, although the expression of catalase mRNA did not change, levels of epidermal catalase from the same patients were lower in lesional and nonlesional skin.

Recent *in vivo* and *in vitro* evidence suggest that the entire epidermis of the affected individual is implicated in vitiligo, particularly keratinocytes and Langerhans cells (39). Indeed, the cellular vacuolation reported by many investigators has been attributed to the build-up of m*M* concentrations of H_2O_2, resulting in H_2O_2-mediated lipid peroxidation (46). Furthermore, it has been shown that, unless cells are treated with antioxidant enzymes (such as catalase), melanocytes and keratinocytes cultured *in vitro* are also susceptible to such oxidative stress (25). Evidence also suggests that H_2O_2 produced in the epidermis is partially transferred to the

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vascular system, where it affects the enzymatic activities and peptides in peripheral blood lymphocytes and red blood cells (19, 41).

It was shown that, although its mRNA expression remains unaltered (42, 43), levels of epidermal catalase are low in vitiligo. Besides catalase, it was found that several other important antioxidant enzymes and biomolecules are also affected (39). The presence of DNA damage in peripheral blood lymphocytes, as well as changes to mitochondria in blood mononuclear cells (8), strongly suggests that systemic oxidative stress may also be implicated. Oxidative stress is defined as the imbalance occurring between the production of free radicals and the ability of the cell to defend itself from oxidative damage, based on the activation of a set of antioxidants and detoxifying enzymes, including superoxide dismutase, catalase, and glutathione peroxidase (18). When this imbalance occurs, oxidatively modified molecules (lipids, proteins, nucleotides) accumulate in the cellular milieu, causing dysfunction and eventually cell death. Recent findings have shown that epidermal and blood samples taken from vitiligo patients display high and low levels of H_2O_2 and catalase, respectively (38) and that antioxidant systems play an important role in the pathogenesis of generalized vitiligo (44). Studies recently carried out in our laboratory suggest that relevant morphological alterations of mitochondria and significant biochemical alterations, such as an increased production of reactive oxygen species (ROS), lipoperoxidation, mitochondrial alterations, and caspase-3 activation, are primarily observed in keratinocytes from perilesional skin rather than in lesional or healthy skin (35). This has led us to suggest that vitiligo perilesional skin represents the substrate where melanocyte death is initiated and that keratinocytes play a central role in vitiligo development. The role of apoptosis in vitiligo remains a controversial topic, however.

Keratinocytes from perilesional vitiligo skin showed the presence of oxidative stress and apoptosis. To investigate the pathways involved in cellular damage, we examined p38 and ERK activation, the NF-kB nuclear translocation, and Smac/DIABLO accumulation.

We decided to further investigate the role played by the p53 tumour suppressor protein, whose constitutive expression is vital for counteracting genotoxic stress (21). Recent results show that *in situ* and *in vitro* levels of epidermal and cytosolic p53 were higher and that levels of 8-oxoguanine are increased in the skin and plasma, indicating significant DNA damage (38).

Despite this mounting evidence of the importance of oxidative stress in the pathogenesis of vitiligo, in the literature there are no data describing antioxidant protection in keratinocytes from perilesional skin patients affected by nonsegmental vitiligo and its possible involvement in the progression of the disease. Here we investigate a possible protective role played by antioxidant species, focusing our attention on natural molecules whose antioxidant properties are well established. Curcumin is a major active component of the food flavor turmeric, which is extracted from the powdered dry rhizome of *Curcuma longa Linn* (Zingiberacee), a perennial herb widely cultivated in tropical regions of Asia. Several studies in recent years have shown that, even at relatively low concentrations, curcumin seems to possess antioxidant, antiproliferative, and anti-inflammatory properties. In addition, curcumin has been found to inhibit NF-kB activation, and studies in animal models have shown that this effect is associated with protection against various stress conditions (13, 36). Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the main ingredient of hot chili pepper, has long been used in spices, food additives, and drugs. It displays anti-inflammatory and analgesic properties, besides to antimutagenic, anticarcinogenic, and antioxidant activities, and is currently used as a topical cream for arthritis pain relief (7). However, the mechanisms underlying its protective effects have not been fully understood.

Materials and Methods

Keratinocyte isolation and cell culture

Punch-biopsies of 6 mm were taken from lesional, perilesional, and healthy skin of 12 patients affected by nonsegmental vitiligo all of which had a similar clinical history with respect to lesion extension and duration of the disease. Lesional, perilesional, and healthy skin is defined as clinically affected skin, skin along the edge of the white patch and clinically unaffected, normally pigmented skin, respectively. Only patients with stable vitiligo were included in this study. None of the patients underwent any kind of treatment (neither systemic nor topical) 6 months before biopsies were obtained. None of the patients we selected had any autoimmune disease in addition to vitiligo, nor did they have autoantibodies against any organs. The clinical information of each patient is summarized in Table 1. Written permission was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local institutional review board. Keratinocytes were isolated according to a previously published method (35). The expression of cytokeratins was evaluated at each passage *in vitro* by a positive staining to pan-cytokeratin-antibody (data not shown) in order to assess the maintenance of the same immunophenotype. At the first passage, the absence of vimentin expression induced us to exclude the presence of any fibroblasts.

Table 1. Clinical Data of Vitiligo Patients

Patients	*Age*	*Clinical type of vitiligo*	*Age of onset/ Stability of vitiligo (years)*[‡]	*Biopsy site*
M[*]1	43	NS[§] generalized	19/7	Trunk
M2	51	NS generalized	6/8.5	Trunk
M3	47	NS generalized	11/10	Abdomen
M4	39	NS generalized	5/8	Pubis
M5	52	NS generalized	25/7	Abdomen
M6	45	NS localized	21/10	Trunk
F[†]1	44	NS generalized	31/8	Abdomen
F2	38	NS generalized	20/7.5	Trunk
F3	49	NS generalized	13/9	Trunk
F4	42	NS generalized	33/8	Pubis
F5	50	NS generalized	27/7	Abdomen
F6	46	NS localized	18/9.5	Trunk

[*]M = Male.
[†]F = Female.
[‡]Vitiligo was considered to be stable as no new lesions or enlargement of old lesions occurred in the last three years.
[§]NS = Non-Segmental Vitiligo.

Cell treatment

Cell treatment was induced by adding curcumin or capsaicin at concentrations of 10 μM for 24 h. To check possible toxic effects of these compounds, untreated cells were also considered and maintained in complete culture medium for the same time. Curcumin, Capsaicin, Bay 11-7082 (NF-kB inhibitor), SB203580 (p38 kinase inhibitor), and PD98059 (MEK inhibitor) were purchased from Sigma. All other reagents were of the highest purity available.

Preparation of cell homogenates and isolation of nuclear fraction

Keratinocytes (1 x 10^6) were washed twice with phosphate buffer saline (PBS), trypsinized, centrifuged, and then resuspended in 100 μl of lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl pH8, 137 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, and 6 M urea supplemented with 0.2 mM PMSF, as well as 10 μg/ml leupeptin and aprotinin. To obtain cell homogenates, samples, after three freeze–thaw cycles, were twice sonicated in ice for 5 seconds and then centrifuged at 14,000 g for 10 min at 4°C. The supernatant was then collected. The protein concentration was determined according to the Bradford method (3). Nuclear fraction was achieved using a nuclear/cytosol fractionation kit according to the manufacturer's instructions (Oncogene Research Products, San Diego, CA).

Lactate dehydrogenase release measurement

Lactate dehydrogenase (LDH) activity, accounting for cell death, was assessed spectrophometrically in the culture medium and in adherent cells (in order to obtain total LDH content) using the LDH assay kit (Roche Diagnostics, Mannheim, Germany). LDH release was calculated as a percentage of total LDH content.

NF-kB, p53, p-ERK, p-p38, and Smac/DIABLO assessment by Western blot

To assess the protein levels of NF-kB, equal amounts of nuclear fraction (60 μg) were diluted in Laemmli's sample buffer with 6 M urea and boiled at 65°C for 5 min. Proteins were separated on 4%–12% SDS-PAGE precast gel (Criterion XT, Bio-Rad Laboratories, Hercules, CA) and were transferred to PVDF Hybond membrane (Millipore Corp., Billerica, MA). The membrane was then incubated overnight at 4°C with a highly specific (rabbit) anti-NF-kB (p65 subunit (C-20): sc-372) polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Equal protein amounts of total homogenate (40 μg for p53, 40 μg for ERK 1, phosphorylated ERK, and Smac/DIABLO, 60 μg for p38 and phosphorylated p38) were diluted in Laemmli sample buffer, boiled for 5 min and separated on 4%–12% SDS-PAGE precast gels (Criterion XT, Bio-Rad Laboratories). Proteins were transferred to PVDF Hybond membranes. The membranes were then incubated overnight at 4°C with (mouse) anti-p53, (rabbit) anti-ERK 1 antibody, (mouse) anti-p-ERK, (mouse) anti-p38, (mouse) anti-p-p38 (Santa Cruz Biotechnology Inc.), (rabbit) anti-Smac/DIABLO (ProSci Inc.). After washing, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. The immunolabeled bands were then detected using a Super-Signal West Dura (Pierce, Rockford, IL) and quantified using the above-mentioned software for image analysis. Results were expressed as ratios between the densitometry of the protein of interest and the densitometry of the loading control (β-actin for homogenate and histone H1 for nuclei).

Determination of intracellular ROS and mitochondrial superoxide

Cells were cultured on glass cover slips and loaded with the ROS-sensitive fluorescent probe H_2DCFDA (2.5 μM) and with MitoSOX (3 μM), dissolved in 0.1% DMSO and Pluronic acid F-127 (0.01% w/v), added to the cell culture media for 15 min at 37°C. The cells were fixed in 2.0% buffered paraformaldehyde for 10 min at room temperature and the DCF and MitoSOX fluorescence analyzed (at an excitation wavelength of 488 nm) using a confocal Leica TCS SP5 scanning microscope (Mannheim, Germany) equipped with an argon laser source for fluorescence measurements. ROS generation was also monitored by flow cytometry. Single-cell suspensions of cells were incubated with H_2DCFDA (1 μM) at 37°C for 15 min and immediately analyzed by flow cytometry.

Total antioxidant capacity

Intracellular total antioxidant capacity (TAC), accounting for ROS scavengers, was measured in cell lysates by a chemiluminescence assay using the photoprotein Pholasin (Abel Antioxidant Test Kit, Knight Scientific Limited, Plymouth, UK), following the manufacturer's instructions. Protein content in the soluble fraction was measured by the method of Bradford (3). The results were calculated using an L-ascorbic acid-based standard curve.

Evaluation of lipid peroxidation

To assess the rate of lipid peroxidation, isoprostane levels were measured in keratinocytes from lesional, perilesional, and healthy skin lysates using the 8-isoprostane EIA kit (Cayman Chemical Company, Ann Arbor, MI), following the manufacturer's instructions. Lipid peroxidation was also investigated in keratinocytes from perilesional skin by confocal scanning microscopy using BODIPY, a fluorescent probe that is intrinsically lipophilic and thus mimics the properties of natural lipids (10). BODIPY 581/591 C_{11} acts as a fluorescent lipid peroxidation reporter that shifts its fluorescence from red to green in the presence of oxidizing agents. Briefly, cells were cultured on glass coverslips and loaded with dye by adding the fluorescent probe BODIPY, dissolved in 0.1% DMSO (5 μM final concentration), to the cell culture media for 30 min at 37°C. The cells were fixed in 2.0% buffered paraformaldehyde for 10 min at room temperature and the BODIPY fluorescence analyzed (at an excitation wavelength of 581 nm) using a confocal Leica TCS SP5 scanning microscope (Mannheim, Germany) equipped with an argon laser source for fluorescence measurements. Moreover, lipid peroxidation was quantified by flow cytometry. Single-cell suspensions were washed twice with PBS and incubated, in the dark, for 30 min at 37°C with BODIPY 581/591 (2 μM) in DMEM. After labeling, cells were washed and resuspended in PBS and analyzed using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA).

Mitochondrial activity assay

Mitochondrial activity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

assay in a 96-well plate (9). After washing with PBS, 100 μl of 0.5 mg/ml MTT solution in PBS was added to the cell cultures and the samples were incubated for 4.0 h at 37°C. Finally, 100 μl of cell lysis buffer (20% SDS, 50% N,N-dimethyl formamide, pH 4.7) was added to each well and the samples were incubated for at least 3 h at 37°C in a humidified incubator, before absorbance values of blue formazan were determined at 590 nm using an ELISA plate reader. Cell viability was expressed as a percentage of MTT reduction.

Mitochondrial permeability transition pore opening

Mitochondrial permeability was measured by calcein fluorescence according to the method described by Petronilli *et al.* (34), albeit with minor modifications. Briefly, calcein-AM freely enters the cell and emits fluorescence upon de-esterification. Co-loading of cells with cobalt chloride quenches the fluorescence in the cell except in mitochondria. This is because cobalt cannot cross mitochondrial membranes (living cells). During induction of mitochondrial permeability transition pore opening (mPTP), cobalt can enter the mitochondria and is able to quench calcein fluorescence (apoptotic cells). Thus, decreased mitochondrial calcein fluorescence can be taken as a measure of the extent of mPTP induction. Briefly, single-cell suspensions were incubated with the fluorescent probes calcein-AM (3 μM) and cobalt chloride (1 mM) for 20 min at 37°C, washed twice with PBS and analyzed using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA).

Assessment of caspase activity FACS analysis

Caspase-3, 8, and 9 activity was quantified by flow cytometry. In brief, single cell suspensions were incubated with FAM-FLICA™ Caspases solution (Caspase FLICA kit FAM-DEVD-FMK) for 1 h at 37°C, washed twice with PBS and analyzed using a FACSCanto flow cytometer (Becton-Dickinson).

Mitochondrial membrane potential

Mitochondrial membrane potential was assessed using tetramethylrhodamine, methyl ester, perchlorate (TMRM). TMRM is a lipophilic potentiometric dye that partitions between the mitochondria and cytosol in proportion to mitochondrial membrane potential ($\Delta\psi$) by virtue of its positive charge. At low concentrations, the fluorescence intensity is a simple function of dye concentration, which is in turn a direct function of mitochondrial potential. Therefore, the accumulation of dye in mitochondria and the intensity of the signal is a direct function of mitochondrial potential. For confocal microscope analysis, cells were cultured on glass coverslips and loaded with dye by adding the fluorescent probe TMRM, dissolved in 0.1% DMSO (100 nM final concentration), to the cell culture media for 20 min at 37°C. The cells were fixed in 2.0% buffered paraformaldehyde for 10 min at room temperature and the TMRM fluorescence analyzed (at an excitation wavelength of 543 nm) using a confocal Leica TCS SP5 scanning microscope equipped with a helium–neon laser source for fluorescence measurements. Mitochondrial membrane potential was also quantified by flow cytometry. Single cell suspensions were washed twice with PBS and incubated, in the dark, for 20 min at 37°C with TMRM (100 nM) in DMEM. After labeling, cells were washed and resuspended in PBS and analyzed using a FACSCanto flow cytometer.

Statistical analysis

All data are expressed as mean ± SD. Comparisons between different groups were performed by Student's t-test. A p value of <0.05 was accepted as statistically significant.

Results

Dose-dependent cytotoxic effects of curcumin and capsaicin

As a preliminary test aimed at evaluating the possible cytotoxicity of antioxidants, dose-dependent measures of LDH release were performed (assessed as an index of cell death) in the presence of increasing concentrations of curcumin and capsaicin. Keratinocytes from perilesional skin were subjected to antioxidant concentrations ranging between 0.1 μM and 100 μM. Only concentrations of curcumin higher than 30 μM are toxic to cells. Capsaicin is found to be nontoxic over the entire range of concentrations tested (Fig. 1A).

Following this, we carried out the MTT test with the two compounds (Fig. 1B). Both curcumin and capsaicin, used at a concentration of 10 μM, induce an increase in mitochondrial activity by 50%. At higher concentrations, curcumin displays a strong inhibitory effect on mitochondrial activity.

Oxidative stress markers and antioxidant protection in keratinocytes from perilesional vitiligo skin

Keratinocytes from healthy, lesional, and perilesional skin were grown in the presence of 10 μM curcumin or 10 μM capsaicin for 24 h. Total antioxidant capacity and lipoperoxidation markers were measured in these cells. Compared to keratinocytes from healthy and lesional vitiligo skin, perilesional cells exhibit a significant increase in the concentration of 8-isoprostanes and a marked reduction in TAC (Fig. 2), which was assayed as an index of the antioxidant defences, confirming the results obtained in our previous investigation (35).

All cells treated with curcumin or capsaicin display a significantly higher TAC with respect to untreated cells, though the extent of this protective effect is maximal in perilesional keratinocytes (Fig. 2A). The antioxidants also cause a significant decrease in the concentration of 8-isoprostanes in keratinocytes from healthy and lesional vitiligo skin (Fig. 2B). ROS production was also investigated by confocal microscope analysis (Fig. 3A), using the fluorescent probe H_2DCFDA. Untreated perilesional cells produce intense green fluorescence, indicating the presence of ROS. Capsaicin- and curcumin-treated perilesional cells are characterized by less marked fluorescence, demonstrating a strong protective effect of these compounds against ROS. These results were confirmed by flow cytometry analysis, as shown in Fig. 3C, and data was also displayed as a histogram (Fig. 3E).

Lipoperoxidation was investigated by confocal microscope analysis using the lipophilic fluorescent probe BODIPY (Fig. 3B), which appears oxidized in untreated perilesional cells but not in antioxidant-treated cells, as confirmed by flow cytometry analysis (Fig. 3D, 3F).

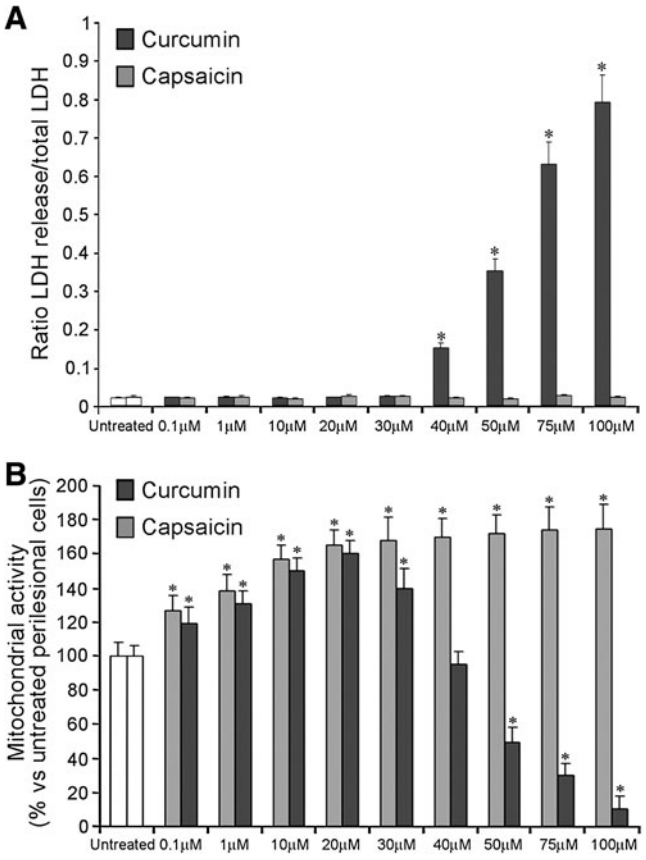

FIG. 1. Cytotoxic effect of antioxidants. (**A**) Dose-dependent measures of LDH release in the presence of increasing concentrations of curcumin and capsaicin. Concentration of curcumin higher than 30 μM are toxic to the cells. Capsaicin is found to be nontoxic over the entire range of concentrations probed. (**B**) Mitochondrial activity in antioxidant-treated keratinocytes from perilesional vitiligo skin, as evaluated using the MTT test. Values are expressed as % *vs* untreated keratinocytes from perilesional skin. The reported values (means $\pm$ s.d.) are representative of four independent experiments, each performed in triplicate. *Significant difference ($p \leq 0.05$) *vs*. untreated perilesional keratinocytes.

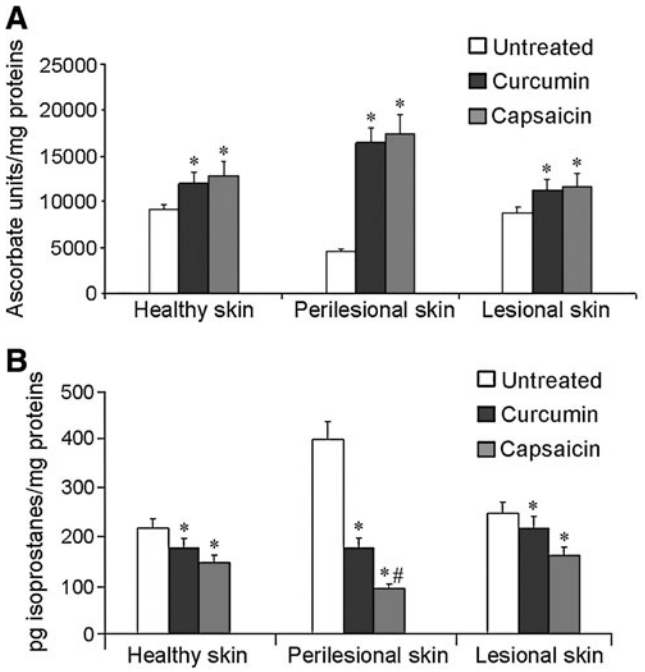

FIG. 2. Quantitative evaluation of total antioxidant capacity (TAC) (A) and 8-isoprostanes (B) in keratinocytes from healthy, perilesional, and lesional skin from vitiligo patients. The reported values (means $\pm$ s.d.) are representative of five independent experiments. *Significant difference ($p \leq 0.05$) *vs*. untreated perilesional keratinocytes. #Significant difference ($p \leq 0.05$) *vs*. curcumin-treated perilesional keratinocytes.

Curcumin and capsaicin protect keratinocytes from perilesional vitiligo skin from mitochondrial damage and apoptosis

In order to ascertain whether curcumin and capsaicin can protect against apoptotic cell death, we analyzed mitochondrial membrane polarization, the mitochondrial permeability transition pore opening and caspase activation. Figure 4A shows confocal microscope analysis of mitochondrial superoxide production, which appears strongly enhanced in untreated perilesional cells but not in antioxidant-treated cells. Mitochondrial membrane polarization, which was assessed by confocal microscopy and flow cytometry (Figs. 4B, 4C, and 4E), was found to be impaired in untreated perilesional keratinocytes. Curcumin effectively restored mitochondrial membrane polarization. Capsaicin treatment showed similar effects, though to a lesser extent. To further confirm this data, mitochondrial permeability transition pore opening was analyzed by flow cytometry (Fig. 4D): curcumin again proved to be more protective compared to capsaicin, as shown in Figure 3F.

Intracellular pathways involved in cellular damage

Our previous study showed the presence of oxidative stress and apoptosis in keratinocytes from perilesional vitiligo skin. To investigate the pathways involved in cellular damage, we examined p38 and ERK activation, the NF-kB nuclear translocation, Smac/DIABLO expression, and p53 accumulation.

Redox-responsive NF-kB is known to be extremely sensitive to various oxidants because essential signaling of NF-kB, NIK/IKK, and MAPKs are regulated by the redox status (31). Numerous stimuli activate NF-kB, including pro-inflammatory cytokines and signals that activate immune receptors, amongst others. In turn, NF-kB regulates the expression of many genes involved in responses ranging from inflammation and immunity, to cell growth, survival, and proliferation. Under normal circumstances, NF-kB activation occurs rapidly and transiently; dysregulated or constitutive NF-kB activity, however, has been functionally linked to the development of diseases, including those characterized by chronic inflammation, autoimmunity, and cancers (20).

In order to better understand the apoptotic pathways activated in our experimental conditions, we measured the expression and activation of Smac/DIABLO and MAP kinases, particularly p38 and ERK. Oxidants can trigger the activation of multiple signaling pathways, including mitogen-activated protein kinases (MAPKs). These MAPK proteins are

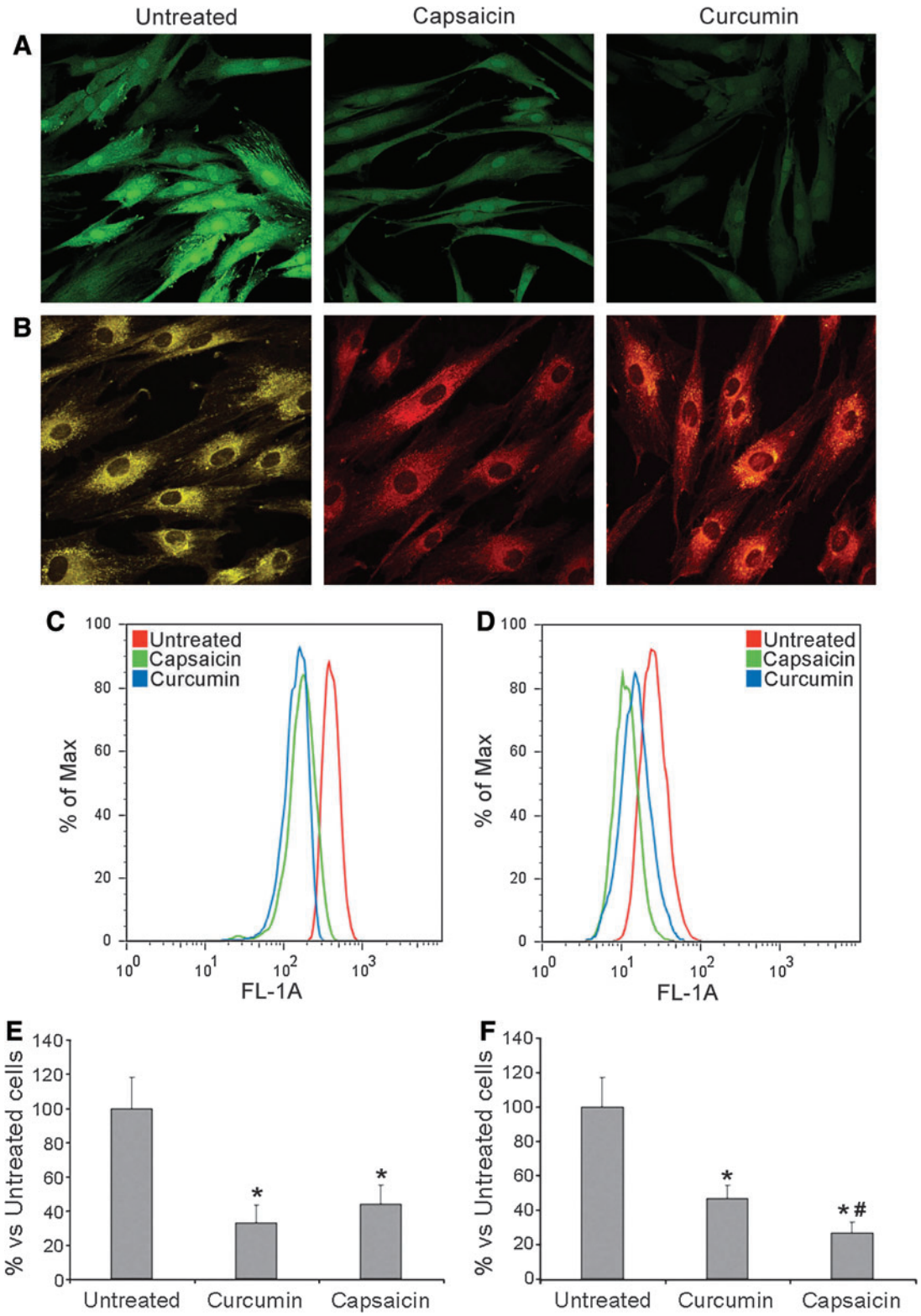

FIG. 3. Confocal microscopy and flow cytometry analysis of oxidative stress markers. (**A**) Confocal microscopy analysis of ROS production in keratinocytes from perilesional vitiligo skin. Curcumin and capsaicin suppress the emission of green fluorescence by H_2DCFDA, which is also evident from flow cytometry analysis (**C**). Lipid peroxidation is investigated in keratinocytes from perilesional skin by confocal scanning microscopy (**B**) and flow cytometry analysis (**D**) using the fluorescent probe BODIPY. Treatments with antioxidants inhibit lipid peroxidation. (**E**) Quantitative analysis of ROS production and (**F**) lipoperoxidation by flow cytometry. The reported values (means ± s.d.) are representative of five independent experiments. *Significant difference ($p \leq 0.05$) *vs.* untreated perilesional keratinocytes. #Significant difference ($p \leq 0.05$) *vs.* curcumin-treated perilesional keratinocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

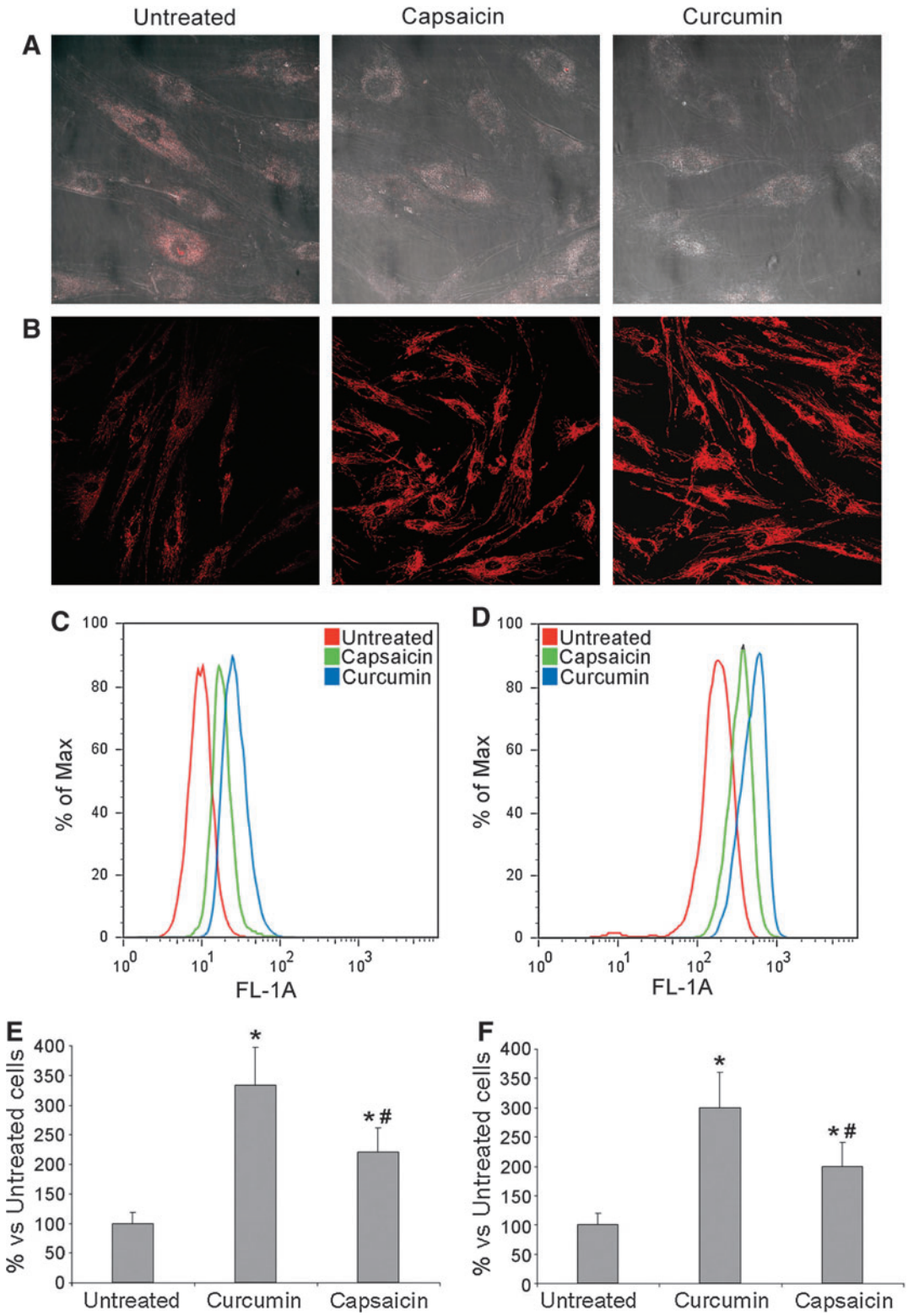

FIG. 4. Mitochondrial damage. Confocal analysis of mitochondrial superoxide (**A**) and mitochondrial membrane depolarization (**B**). Curcumin treatment inhibited mitochondrial superoxide production and restored mitochondrial membrane polarization. We also analyze mitochondrial permeability transition pore opening (**C**) and mitochondrial membrane polarization by flow cytometry (**D**): curcumin results to be more protective when compared to capsaicin. (**E**) Quantitative analysis of mitochondrial membrane polarization and (**F**) mitochondrial permeability transition pore opening by flow cytometry. The reported values (means $\pm$ s.d.) are representative of five independent experiments. *Significant difference ($p \leq 0.05$) *vs.* untreated perilesional keratinocytes. #Significant difference ($p \leq 0.05$) *vs.* curcumin-treated perilesional keratinocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

mediators of signal transduction from the cell surface to the nucleus and play a major role in triggering and coordinating gene responses. ERKs are predominantly activated by mitogenic signals, whereas JNK and p38 are primarily activated by environmental stress, such as UV radiation, inflammatory cytokines, heat shock, and DNA-damaging agents. Phosphorylation of JNK and p38 has a role in cellular differentiation, inflammatory responses and apoptosis (17). Another protein implicated in apoptosis is the second mitochondria-derived activator of caspase (Smac/DIABLO), a mammalian mitochondrial protein that is able to interact with and thereby antagonize inhibitor of apoptosis proteins (IAPs). It has been shown that Smac/DIABLO is encoded by a nuclear gene and is imported into mitochondria; upon induction of most cell death-related stimuli, Smac/DIABLO is released into the cytosol and triggers cell death by inhibiting the caspase-inhibiting actions of the IAPs (47). By binding to IAPs, Smac/DIABLO either displaces active caspases or prevents the binding of IAPs to active caspases, thus promoting cell death.

We also focused our attention on p53 accumulation. An overexpression of p53 has been shown in vitiligo (40). p53 functions as a transcription factor to regulate target genes involved in various processes including apoptosis, but this topic remain controversial in vitiligo.

In untreated keratinocytes from perilesional vitiligo skin, we observed high levels of activated p38, NF-kB p65 subunit, p53, and Smac/DIABLO proteins. In contrast low levels of ERK phosphorylation were found (Fig. 5).

To investigate the functional relationship between these different pathways, we used specific inhibitors and evaluated possible alterations of each pathway. The inhibition of p38 kinase with SB203580 caused an increase in ERK phosphorylation and a marked inhibition in NF-kB, p53, and Smac/DIABLO cellular pathways, indicating that p38 activity is necessary to increase NF-kB p65 subunit nuclear translocation, p53 and Smac/DIABLO accumulation. Inhibition of NF-kB with Bay 11-7082 abrogated p53 and Smac/DIABLO accumulation but did not affect p38 and ERK phosphorylation, indicating that NF-kB activity is necessary to increase p53 and Smac/DIABLO protein levels in response to oxidative stress. Moreover, we can hypothesize that NF-kB is a downstream event of p38 and ERK pathways and downstream of p53 and Smac/DIABLO. We next examined the role of ERK in perilesional keratinocytes cellular damage and apoptosis. Inhibition of ERK phosphorylation with MEK inhibitor PD98059 did not affect p38 phosphorylation and NF-kB activation, but decreased p53 accumulation, indicating that p53 is a downstream signal of MAPK pathway. Therefore the above results clearly indicate that ROS-induced p38 and ERK-1/2 kinases have opposing effects on p53 accumulation and that phosphorylation of p38 is necessary to induce NF-kB p65 nuclear translocation and p53 and Smac/DIABLO accumulation. Moreover, the presence of Smac/DIABLO accumulation confirms the presence of apoptosis in our cellular model.

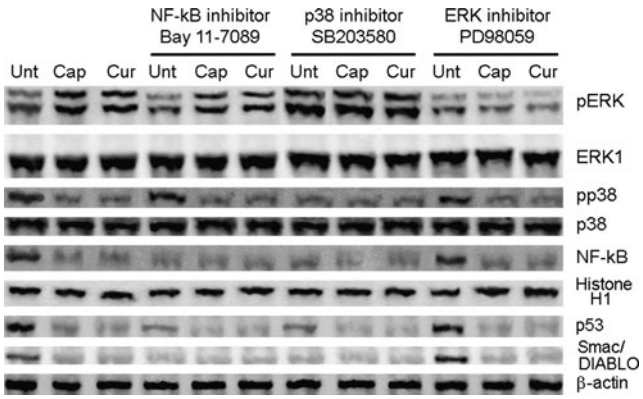

FIG. 5. Western blot analysis of NF-kB, p53, ERK, p38, and Smac/DIABLO in keratinocytes from perilesional vitiligo skin. Curcumin and capsaicin inhibit NF-kB nuclear translocation, p53 accumulation, p38, and Smac/DIABLO activation and promote ERK phosphorylation. To investigate the relationships among these pathways Bay 11-7082 (NF-kB inhibitor), SB203580 (p38 kinase inhibitor), PD98059 (MEK inhibitor) were used. Cap, capsaicin-treated perilesional keratinocytes, Cur, curcumin-treated perilesional keratinocytes, Unt, untreated perilesional keratinocytes.

Curcumin and capsaicin protect keratinocytes from perilesional vitiligo skin from cellular damage

To examine whether curcumin and capsaicin are involved in the NF-kB pathway, we investigated whether these compounds inhibited the activity of the NF-kB subunit p65 in keratinocytes from perilesional skin of vitiligo patients. As shown in Figure 5, the high levels of nuclear NF-kB subunit p65 significantly decreased after treatment with antioxidants, indicating that these compounds suppress the activity of NF-kB and the subsequent inflammatory response. In particular, 10 μM curcumin pretreatment induces a marked reduction in NF-kB levels.

Curcumin and capsaicin increase ERK phosphorylation, thus inhibiting apoptosis. In untreated keratinocytes from perilesional vitiligo skin, increased levels of activated p38 are evident, and treatment with curcumin and capsaicin reduce p38 phosphorylation, indicating strong inhibition of this stress-activated pathway. The same cells exhibit a marked activation of Smac/DIABLO, while curcumin and capsaicin treatments strongly inhibited this pathway (Fig. 6). To investigate which apoptotic pathways are activated, we quantified the activity of caspase-3, 8, and 9 by flow cytometry. Figure 6 shows marked activation of caspases-3 and 9 in keratinocytes from perilesional vitiligo skin and shows curcumin treatment to be the most effective in attenuating this effect. Caspase-8 is also activated in perilesional cells, but to a lesser extent than caspase-3 and caspase-9. In this case, capsaicin shows better protection than curcumin. In addition, curcumin and capsaicin prevent p53 accumulation.

Discussion

In this study, we shed light on the signaling pathways that contribute to cell damage in perilesional keratinocyte from vitiligo skin and the protective effects of curcumin and capsaicin treatments. In particular, we investigated cellular pathways that contribute to keratinocyte apoptosis and the NF-kB inflammatory pathway.

In 1963, Fitzpatrick and Breathnach found that melanin pigmentation of the skin was due to the interaction of epidermal melanocytes with keratinocytes that acquired the melanosomes secondarily and served in their transport (14).

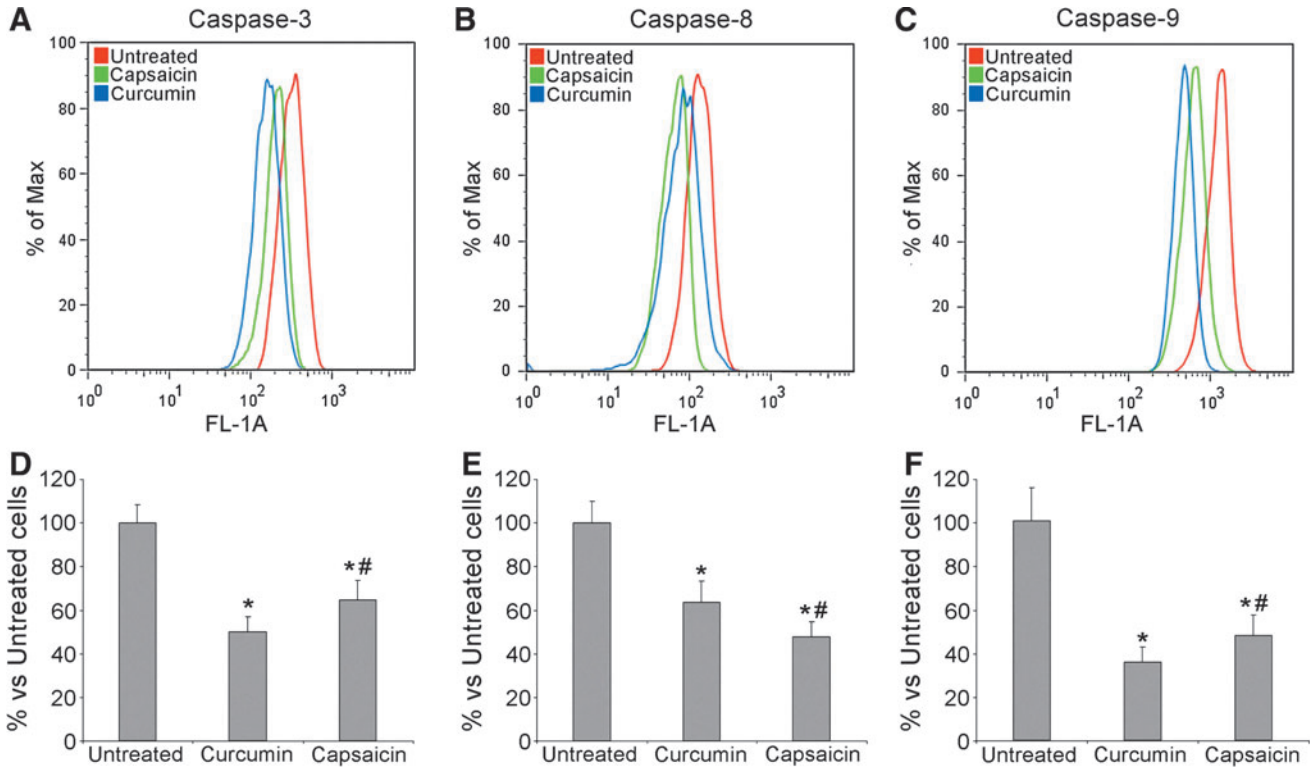

FIG. 6. Flow cytometry analysis of caspases-3, 8, and 9 activation in keratinocytes from perilesional vitiligo skin. Curcumin and capsaicin inhibit caspase-3 (**A**, **D**) and 9 activation (**C**, **F**), with curcumin treatment the most effective in attenuating this effect. Caspase-8 is also activated in perilesional cells (**B**, **E**), but to a lesser extent than caspase-3 and caspase-9. In this case, capsaicin shows better protection than curcumin. The reported values (means ± s.d.) are representative of five independent experiments. *Significant difference ($p \leq 0.05$) *vs.* untreated perilesional keratinocytes. #Significant difference ($p \leq 0.05$) *vs.* curcumin-treated perilesional keratinocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

The epidermal melanin unit, which consists of an epidermal melanocyte and the group of keratinocytes with which it maintains functional contact, was proposed to act as the fundamental integrated multicellular system for melanin pigmentation. Several hypotheses have been proposed for the mechanisms involved in the degeneration of vitiligo melanocytes, one of which is that keratinocytes and their products are necessary for the function of melanocytes in the epidermis (29).

Structural alterations in keratinocytes have been related to functional changes in vitiligo patients (22). Keratinocytes produce several factors that support the growth and melanization of nearby melanocytes, such as basic fibroblast growth factor (bFGF), endothelin-1 (ET-1), and stem cell factor (SCF) (30). Apoptotic keratinocytes may not produce SCF, subsequently impinging on melanocyte survival in vitiligo lesions. In a recent study, Moretti *et al.* (27) showed the presence of both TNF-α and IL-6 transcripts in perilesional biopsies, suggesting that melanocytes can be inhibited by these cytokines and that keratinocyte apoptosis is associated with vitiligo skin. The results of the present study confirm these findings and also indicate that perilesional keratinocytes exhibit biochemical alterations and clear signs of oxidative stress, even if obtained from subjects with stable vitiligo. An increase in ROS production due to mitochondrial dysfunction appears to be an important mechanism for the onset of oxidative stress in these cells where impaired mitochondrial activity, mitochondrial depolarization, and mitochondrial permeability transition pore opening were associated to a significant increase in the mitochondrial superoxide production. Perilesional keratinocytes also showed a considerable activation of caspase-3, 8, and 9, which represents a well known marker of apoptosis (35). Having said this, the molecular mechanisms that trigger ROS-induced apoptosis are still poorly understood.

A number of studies suggest that mitochondrial integrity is controlled by p53, a tumor suppressor protein whose activity regulates downstream activation of apoptosis; this topic have proved controversial in the field of vitiligo, however. In a recent study, Schallreuter's group demonstrated that the presence of overexpressed p53 throughout the epidermis is associated with low catalase expression, confirming the presence of oxidative stress and H_2O_2 accumulation. In contrast, their results showed there is no apoptosis in vitiligo (38). As yet, the mechanism by which p53 regulates apoptosis remains to be elucidated.

The build-up of p53 is implicated in a number of cellular responses. The mechanism that is primarily responsible for p53 build-up is phosphorylation since, in its phosphorylated state, p53 is not targeted for ubiquitination or degradation, thereby prolonging its half-life (15). p53 can be directly or indirectly phosphorylated by several members of the MAP kinase family, including c-Jun N-terminal kinase (16), p38 kinase (32), and ERK (49). While the p38 kinase-dependent build-up of p53 was not investigated in this study, we propose that ROS-activated p38 kinase directly or indirectly phosphorylates p53 in keratinocytes, thereby leading to its build-up.

To determine the mechanism by which MAP kinases regulate apoptosis in keratinocytes from perilesional vitiligo skin, we analyzed ERK-1/2 and p38 kinase activities. We also show that the varying effects of the two proteins on apoptosis are due to converse regulation of p53 build-up. We show, for example, that ERK-1/2 and p38 kinases have opposing regulatory effects on apoptosis. Using specific MAPK subtype inhibitors, we demonstrated that p38 and ERK-1/2 kinase activation act as pro- and anti-apoptotic signals, respectively. The build-up of p53 tumour suppressor and Smac/DIABLO proteins may explain the differing effects of p38 and ERK-1/2 kinases on keratinocyte apoptosis.

Transcriptional and post-translational modification of p53 plays an essential role in the stabilization and activation of p53. A number of serine/threonine kinases can directly phosphorylate p53, affecting its activity and subsequent biological functions (4).

Whereas many studies highlight the importance of p38 kinase in regulating p53 activity, the extent to which ERK-1/2 kinase is involved in p53 phosphorylation and/or build-up is less clear. Previous studies have shown that, depending on cell type and extracellular stimuli, ERK activity can cause a build-up of p53 (33). Equally, regulation of p53 build-up may also protect against apoptotic cell death (1).

The ERK pathway is mainly activated in response to mitogens and growth factors, and has long been associated with cell proliferation and survival (37). The ERK pathway has several effects on key molecules involved in the inhibition of apoptosis. However, we clearly show that, by reducing the build-up of p53, ERK-1/2 acts as an anti-apoptotic signal in keratinocytes from perilesional vitiligo skin. Further experiments are needed to determine whether ERK-1/2 and p38 kinase-mediated regulation of p53 build-up is due to phosphorylation of the latter. To our knowledge, however, this is the first study that links ROS-induced keratinocyte apoptosis with p53 and its opposing phosphoryl regulation by ERK-1/2 and p38 kinase.

In keratinocytes from perilesional vitiligo skin, we demonstrate the presence of caspase-9 and p38 activation and little ERK phosphorylation, and that treatment with curcumin and capsaicin enhances the ERK pathway and reduces caspase-9 activation. This suggests that ERK-caspase-9 pathway crosstalk is present in our cell model. Interestingly, curcumin and capsaicin inhibited p38 phosphorylation in our keratinocytes and there is evidence to suggest that inhibition of the ERK pathway is due to p38 signaling (23). Particularly in human keratinocytes, direct interaction between p38 isoforms and ERK is proposed to inhibit ERK phosphorylation and activity (12). In normal human foreskin keratinocytes, p38 and ERK were isolated in complex, and activation of p38 was associated with inhibition of ERK phosphorylation (11). However, the molecular mechanism by which p38 binds to ERK was not investigated in our study.

For the first time, our results clearly indicate the activation of MAPK pathways in keratinocytes from perilesional vitiligo skin, as shown by p38 and ERK phosphorylation. Upon treatment with curcumin and capsaicin, p38 phosphorylation decreased whereas ERK phosphorylation increased, suggesting that MAPK pathways are responsible for cellular damage in perilesional keratinocytes from vitiligo skin. This study shows that ROS-induced p38 kinase triggers activation of the transcription factor NF-kB. Our results are supported by findings reported in other studies, which suggest that p38 kinase may play a role in regulating NF-kB activation in other experimental models (5). Transcriptional activation of p53 and/or other pro-apoptotic genes may be responsible for the pro-apoptotic effects of activated NF-kB (50).

Given the immense relevance that the p53 and NF-kB pathways have in cellular physiology, it is not surprising that crosstalk between these two transcriptional regulatory networks has been identified and studied extensively (45). How exactly NF-kB and p53 interact to regulate apoptosis is a matter of controversy. It has been suggested that NF-kB can act either upstream or downstream of p53 during apoptosis, however (50).

In this study we show that inhibiting NF-kB activation led to a decrease in p53 activity (Fig. 6), demonstrating that activated NF-kB modulates expression of pro-apoptotic p53 in perilesional vitiligo skin. The molecular mechanisms by which p38 kinase activation, p53 build-up and caspase-3 activity are lowered remains to be elucidated.

In summary, we show that ROS-induced p38 and ERK-1/2 kinases have opposing, pro- and anti-apoptotic effects, respectively, which are closely linked to the build-up of p53.

In this study, we showed that NF-kB p65 fragment is present in nuclear keratinocytes from perilesional vitiligo skin, with curcumin and, to a lesser extent, capsaicin, capable of inhibiting these proinflammatory pathways. In their HaCaT cell line, Cho *et al.* (6) showed that the inhibitory effect of curcumin on cytokine expression was associated with the suppression of MAPKs and NF-kB, suggesting that curcumin could be used as an immunomodulatory agent in inflammatory skin diseases. Accordingly, in keratinocytes from our perilesional vitiligo skin samples, curcumin was found to inhibit p38 and NF-kB activation; in contrast, curcumin increased ERK phosphorylation.

We observe that Smac/DIABLO in keratinocytes from perilesional vitiligo skin contributes to apoptosis, while curcumin and capsaicin treatments strongly inhibit this intrinsic apoptotic pathway (Fig. 6). Moreover, curcumin can better protect against mitochondrial damage, Smac/DIABLO activation and ERK activation than capsaicin, resulting in prevention of apoptosis through caspase-9 inhibition. Interestingly, curcumin and capsaicin also showed a strong inhibitory effect on caspase-8 activation.

Antioxidants have been shown to attenuate the activation of MAPKs (48), thus indicating that the MAPK signaling pathway is an important target for ROS. It is well-documented that nuclear factor kappa B (NF-kB) transcription factors are a downstream target of the MAPK signal transduction pathway and that activation of NF-kB has a crucial role in many inflammatory diseases, including vitiligo. In keratinocytes from perilesional vitiligo skin we observed strong activation of NF-kB pathways, which is responsible for inflammatory processes *in vivo*. This activation is markedly reduced by curcumin and capsaicin treatment.

In conclusion, our data show that ROS production and mitochondrial damage in keratinocytes from perilesional vitiligo skin trigger apoptosis via Smac/DIABLO and MAPK pathways. We also show that NF-kB is markedly activated in perilesional keratinocytes. Curcumin treatment protects cells from mitochondrial damage and apoptosis by: (i) inhibiting caspase and Smac/DIABLO activation, inhibiting p38 phosphorylation and increasing ERK activity, (ii) restoring mito-

chondrial permeability and mitochondrial membrane potential, and (iii) inhibiting NF-kB activation. Considering the consequences of aberrant NF-kB and MAPK signaling in keratinocytes from perilesional vitiligo skin, a major goal of our work is to develop specific inhibitors of these pathways.

Curcumin and capsaicin may therefore represent a valid approach to limit keratinocyte damage in perilesional vitiligo skin and could be used as a therapeutical tool to protect against vitiligo progression.

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Author Disclosure Statement

The authors state that no competing financial interests exist.

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Abbreviations Used

AP-1 = activator protein 1
Bax = Bcl-2–associated X protein
bFGF = basic fibroblast growth factor
Bim = Bcl-2 interacting mediator of cell death
BODIPY = boron-dipyrromethene
Calcein-AM = calcein acetoxymethyl ester
Caspase = cysteine-aspartic protease
$\Delta\psi$ = mitochondrial membrane potential
DEVD = Asp-Glu-Val-Asp
DMEM = Dulbecco's Modified Eagle's Medium
DMSO = dimethyl sulfoxide
EDTA = ethylenediaminetetraacetic acid
EIA = enzyme immunoassay
ELISA = enzyme-linked immunosorbent assay
ERK = extracellular signal-regulated kinase

Abbreviations Used (cont.)

ET-1 = endothelin-1
FACS = fluorescence-activated cell sorting
FAM = fluorescein acetoxymethyl ester
FLICA = Fluorochrome Inhibitor of Caspases
FMK = fluoromethyl ketone
H_2DCFDA = 2′, 7′-dichlorofluorescein diacetate
HaCaT = Human adult low Calcium Temperature keratinocytes
IAPs = inhibitor of apoptosis proteins
IKK = inhibitor of kappa B kinase
IL-1β = interleukin-1 beta
IL-6 = interleukin-6
IL-8 = interleukin-8
JNK = Jun N-terminal kinase
LDH = lactate dehydrogenase
MAPK = mitogen-activated protein kinase
Mcl-1 = myeloid cell leukemia sequence 1
MEK = mitogen activated protein kinase or extracellular signal-regulated kinase
mPTP = mitochondrial permeability transition pore
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-kB = nuclear factor kappaB
NIK = nuclear factor kappaB-inducing kinase
PAGE = polyacrylamide gel electrophoresis
PBMCs = peripheral blood mononuclear cells
PBS = buffered saline solution
PMSF = phenylmethanesulphonylfluoride
PVDF = polyvinylidene fluoride
ROS = reactive oxygen species
RPMI = Roswell Park Memorial Institute medium
SCF = stem cell factor
SD = standard deviation
SDS = sodium dodecyl sulphate
Smac/DIABLO = second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
TAC = total antioxidant capacity
TMRM = tetramethylrhodamine, methyl ester, perchlorate
TNF-α = tumor necrosis factor-alpha