Hypoxia-inducible Factor-1α, a Key Factor in the Keratinocyte Response to UVB Exposure

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Hypoxia-inducible factor-1 (HIF-1) is a major transcription factor sensitive to oxygen levels, which responds to stress factors under both hypoxic and nonhypoxic conditions. UV irradiation being a common stressor of skin, we looked at the effect of UVB on HIF-1α expression in keratinocytes. We found that UVB induces a biphasic HIF-1α variation through reactive oxygen species (ROS) generation. Whereas rapid production of cytoplasmic ROS down-regulates HIF-1α expression, delayed mitochondrial ROS generation results in its up-regulation. Indeed, activation of p38 MAPK and JNK1 mediated by mitochondrial ROS were required for HIF-1α phosphorylation and accumulation after UVB irradiation. Our experiments also revealed a key role of HIF-1α in mediating UVB-induced apoptosis. We conclude that the broad impact of the HIF-1 transcription factor on gene expression could make it a key regulator of UV-responsive genes and photocarcinogenesis.

Solar UV radiation is the major risk factor for the development of skin cancer, the most common malignancy in the world. Several factors participate in the human natural photoprotective barrier, including constitutive and UV-induced pigmentation, stratum corneum thickness, UV-induced immune responses, UV-induced apoptosis, antioxidant defense, and DNA repair systems (1, 2). To develop a strategy for the prevention and the therapy of skin cancer, relationships between the various cellular signaling pathways that modulate cellular responses to UV irradiation in skin cells require a more in-depth understanding. Within this complex group of UV-induced cellular responses, the role of ROS3 appears central with dual effects (3). On one hand, an increase in ROS concentrations following UVB irradiation leads to cytotoxicity through protein, lipid, and DNA oxidation both in vitro and in vivo (4–7). Reduction of the deleterious effects of UV-induced ROS by reinforcement of the antioxidant defense supports this notion (8–10). On the other hand, ROS can also act as second messenger molecules mediating the response of cells to UVB (11–14). We have recently demonstrated that UVB induces ROS production in two distinct waves. Whereas the second peak in ROS concentration has a direct effect on apoptosis, the first peak could mediate signal transduction (10).

In this study, we addressed the possible role of ROS as second messengers after UVB irradiation and their potential relationship with a stress-inducible transcription factor, HIF-1. This factor, so named for its exquisite sensitivity to oxygen levels in the cell environment, participates in the regulation of numerous genes involved in angiogenesis, glycolysis, apoptosis, migration, and metastasis (15–18). We decided to look at HIF-1α expression within the context of ROS production in keratinocytes, in response to UVB irradiation, because ROS were already known to influence HIF-1α regulation and hypoxia-induced apoptosis (19). Moreover, involvement of HIF in the modulation of cell responses to growth factors under normoxia in a ROS-dependent manner (19, 20) and UVB-mediated induction of VEGF (one of the major HIF-1α target genes) (21–23) lend further support to our hypothesis.

HIF-1α is a heterodimeric factor consisting of two α and β subunits (24). In normoxia, HIF-1α is rapidly targeted for ubiquitination and proteasomal degradation after binding to the von Hippel-Lindau protein. The hydroxylation of HIF-1α mediated by prolyl hydroxylases is a prerequisite for the association of HIF-1α with von Hippel-Lindau protein (15, 25–27). Reduction in prolyl hydroxylase activity under hypoxic conditions results in the stabilization and the accumulation of HIF-1α. Hypoxia-mediated ROS modulation and post-transcriptional modification (mainly phosphorylation) of HIF-1α has also been shown to be important in its stabilization and/or transcriptional activation process (28, 29). When stabilized, HIF-1α can be involved in hypoxia-induced apoptosis via either direct or indirect interaction with p53 or through the induction of pro- or antiapoptotic proteins (30). DNA damage and genetic instability under severe hypoxia, resulting in p53 activation, has been shown that has an important role in the modulation of HIF-1α and the activation of the apoptotic pathways (30, 31).
In this paper, we report the implication of UVB-induced ROS as second messengers in apoptosis through the modulation of HIF-1α, suggesting a novel and key role of HIF-1α in the cell response to UVB and a putative involvement in photocarcinogenesis.

**EXPERIMENTAL PROCEDURES**

**Source of Keratinocytes and Irradiation Procedure**—Keratinocytes were isolated from normal human skin in patients undergoing plastic surgery, grown in MCDB153 medium, and irradiated at a dose of 200 mJ/cm² using a Biotronic device (Vilber Lourmat, Marne la Vallée, France), equipped with a dosimeter, in which the UVB lamp emitted a continuous spectrum between 280 and 380 nm (major peak at 312 nm), as already described (9, 10).

**Lentiviral Vector Constructs and Keratinocyte Transduction**—The lentivectors used for transduction (TPCatalaseW, TPcuZnSODW, and TPmnsODW; see supplemental Fig. S2A) have been previously described (9, 10). Briefly, the different lentiviral vectors were constructed by inserting catalase, CuZn-SOD, and Mn-SOD cDNAs into the multiple cloning site of the TPW vector downstream of the human phosphoglycerate kinase promoter. TPW was also used as the backbone for the construction of the ThshHIF1W by replacement of human phosphoglycerate kinase by a cassette containing the H1 promoter followed by an shRNA sequence (GATGTTAGCTC-CCTATATCCC) targeting the HIF-1α mRNA (see supplemental Fig. S3A). Lentiviral particles were produced by transient transfection of 293T cells using a calcium phosphate transfection technique as previously described (9, 10). Retroviral vector pRS-p53, hereafter called shp53, was a generous gift from Dr. R. Agami (32). Determination of the titer of each viral supernatant was performed by assessing enhanced green fluorescent protein expression by flow cytometry and enzyme-linked immunosorbent assays of p24.

For transduction, keratinocytes (5 × 10⁵ cells/T25 flask) were incubated for 24 h in complete medium. Prior to infection, medium was removed, and cells were incubated with viral supernatants for 24 h at 37 °C in the presence of 8 μg/ml protamine sulfate. After 5 days, the percentage of enhanced green fluorescent protein-positive cells was determined by cytofluorimetry (9, 10).

**Determination of Catalase, CuZn-SOD, and Mn-SOD Activities**—All of these assays were performed as previously described (9, 10) using the SOD assay kit-WST (Dojindo Labs, Ozyme, Orsay, France) was added. After a 15-min incubation at 30 °C, reactions were stopped by adding an equal volume of 2 × SDS (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM dithiothreitol). Proteins were resolved in SDS-PAGE and revealed by immunoblot analysis.

**HIF-1α DNA Binding Activity Analysis**—Nuclear proteins were prepared with the TransAMTM nuclear extract kit in accordance with the manufacturer’s instructions (Active Motif Europe, Rixensart, Belgium). HIF-1α activation was determined with the TransAMTM enzyme-linked immunosorbent assay kit. In brief, 15 μg of nuclear extract was added to each well of a 96-well plate coated with an immobilized oligonucleotide corresponding to the HRE consensus sequence. One hour later, wells were incubated with HIF-1α antibody for 1 h followed by 1 h of incubation with horseradish peroxidase-conjugated antibody. Thirty minutes after the addition of developing solution, the reaction was stopped by the addition of H₂SO₄, and the final A₄₅₀ was read on a microplate reader.

**Measurement of Intracellular ROS**—The intracellular production of ROS was assessed using the CM-H₂DCF-DA probe (Molecular Probes) or the MitoSoX™ red mitochondrial superoxide indicator (Molecular Probes). Briefly, after the addition of CM-H₂DCF-DA (5 μM) or MitoSOX (5 μM), cells were incubated for 15 min at 37 °C in the dark. Whereas CM-H₂DCF is oxidized by cytoplasmic ROS to the highly green fluorescent CM-DCF compound, MitoSOX is targeted to the mitochondria and oxidized by superoxide to a red fluorescence component. After two washes with phosphate-buffered saline, the cells were detached by trypsin-EDTA and immediately analyzed by flow cytometry. Ten thousand individual data points were collected for each sample. To observe ROS production by fluorescence microscopy, keratinocytes grown on glass coverslips in 3.5-cm wells each sample. To observe ROS production by fluorescence microscopy, keratinocytes grown on glass coverslips in 3.5-cm wells were loaded with CM-H₂DCF-DA or MitoSOX as mentioned above. After two washes in phosphate-buffered saline, cells were observed and photographed under a fluorescence microscope.

**RNA Extraction and Quantitative Real Time Reverse Transcription-PCR**—Total cellular RNA was extracted using TRIzol® (Invitrogen) according to the procedure of the manufacturer. RNA integrity was confirmed by electrophoresis on ethidium bromide-stained 1% agarose gels. Total cellular RNA...
(5 μg) was reverse transcribed at 42 °C for 60 min in 28 μl containing 0.07 units/μl reverse transcriptase, 7.3 mM dithiothreitol, 0.5 mM each deoxynucleotide triphosphates (dNTPs), and 3.6 μM oligo(dT) primer (Invitrogen). Reactions were stopped by heat inactivation for 10 min at 92°C. Primer sequences used for PCR were as follows: HIF-1α, forward primer (GGGAGGATCACCCGCTCCC) and reverse primer (CACTCAGTGGGCTCGAGAAC); Glut-1, forward primer (GCTCACTGACCTCAAGGAC) and reverse primer (GAGCATCAGCAGCAGCAGCAGT); PK-2, forward primer (GGGTGCGACGACCCTCAG) and reverse primer (GTACGATGGGCTTCTG); tubulin, forward primer (GAGTTCAGTCCATCCATCAGTT) and reverse primer (TAGAGCTCCCAGCAGG). Real time PCR was carried out using the Syber Green method with the Stratagene system. The reactions were cycled 40 times after initial polymerase activation (50 °C, 2 min) and initial denaturation (95 °C, 15 min) using the following parameters: denaturation at 95 °C for 15 s, annealing at 60 °C for 12 s, and extension at 60 °C for 20 s. A final cycle of fusion (95 °C, 30 s; 60 °C, 30 s; 95 °C, 30 s) terminated these reactions. The standard curve demonstrated a linear relationship between the cycle threshold (Ct) values and the cDNA concentration. The relative expression of each gene was assessed by considering the Ct values and efficiency of the primers and then normalized using the expression levels of tubulin.

Statistics—Statistical analyses were performed using a one-way analysis of variance test, followed by a post hoc Tukey’s test, and a p value of <0.05 was considered significant. Results are presented as means ± S.D.

RESULTS

Kinetics of HIF-1α Expression following UVB Irradiation—To determine whether UVB irradiation could play a role on HIF-1α expression level and activity, we first analyzed HIF-1α protein levels in UVB-irradiated keratinocytes using Western blot analysis. Results showed a dramatic decrease of HIF-1α protein immediately (<0.5 h) after irradiation, followed by a gradual increase from 2.5 to 10 h postirradiation (Fig. 1A). The biphasic variation in HIF-1α protein level was correlated with the variation of HIF-1α DNA binding measured by a TransAM™ HIF-1α enzyme-linked immunosorbent assay kit (Fig. 1B). In order to test whether HIF-1α was modified at the transcriptional level, we performed real time quantitative reverse transcription-PCR on HIF-1α mRNA. As shown in Fig. 1C, a near mirror effect on the respective levels of protein and HIF-1α mRNA was observed. Indeed, HIF-1α mRNA significantly decreased, whereas the protein increased (from 5 to 10 h postirradiation). Taken together, our results suggest that the variations in the amount of HIF-1α protein observed in response to irradiation may occur through the regulation of the protein synthesis or stability.

ROS Involvement in HIF-1α Variations following UVB Irradiation—Since ROS generated via cytoplasmic or mitochondrial complexes have been described to be involved in HIF-1α protein degradation or stabilization in response to hypoxia, respectively (19, 33), we wondered whether ROS generation could be responsible for the biphasic effect of UVB irradiation on HIF-1α. To this end, we measured both cytoplasmic and mitochondrial steady-state level of ROS in UVB-irradiated keratinocytes, using CM-H2DCF-DA and MitoSOX probes, respectively (Fig. 2A; see also supplemental Fig. S1). UVB irradiation induced an immediate strong cytoplasmic ROS accumulation, which rapidly declined to basal levels 1 h postirradiation. A second progressive and more moderate increase in cytoplasmic ROS level was observed from 3 to 9 h postirradiation.
tion (Fig. 2A and supplemental Fig. S1A). By contrast, measurement of mitochondrial ROS showed a single peak from 1 to 3 h after UVB (Fig. 2A and supplemental Fig. S1B). These results suggest that intracellular ROS production in response to UVB irradiation is a complex biphasic and compartmented process that could be linked to the biphasic degradation and accumulation of HIF-1α in irradiated keratinocytes.

To further investigate the respective effects of cytoplasmic and mitochondrial ROS on HIF-1α protein modulation, we used either transduced keratinocytes overexpressing antioxidant enzymes, including catalase, CuZn-SOD, or Mn-SOD (see supplemental Fig. S2), or cells treated with diphenylene iodonium (DPI; 2.5 μM), an inhibitor of cytoplasmic NADPH oxidase, or N-acetyl cysteine (NAC; 10 mM), a precursor of glutathione that is the substrate of glutathione peroxidases. It is worthy of mention that catalase, CuZn-SOD, and Mn-SOD locate in peroxisomes, cytoplasm, and mitochondria, respectively, and that glutathione peroxidase is present in both cytoplasmic and mitochondrial compartments (34). First of all, cytoplasmic and mitochondrial ROS levels were analyzed at various times after irradiation (Fig. 2, B and C). Measurement of cytoplasmic ROS indicated a significant decrease in endogenous ROS levels in nonirradiated conditions as compared with nontreated cells. Following irradiation, catalase and Mn-SOD overexpression as well as NAC treatment prevented the late increase in cytoplasmic ROS levels without abrogation of the first peak. In contrast, pretreatment of keratinocytes with DPI inhibited the initial peak in cytoplasmic ROS levels but did not affect the late increase. The CuZn-SOD overexpression had no significant effect on ROS levels following irradiation. Assessment of mitochondrial ROS showed that only Mn-SOD overexpression or NAC treatment prevented increase in mitochondrial ROS after irradiation. Overall, data suggest that the source of the primary increase in ROS levels is mostly dependent on the activity of a cytoplasmic NADPH oxidase, whereas the second increase in ROS levels originates from mitochondria.

Next, the HIF-1α protein level was analyzed after irradiation of transduced or treated keratinocytes. Results showed that neither catalase nor CuZn-SOD overexpression affected the biphasic profile of HIF-1α expression. By contrast, overexpression of mitochondrial Mn-SOD completely abrogated the increase of HIF-1α normally observed 10 h postirradiation (Fig. 2D).
Effect of UVB Irradiation on the Phosphorylation Status of HIF-1α—In addition to the variations of HIF-1α protein level, UVB irradiation modified the migration pattern of HIF-1α (Fig. 1A and 2D), suggesting that UVB could affect post-translational modifications. In order to better characterize the different protein bands, we treated protein extracts from irradiated and nonirradiated keratinocytes with alkaline phosphatase. Converting the upper band into the lower migrating band upon phosphatase treatment indicated that this band, which appears after irradiation, corresponds to a phosphorylated form of the protein (Fig. 3A). Moreover, the irradiation of keratinocytes with increasing doses of UVB demonstrated a dose-dependent accumulation of the phosphorylated form of HIF-1α (Fig. 3B). We looked at the p53 and the serine 15-phosphorylated p53 accumulation as a control for the dose response of cells to irradiation (Fig. 3B).

Since several studies showed that both UV and hypoxia can activate the MAPK signaling cascade, including ERK, p38 MAPK, and JNK pathways (19, 33, 35–37), these regulators could influence HIF-1α phosphorylation. To address this issue, keratinocytes were treated with ERK, p38 MAPK, or JNK inhibitors prior to UV irradiation. As shown in Fig. 3C, all inhibitors used were efficient in inhibiting its own pathway. Regarding the HIF-1α expression, ERK inhibitor had no effect on its status at any time after irradiation. By contrast, JNK and p38 MAPK were obviously required for the accumulation of HIF-1α, since their inhibition prevented up-regulation of HIF-1α in the phosphorylated and also nonphosphorylated forms. Interestingly, nonirradiated keratinocytes treated with either p38 or JNK inhibitors exhibited a lower amount of phosphorylated HIF-1α in comparison with nontreated cells, which suggested that both kinases are also involved in phosphorylation of HIF-1α. To further confirm the role of MAPK in the phosphorylation of HIF-1α following irradiation, we analyzed HIF-1α status in keratinocytes after inhibition of HIF-1α degradation. To this aim, keratinocytes were treated with MG132, a proteasome inhibitor, and then were subjected to different MAPK inhibitors (Fig. 3D). Assessment of HIF-1α protein following irradiation indicated that in the

2D) without affecting the early decrease of HIF-1α. These results suggested a role for the mitochondrial ROS in HIF-1α stability after irradiation. Blocking the late accumulation of HIF-1α following irradiation upon pretreatment with NAC further confirmed this notion. Interestingly, DPI treatment of keratinocytes prior to irradiation completely abrogated the immediate HIF-1α down-regulation in response to UVB but did not affect its late accumulation. Taken together, these data suggest that ROS generated by NADPH oxidase activity in the cytoplasm immediately after UVB irradiation participates in HIF-1α down-regulation. In contrast, the transient later production of mitochondrial ROS is involved in the long lasting HIF-1α stabilization and accumulation.
presence of MG132 and inhibitor of either p38 or JNK kinase, HIF-1α was not phosphorylated. However, the amount of HIF-1α in this condition was lower than that found in cells treated only with MG132. Taken together, these results confirm that activation of p38 MAPK and JNK1 is required for accumulation of HIF-1α in UVB-irradiated keratinocytes.

To determine whether the activation of MAPKs was mediated through UVB-induced ROS production, activation of ERK, p38, and JNK was assessed by Western blot analysis in catalase-, CuZn-SOD-, and Mn-SOD-transduced cells and NAC- or DPI-treated keratinocytes (Fig. 2D). Our results indicated a clear decrease in the activation of those three MAPK pathways following irradiation in both Mn-SOD-transduced cells and NAC-treated keratinocytes. In contrast, catalase overexpression resulted in selectively decreased ERK activation. Neither overexpression of CuZn-SOD nor the treatment with DPI provided a significant effect in UVB-induced ROS-mediated MAPK activation. Overall, our results show that the activation of JNK and p38 MAPK through UVB-induced mitochondrial ROS production is required for HIF-1α accumulation.

Transcriptional Activity of HIF-1α after UVB Irradiation—To demonstrate that the variation of HIF-1α observed following irradiation has a biological significance, we tested the transcriptional activity of HIF-1α by assessing the expression of HIF-1α well characterized target genes, VEGF-A, Glut-1, and PFK-2 (Fig. 4). As expected, quantitative reverse transcription-PCR analysis indicated a marked decrease in mRNA level of all three genes in response to irradiation. This down-regulation was followed by a progressive increase of the expression of these genes 5 h postirradiation (i.e. when HIF-1α protein accumulated). In order to determine whether this effect is dependent upon HIF-1α accumulation in irradiated cells, we used an shRNA strategy that leads to HIF-1α mRNA degradation. Keratinocytes were transduced with lentivectors expressing a shRNA directed against HIF-1α (see supplemental Fig. S3A) or the red fluorescent protein (data not shown) as a negative control. The shHIF-1α inhibited more than 99% of HIF-1α accumulation in keratinocytes when treated with CoCl2, an inhibitor of prolyl hydroxylase-mediated HIF-1α degradation (see supplemental Fig. S3B). In shHIF-1α-transduced cells, a dramatic reduction of the expression of all three mRNA prior to irradiation was observed, indicating that their basal expression in keratinocytes was mainly HIF-1α-dependent. A statistical analysis (post hoc Tukey’s test), showed a significant difference (p < 0.05, n = 3) for all three mRNA expression profiles over time, between nontransduced and transduced cells, indicating that the expression of mRNAs upon irradiation was at least partially dependent on HIF-1α. However, the increase in the messengers of all three target genes was still observed after UVB irradiation (10–20 h) in keratinocytes lacking HIF-1α expression.

This phenomenon may be attributed to residual HIF-1α protein stabilized by UVB or other UV-induced transcriptional factors. Overall, these results suggest that the variation of HIF-1α in response to UVB irradiation does not only lead to the modulation of these target genes, involved in glucose metabolism and angiogenesis, but may also potentially affect other target genes involved in cell metabolism and survival.

Implication of HIF-1α in UVB-induced Apoptosis—Since we had previously demonstrated that UVB irradiation provokes ROS induced apoptosis (10), we wondered whether HIF-1α activation by ROS could be implicated in this process. To answer this question, cells were transduced with vectors expressing either HIF-1α shRNA or p53 shRNA, as a control of the inhibition of UV-induced apoptosis. Cells were then UVB-irradiated, and apoptosis was measured 20 h after irradiation by flow cytometry using VAD-fmk, which binds to activated caspases, and PI as an indicator of plasma membrane permeability. The percentages of apoptotic (VAD-fmk+/PI−) and late apoptotic plus necrotic cells (VAD-fmk+/PI+) indicated that the down-regulation of HIF-1α reduced the apoptotic cells by 40% (Fig. 5, A and B). To further characterize the mechanism by which HIF-1α mediates UV-induced apoptosis, we assessed activation of caspases separately by Western blot (Fig. 5C). Caspase-3, -8, and -9 were cleaved in normal cells 20 h postirradiation, whereas there was a clear reduction in the activation of all three caspases in cells expressing either HIF-1α or p53 shRNA, which corroborated the reduction of apoptotic cells observed by flow cytometry.

Because an interaction between HIF-1α and p53 has been implicated as one of the mechanisms by which HIF-1α is involved in hypoxia-induced apoptosis (30), we investigated the effect of this potential interaction in our system. Intriguingly, total p53 protein as well as phosphorylated p53 accumulation was significantly reduced in cells transduced with shHIF-1α both before and after irradiation (Fig. 5, C and D). In the same way, the HIF-1α protein level was lower in keratinocytes transduced with shp53. These results, suggesting a particular regulatory cross-talk between the two proteins, need to be deepened by complementary investigations.
Besides being already recognized as the main transcription factor induced under hypoxic conditions, HIF-1α has recently been shown to respond to other stress factors under normoxia. In both situations, ROS have been implicated as the main regulators of HIF-1α. Consistent with this notion, we have highlighted for the first time the mechanisms contributing to the modulation of HIF-1α in response to UVB-induced ROS production and the effect of HIF-1α on UVB-induced apoptosis.

**Biphasic Effect of UVB on HIF-1α**—Until now, two mechanisms regulating HIF-1α expression and activation have been documented. In response to hypoxia, HIF-1α protein is stabilized thanks to the inhibition of prolyl hydroxylase activity and, consequently, the inhibition of ubiquitin-mediated degradation (33, 36). However, in response to growth factor stimulation, such as epidermal growth factor, HIF-1α protein synthesis is upregulated (25, 38). Our results demonstrate that HIF-1α variations following irradiation take place at both protein and mRNA levels with an unexpected opposite effect; an increase in the protein level was observed while the messenger decreased. A regulatory loop has been previously reported in prolonged hypoxia (39, 40). Indeed, an endogenous antisense HIF-1α transcript, complementary to HIF-1α mRNA and constitutively expressed in human tissues and tumors, has been reported to contain several putative HRE sequences in its promoter (41). Thus, it is likely that the late accumulation of HIF-1α protein following UV irradiation leads to an HIF overexpression, which consequently increases HIF-1α mRNA degradation.

The expression kinetic of HIF-1α protein after irradiation reveals a prompt degradation process, whereas accumulation takes a long time to be initiated. Thus, we assume that the degradation, which correlates with the down-regulation of the HIF-1α target genes (VEGF, Glut, and PFK), may be crucial for the modulation of genes involved in the complex cellular response to UVB. To our knowledge, this report is the first to show that a down-regulation of HIF-1α, in addition to its up-regulation, is important to transduce signals upon a stress.

**Different Origins of UVB-induced ROS and Effects of ROS on HIF-1α Expression**—Under physiological conditions, ROS are generated by mitochondria via enzyme complexes involved in oxidative phosphorylation. Numerous cytosolic enzymes, including cyclooxygenase, nitric-oxide synthase, xanthine oxidase, and the plasma membrane-bound NADPH oxidase, can...
also generate ROS. We have recently demonstrated that UVB irradiation induces ROS production at two distinct stages and that the primary increase in ROS levels detected immediately upon irradiation is dependent on NADPH oxidase activity (10). Here, we expanded this knowledge by showing that the second increase in ROS derives mainly from mitochondria.

A detailed analysis reveals that the biphasic effect of UVB on HIF-1α is directly related to cytoplasmic and mitochondrial ROS generation. HIF-1α stabilization, translocation, and activation mediated by ROS has already been evoked as a potential regulatory mechanism (19, 33, 36, 37). Besides the well known mechanism of direct ROS effects on prolyl hydroxylase activity, two additional models for regulation of HIF-1α in hypoxia have been proposed. The first model suggests that converting O2 into ROS by a NADPH oxidase of unknown cell localization leads to HIF-1α degradation in normoxia. Based on this model, a decline in oxygen levels would result in a reduced formation of ROS and consequently an increased HIF-1α. According to the second model, the increase in mitochondrial ROS formation by hypoxia leads to HIF-1α stabilization. Our results suggest that both mechanisms may be involved in the biphasic effect of UVB irradiation on HIF-1α regulation in keratinocytes. Indeed, an immediate ROS production after UVB irradiation deriving from cytoplasmic NADPH oxidase activity leads to HIF-1α down-regulation, whereas the later production of mitochondrial ROS is involved in the long lasting HIF-1α accumulation.

**Effect of MAPKs on HIF-1α**—There is accumulating evidence that hypoxia and nonhypoxia stimuli activate MAPKs, which then regulate HIF-1α expression. Up-regulation of HIF-1α mediated by ERK1/2 activation is observed following hypoxia (42), treatment with interleukin-1β, or treatment with prostaglandin E2 (19, 33, 36, 37). Moreover, there is an absence of HIF-1α stabilization in p38α-deficient cells (43) or in JNK inhibitor-treated cells (44) upon hypoxia. Conversely, overexpression of p38 upstream kinases MKK3 and MKK6 under both normoxic and hypoxic conditions leads to stabilization of HIF-1α (45). Interestingly, it has been also shown that ROS are key elements in HIF-1α induction through MAPK activation in hypoxic (28, 46) and nonhypoxic (19, 33, 36, 37) conditions. Regarding UV, many reports indicate that UVB stimulates JNK, p38 kinase, and ERK (35) and that UVB-induced ROS are involved in the activation of MAPK signal transduction pathways (47). In agreement with these results, we found that ROS-mediated activation of JNK and p38 MAPK following UVB irradiation are important for phosphorylation and accumulation of HIF-1α. Moreover, we found that MAPK activation originates from a mitochondrial signal, since the overexpression of the two cytoplasmic antioxidant enzymes, catalase and CuZn-SOD, never affected the HIF-1α, JNK, and p38 phosphorylation status. It is likely that the mitochondrial ROS signal is transferred by a not yet characterized factor. Then this factor enables the induction of a common activator of JNK and p38 MAPK, such as the apoptosis-signaling kinase 1, known to be activated by both oxidative stress and UVB irradiation (47). Consequently, activation of JNK and p38 MAPK leads to the phosphorylation and probably stabilization of HIF-1α, leading to its accumulation. Concomitantly, MAPK activation may result, through the inactivation of prolyl hydroxylase (28, 46), in a reinforced increase of HIF-1α (Fig. 6). This model would account for the down-

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**FIGURE 6. Schematic representative of the proposed model for the effect of UVB irradiation on HIF-1α protein.**
regulation of HIF-1α in keratinocytes treated with JNK or p38 inhibitors.

To explain the rapid down-regulation of HIF-1α following irradiation, the direct effect of cytoplasmic ROS on prolyl hydroxylase mentioned by Gerald et al. (37) could not be proposed in this model. Indeed, the authors have shown that ROS inhibit prolyl hydroxylases and therefore stabilize HIF-1α, whereas our results clearly demonstrate a destabilization of HIF-1α by the cytoplasmic ROS. These data suggest that another dominant mechanism leading to HIF-1α degradation operates quickly in response to UVB. This mechanism may involve a direct HIF-1α hydroxylation by ROS, a blockade of protein synthesis following UVB irradiation, or a rapid redistribution of oxygen into the cytoplasm as a result of the inhibition of mitochondrial respiration (48, 49) by UVB. These hypotheses deserve to be investigated in further studies.

The Role of HIF-1α in UVB-induced Apoptosis—In hypoxic conditions, HIF-1α can either induce or inhibit apoptosis, depending on the cell type (30). On the one hand, HIF-1α-mediated overexpression of the proapoptotic proteins, such as Bcl-2-binding proteins (BNIP3 and NIX), or stabilization of p53 has been implicated as the two main mechanisms mediating the proapoptotic effects of HIF-1α. On the other hand, the intrinsic proapoptotic function of HIF-1α in pancreatic cell lines has been reported (30). Our results indicate that HIF-1α has a proapoptotic effect in our experimental conditions. The correlation between HIF-1α silencing and the reduction of different caspase activities after irradiation suggests that HIF-1α intervenes on both the extrinsic (caspase-8 activation) and the intrinsic (caspase-9 activation) apoptotic pathways. Furthermore, the direct relation between HIF-1α and p53 at both the protein level (Fig. 5, C and D) and mRNA level (data not shown) suggests that a complex cross-talk between HIF-1α and p53 may be involved in mediating UVB-induced apoptosis. Further studies are needed to uncover the precise mechanism by which HIF-1α modulates UVB-induced apoptosis.

Conclusion and Perspectives—The HIF-1 transcription factor is a key element in the regulation of different genes involved in cell survival, apoptosis, cell motility, cytoskeletal structure, cell adhesion, and energy metabolism. Thus, it is likely that its spatial-temporal repression and activation has a substantial influence on the regulation of UV-responsive genes that influence physiologic host responses, such as constitutive pigmentation (50) and the tanning process (51). The mildly hypoxic microenvironment of skin, leading to an increase in HIF-1α protein (52), could further favor a specific role of this protein in skin physiology. Considering the effect of MAPKs in restoration of the skin barrier, modulation of apoptosis, and control of inflammatory responses upon ultraviolet irradiation (53), a pivotal role of HIF-directed signaling may reasonably be anticipated in these processes. In pathophysiology, the essential role of HIF-1 in tumoral angiogenesis and invasion (17, 54–56) could make it a key element as not only an effector in UVB-induced apoptosis but also a factor regulating the developmental and progressive stages of skin cancers. Last, the potential presence of the HIF transcription factor at the center of many pathways triggered by UVB irradiation makes it a promising therapeutic target for the prevention of photocarcinogenesis.

To this end, the role of HIF in cellular responses to acute and chronic UV irradiation must be defined.

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REFERENCES

1. Matsumura, Y., and Ananthaswamy, H. N. (2002) Expert. Rev. Mol. Med. 2002, 1–22
2. Sander, C. S., Chang, H., Hamm, F., Elsner, P., and Thiele, J. J. (2004) Int. J. Dermatol. 43, 326–335
3. Heck, D. E., Gerecke, D. R., Vetranio, A. M., and Laskin, J. D. (2004) Toxicol. Appl. Pharmacol. 195, 288–297
4. McArdle, F., Rhodes, L. E., Parslew, R., Jack, C. I., Friedmann, P. S., and Jackson, M. J. (2002) Free Radic. Biol. Med. 33, 1355–1362
5. Punnonen, K., Punta, A., and Ahotupa, M. (1991) Photochem. Photobiol. 53, 3–6
6. Shinoda, Y., Wilt, E., Han, D., Tseng, B., Aziz, T., Nguyen, L., and Packer, L. (1994) Photochem. Photobiol. 59, 183–191
7. Tyrrell, R. M. (1995) Biochem. Soc. Symp. 61, 47–53
8. Maalouf, S., El-Sabban, M., Darwiche, N., and Gali-Muhtasib, H. (2002) Mol. Carcinog. 34, 121–130
9. Revzani, H. R., Cario-Andre, M., Pain, C., Ged, C., de Verneuil, H., and Taieb, A. (2007) Cancer Gene Ther. 14, 174–186
10. Revzani, H. R., Mazurier, F., Cario-Andre, M., Pain, C., Ged, C., Taieb, A., and de Verneuil, H. (2006) J. Biol. Chem. 281, 17999–18007
11. Garmyn, M., and Degrefe, H. (1997) J. Photochem. Photobiol. B 37, 125–130
12. Jayaraman, L., Murthy, K. G., Zhu, C., Curran, T., Xanthoudakis, S., and Prives, C. (1997) Genes Dev. 11, 558–570
13. Brenneisen, P., Wenk, J., Klitz, O. W., Wlaschek, M., Briviba, K., Krieg, T., Sies, H., and Scharfetter-Kochanek, K. (1998) J. Biol. Chem. 273, 5279–5287
14. Beal, S. M., Lee, Y. S., and Kim, J. A. (2004) Biochimie (Paris) 86, 425–429
15. Bardos, J. I., and Ashcroft, M. (2005) Biochim. Biophys. Acta 1755, 107–120
16. Harris, A. L. (2002) Nat. Rev. Cancer 2, 38–47
17. Maxwell, P. H., Dachs, G. U., Gleadle, J. M., Nicholls, L. G., Harris, A. L., Stratford, I. J., Hankinson, O., Pugh, C. W., and Ratcliffe, P. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8104–8109
18. Semenza, G. L. (2001) Cell 107, 1–3
19. Kietzmann, T., and Gorlach, A. (2005) Semin. Cell Dev. Biol. 16, 474–486
20. Taddei, J. I., and Land, S. C. (2001) FEBS Lett. 509, 269–274
21. Blaudschun, R., Brenneisen, P., Wlaschek, M., Meewes, C., and Scharfetter-Kochanek, K. (2000) FEBS Lett. 474, 195–200
22. Kim, M. S., Kim, Y. K., Eun, H. C., Cho, K. H., and Chung, J. H. (2006) J. Invest. Dermatol. 126, 2697–2706
23. Mildner, M., Weninger, W., Trautinger, F., Ban, J., and Tschachler, E. (1999) Photochem. Photobiol. 70, 674–679
24. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5510–5514
25. Bardos, J. I., Chau, N. M., and Ashcroft, M. (2004) Mol. Cell. Biol. 24, 2905–2914
26. Lee, J. W., Bae, S. H., Jeong, J. W., Kim, S. H., and Kim, K. W. (2004) Exp. Mol. Med. 36, 1–12
27. Fandrey, J., Gorr, T. A., and Gassmann, M. (2006) Cardiovasc. Res. 71, 642–651
28. Bell, E. L., Emerling, B. M., and Chandel, N. S. (2005) Mitochondrion 5, 322–332
29. Brahimi-Horn, C., Mazure, N., and Pouyssegur, J. (2005) Cell Signal. 17, 1–9
30. Greiner, A. E., and van der Wall, E. (2004) J. Clin. Pathol. 57, 1009–1014
31. Kaluzova, M., Kaluz, S., Lerman, M. I., and Stanbridge, E. J. (2004) Mol. Cell. Biol. 24, 5757–5766
32. Bruumkamp, T. R., Bernard, R., and Agami, R. (2002) Cancer Cell 2, 243–247
33. Michiel, C., Minet, E., Mottet, D., and Raes, M. (2002) Free Radic. Biol.
HIF-1α in Cellular Response to UVB Irradiation

34. Mates, J. M. (2000) Toxicology 153, 83–104
35. Bode, A. M., and Dong, Z. (2003) Sci STKE 2003, RE2
36. Semenza, G. L. (1999) Cell 98, 281–284
37. Gerald, D., Berra, E., Frapart, Y. M., Chan, D. A., Giaccia, A. J., Mansuy, D., Pouyssegur, J., Yaniv, M., and Mecha-Grigoriou, F. (2004) Cell 118, 781–794
38. Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C., and Semenza, G. L. (2001) Mol. Cell. Biol. 21, 3995–4004
39. Thrash-Bingham, C. A., and Tartof, K. D. (1999) J. Natl. Cancer Inst. 91, 143–151
40. Uchida, T., Rossignol, F., Matthay, M. A., Mounier, R., Couette, S., Clottes, E., and Clerici, C. (2004) J. Biol. Chem. 279, 14871–14878
41. Rossignol, F., Vache, C., and Clottes, E. (2002) Gene (Amst) 299, 135–140
42. Minet, E., Michel, G., Mottet, D., Raes, M., and Michiels, C. (2001) Free Radic. Biol. Med. 31, 847–855
43. Corre, S., Primot, A., Sviderskaya, E., Bennett, D. C., Vaulont, S., Goding, C. R., and Galibert, M. D. (2004) J. Biol. Chem. 279, 51226–51233
44. Bedogni, B., Welford, S. M., Cassarino, D. S., Nickoloff, B. J., Giaccia, A. J., and Powell, M. B. (2005) Cancer Cell 8, 443–454
45. Hildesheim, J., Awowad, R. T., and Fornace, A. J., Jr. (2004) J. Invest. Dermatol. 122, 497–502
46. Ryan, H. E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J. M., and Johnson, R. S. (2000) Cancer Res. 60, 4010–4015
47. Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L., and Simons, J. W. (1999) Cancer Res. 59, 5830–5835
48. Erler, J. T., Cawthorne, C. J., Williams, K. J., Koritzinsky, M., Wouters, B. G., Wilson, C., Miller, C., Demonacos, C., Stratford, I. J., and Dive, C. (2004) Mol. Cell. Biol. 24, 2875–2889