Central Role of Ferrous/Ferric Iron in the Ultraviolet B Irradiation-mediated Signaling Pathway Leading to Increased Interstitial Collagenase (Matrix-degrading Metalloprotease (MMP)-1) and Stromelysin-1 (MMP-3) mRNA Levels in Cultured Human Dermal Fibroblasts*

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Reactive oxygen species (ROS) are important second messengers for the induction of several genes in a variety of physiological and pathological conditions. Ultraviolet B (UVB) irradiation has recently been shown to generate lipid peroxidation products and hydroxyl radicals (HO·) with detrimental long term effects like cancer formation and premature aging of the skin. Here, we addressed the question of whether ferrie/ferrous iron via the generation of ROS may mediate the UVB response, finally leading to connective tissue degradation, a hallmark in carcinogenesis and aging. Therefore, we studied the involvement of iron and ROS in the modulation of Jun N-terminal kinase 2 (JNK2) activity, c-jun and c-fos mRNA levels, key signaling steps in the transcriptional control of matrix-degrading metalloprotease (MMP)-1/interstitial collagenase and MMP-3/stromelysin-1 after UVB irradiation of human dermal fibroblasts \textit{in vitro}. The iron-driven generation of lipid peroxides and hydroxyl radicals were identified as early events in the downstream signaling pathway of the UVB response leading to a 15-fold increase in JNK2 activity, a 3.5-fold increase in c-jun, to a 6-fold increase in MMP-1, and a 3.8-fold increase in MMP-3 mRNA levels, while virtually no alteration of c-fos mRNA levels were observed. Diminished generation of reactive oxygen species resulted in a significant reduction of JNK2 activity, c-jun, MMP-1, and MMP-3 mRNA levels after UVB irradiation compared with UVB-irradiated cells. Collectively, we have identified the iron-driven Fenton reaction and lipid peroxidation as possible central mechanisms underlying signal transduction of the UVB response.

Cells have evolved exclusive regulatory networks for the most common heavy metal, iron, which plays an ambivalent role in biology. While iron is required as cofactor for many biological reactions, its toxicity threatens cellular integrity (1). Besides a transferrin-independent iron-uptake at the cellular level, the transferrin/ferritin system as a post-transcriptional regulatory circuit has evolved that coordinates iron uptake, storage, and utilization by involvement of the iron regulatory protein (2–4). At the molecular level, a well regulated equilibrium between mainly stored and less "free" ferric/ferrous iron positively influences ribonucleotide reductase in several cell lines, which catalyzes reduction of the four common ribonucleotides to their corresponding deoxyribonucleotides, an essential step in DNA synthesis, normal cell growth, and viability (5, 6). Furthermore, in many cell lines iron is involved in the regulation of the protein kinase C gene expression, which plays a central role in signal transduction pathways (7).

Iron exerts its toxicity through a series of reactions with reactive oxygen species called the modified Haber-Weiss or Fenton reaction (Fe³⁺ + H₂O₂ → Fe²⁺ + HO· + HO₂⁻), generating the highly toxic hydroxyl radical (HO·). The generation of hydroxyl radicals via Fenton chemistry represents one of the most important mechanisms in various pathological conditions. Hydroxyl radicals can lead to DNA damage and impairment of normal DNA and protein synthesis and cell proliferation and thus has been thought to be causally involved in the multistep process of carcinogenesis (8, 9). Furthermore, ferrous/ferric iron has a decisive function in lipid peroxidation processes by direct reaction with unsaturated fatty acids or reaction with preformed lipid hydroperoxides to form chain-carrying alkoxyl and peroxyl radicals, leading to severe damage of cellular integrity (10–13). In addition, the initiation of lipid peroxidation often depends on the occurrence of the HO· radical, suggesting that iron-catalyzed Fenton reaction and lipid peroxidation processes work in cooperation (14, 15).

Ultraviolet B (UVB) irradiation has been discussed as a further source for the initiation of lipid peroxidation, and, in fact, a UVB-dependent generation of hydroxyl radicals and lipid peroxides has been demonstrated in human keratinocytes and fibroblast cultures (16, 17). The iron content is subst...
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Reagents—Diethylthiocuprate (DDC), an inhibitor of copper/zinc superoxide dismutase; bathionium sulfoxime (BSO), an indirect inhibitor of glutathione peroxidase; amnionitriol (ATZ), an inhibitor of catalase; butylated hydroxytoluene (BHT) and vitamin E derivative, Trolox, both inhibitors of lipid peroxidation; MeSO and mannitol, both scavengers of hydroxyl radicals; Fe(III) citrate; and the iron chelator deferoxamine (DFO) were obtained from Sigma (Deisenhofen, Germany). The iron chelator N-2-hydroxybenzyl N’-benzylethylenediaminodiacetic acid (HBED) (46) was provided by H. Sies. The MMP-1 probe used was a 920-base pair HindIII/SmaI fragment of human collagenase cDNA (47), and the MMP-3 probe was a 160-base pair EcoRI/Xho1 cDNA fragment of human stromelysin-1 (48). The probe for c-fos was a 1400-base pair HindIII/BamHI fragment of the cDNA clone h3c-1 (49), and the probe for c-jun was an 800-base pair BglII/Nco1 fragment currently inserted in pUC 18 (50). An 8-mer oligonucleotide (5’-ACG GTA TCT GAT CGT CTT CGA ACC-3’) (51) for the 18 S rRNA was synthesized (Pharmacia, Freiburg, Germany).

Cell Cultures—Dermal foreskin fibroblasts were established by outgrowth from biopsies of healthy human donors (52) with an average age between 3 and 6 years and cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with glutamine (2 mM), penicillin (400 units/ml), streptomycin (50 μg/ml), 10% fetal calf serum (Biochrom, Berlin, Germany), but without sodium ascorbate, and grown on plastic tissue culture dishes in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were used at passages 7–16, corresponding to cumulative population doubling levels 14–34 (53, 54). Confluent cells were preincubated for 18 h with different chemical compounds, subsequently irradiated with total UVB at a dose of 200 J/m² (29), and incubated for 24 h prior to Northern blot analysis.

Light Source and UV Irradiation—A 1000-watt xenon high pressure UV source was used in conjunction with a monochromator with holographic grating (Dermolux UMW, Fa. Müller, Moosimming, Germany). For the experiments, the total UVB spectrum (280–320 nm) was used. Dose rates were determined by an integrated thermopile that is equally sensitive in the range from 270 up to 4000 nm. The dose rates on the cell surface were 3.4 milliwatts/cm² for the total UVB spectrum. To guarantee a constant intensity and spectral distribution, dosimetry and spectroradiometric analyses were performed prior to experiments with an OL-754 UV/visible light spectroradiometer (Optronix, Orlando, FL). Confluent fibroblast monolayer cultures were rinsed twice with phosphate-buffered saline, and all irradiations were performed under a thin layer of phosphate-buffered saline. There was negligible loss in viability of cell populations compared with cells held under similar conditions without irradiation. Following UV irradiation, cells were washed with phosphate-buffered saline and cultivated in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and supplements for various periods of time.

Cytotoxicity Assay—The viability of the dermal fibroblasts was measured 48 h after incubation with BSO, DDC, ATZ, DFO, HBED, Fe(III) citrate, Fe(OH)₃-dextrin, mannitol, MeSO, and Trolox. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was used for the quantification of living metabolically active cells. Mitochondrial dehydrogenases metabolize MTT to purple formazan dye (55). Cytotoxicity was calculated as the percentage of formazan formation in cells treated with the agents compared with mock-treated controls.

RNA Extraction and Northern Blot Analysis—Total RNA was isolated and analyzed by Northern blot hybridization with specific cDNA probes or oligonucleotides for interferential collagenase (MMP-1), stromelysin-1 (MMP-3), c-fos, c-jun, and 18 S rRNA as described elsewhere (27, 29, 51). Briefly, after extraction of total RNA, equal amounts (5 μg) were fractionated on a 0.9% 2.2 M formamide/3 M urea gel. After diffusion transfer to nitrocellulose filters and vacuum baking (Schleicher & Schuell, Dassel, Germany), hybridizations were performed using denatured α-²²P-labeled cDNA probes. For 3’-end labeling of the 24-mer 18 S rRNA probe, 5 × terminal deoxynucleotidyltransferase buffer (0.5 mM potassium cacodylate (pH 7.2), 10 mM cobalt chloride (CoCl₂), 1 mM dithiothreitol), 10 pmol of 3’-ends (equal to 80 ng of

**EXPERIMENTAL PROCEDURES**

Due to promoter similarities, a variety of matrix-degrading metalloproteases, like the interstitial collagenase (MMP-1) (30), stromelysin-1 (MMP-3) (31), and matrilysin (MMP-7) (32), have been shown to be similarly regulated in different experimental settings. Accordingly, MMP-1 and MMP-3 have been found to be similarly induced upon UVA and UVB irradiation (27, 29, 33). The promoter of MMP-1 carries five AP-1 sites (30), and that of MMP-3 has a single AP-1 site (31); these sites are transactivated by binding of the newly synthesized and heterodimerized Fos and Jun, which constitute the AP-1 transcription factor, responsible for c-fos transcription via the Jun N-terminal kinases 1 and 2 (JNK1, JNK2) phosphorylation (38). There is some evidence that ROS are involved in activation of ERKs preceding phosphorylation of the TCF/Elk-1 (39). Furthermore, the Jun N-terminal kinases 1 and 2 (JNK1, JNK2) phosphorylate both components of the heterodimeric c-Jun/ATF2 (activating transcription factor), responsible for c-jun transcription, and c-Jun of the AP-1 complex (35, 40, 41). Research on the regulation of the activity of kinases, of c-jun and of c-fos transcription by extracellular and environmental stimuli like growth factors and ROS, is a matter of increasing interest and relevance (42–44), since it may provide ultimate clues for mechanisms underlying connective tissue degradation in pathological states.

In view of the compelling evidence for an increase in the intensity of UVB irradiation due to stratospheric ozone depletion (45), we have focused our studies on the regulation of the UVB induction of two major matrix-degrading metalloproteases, the interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3). While MMP-1 mainly cleaves interstitial collagenases, the major structural components of the dermis, MMP-3 preferentially degrades proteoglycans as well as structural glycoproteins (25). Here, we provide circumstantial evidence that the iron-driven generation of hydroxyl radicals and lipid peroxides substantially affects JNK2 activity and c-jun transcription, two key steps of the signaling pathway, leading to MMP-1 and MMP-3 mRNA induction after UVB irradiation, while c-fos transcription is unaffected. In addition, we have outlined preventive strategies that may stimulate further development of protective agents to prevent iron-dependent pathological processes such as photoaging and tumor progression.

The Jun N-terminal kinases 1 and 2 (JNK1, JNK2) phosphorylate both components of the heterodimeric c-Jun/ATF2 (activating transcription factor), responsible for c-fos transcription via the serum response factor, and a ternary complex formed using denatured 18 S rRNA probe, 5’-GCT TCT GAT CGT CTT CGA ACC-3’ (51) for the 18 S rRNA was synthesized (Pharmacia, Freiburg, Germany).

Cell Cultures—Dermal foreskin fibroblasts were established by outgrowth from biopsies of healthy human donors (52) with an average age between 3 and 6 years and cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Eggenstein, Germany) supplemented with glutamine (2 mM), penicillin (400 units/ml), streptomycin (50 μg/ml), 10% fetal calf serum (Biochrom, Berlin, Germany), but without sodium ascorbate, and grown on plastic tissue culture dishes in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were used at passages 7–16, corresponding to cumulative population doubling levels 14–34 (53, 54). Confluent cells were preincubated for 18 h with different chemical compounds, subsequently irradiated with total UVB at a dose of 200 J/m² (29), and incubated for 24 h prior to Northern blot analysis.

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DNA of the 24-mer), 1.5 μCi of [α-32P]dCTP/μl of reaction volume, and 1 unit/μl terminal deoxynucleotidyltransferase (Life Technologies) were incubated at 37 °C for 1 h. Sequential hybridizations with different probes were performed. Denstometric analysis was carried out using the ScanPackII system (Biometra, Göttingen, Germany).

**Determination of the Lipid Peroxidation End Products Malondialdehyde (MDA) and 4-Hydroxy-2E-nonenal (4-HNE)—** Lipid peroxidation assay was performed according to the manufacturer’s protocol (Calbiochem-Novabiochem, Bad Soden, Germany) with minor modifications. 4–5 × 10⁶ cells were collected in 500 μl of phosphate-buffered saline containing 200 μM BHT 0.5 h after irradiation and centrifuged at 500 × g for 5 min at 4 °C. The reaction was stopped on ice, and the absorbance (A) of the blue to purple color was measured at 568 nm. The equation MDA = A × 5/ε gives the concentration where ε is the apparent molar extinction coefficient obtained from the standard curve.

**Determination of the Activity of c-Jun N-terminal Kinase 2 (JNK2)—** C-Jun N-terminal kinase 2 was immunoprecipitated for 2–3 h on ice from cell lysates (50 μg of total protein) in 200 μl of radioimmuno precipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.2 mM sodium vanadate (Na₃VO₄), 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 2 μg/ml leupeptin) using 10 μl of an anti-JNK2 antiserum (rabbit; diluted 1:10 in distilled water), which we gratefully received from P. E. Shaw (Nottingham, United Kingdom). Immune complexes were collected at 4 °C overnight with 35 μl of Protein A-Sepharose 6MB (Pharmacia) preequilibrated in radioimmune precipitation lysis buffer. The pelleted immune complex/Protein A conglomerate was washed with both ice-cold radioimmune precipitation buffer and kinase buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol) three times each. 35 μl of kinase buffer was added to the pellets, followed by 5 μl of 150 μM ATP, 6 μl (1 μg/μl) glutathione S-transferase-c-Jun (residues 1–79, 37 kDa) fusion protein (Alexis, Grünberg, Germany) (57), and 5 μl (5 μCi) of [γ-32P]ATP (5 Ci/μmol). The mixture was incubated at 37 °C for 25 min and repeatedly vortexed during this incubation time. The reaction was stopped by the addition of gel loading buffer (58) followed by denaturation for 5 min at 95 °C. The samples were centrifuged for 5 min, and the supernatants were analyzed by electrophoresis on a 15% SDS-polyacrylamide gel. After the gel was dried, the phosphorylated JNK2 substrate glutathione S-transferase-c-Jun was identified by autoradiography.

**RESULTS**

**Cytotoxicity of Different Chemical Compounds Involved in the Accumulation or Detoxification of Reactive Oxygen Species—** We intended to avoid interference of cytotoxicity from chemical agents. Therefore, the MTT assay was used to determine the optimal concentrations at which more than 75% of the mitotic fibroblasts survived at least 48 h after incubation with the different agents with no change in morphology (Fig. 1, A–C). The optimal concentration for both iron chelators DFO and HBED (46) and for the iron(III) compounds Fe(OH)₃-dextrin and Fe(III) citrate was 1 mM (Fig. 1A). For the hydroxyl radical scavengers, Me₆SO (DMSO) in figures and mannoitconcentrations of 30 and 3 mM, respectively, fit the above mentioned requirement (Fig. 1B). Trolox, a water-soluble vitamin E derivative with chain-breaking capacity for lipid peroxidation, was used at a concentration of 100 μM, and BHT was used at a concentration of 10 μM (Fig. 1C), DDC, an inhibitor of copper/zinc superoxide dismutase, was used at a concentration of 10 μM, and ATZ, the catalase-inhibiting agent, was used at a concentration of 50 μM. BSO, the agent indirectly inhibiting the glutathione peroxidase, was used at a concentration of 50 μM (59).

**Iron is the Limiting Step in Controlling the Extent of the UVB Response—** We have used several experimental approaches to study the role of intracellular reactive oxygen species and iron in the signal transduction pathway of the UVB response in terms of MMP-1 and MMP-3 induction. First, the copper/zinc superoxide dismutase was inhibited with diethyl dithiocarbamate, thus increasing the intracellular concentration of superoxide anions. Second, the enzymes that detoxify intracellular hydrogen peroxide like the catalase and, independently, the glutathione peroxidase were inhibited with aminotriazol (for inhibition of catalase) and buthionine sulfoximine (for indirect inhibition of glutathione peroxidase). Both inhibitors have been used alone and in combination, leading to an increase in the concentration of intracellular hydrogen peroxide. Third, iron chelators, like DFO or HBED have been used to block the Fenton reaction, resulting in a decrease in the formation of hydroxyl radicals and lipid peroxides. Mechanistically, the Fenton reaction strongly depends on the presence of unchelated iron, in that ferrous iron (Fe²⁺) becomes oxidized to ferric iron (Fe³⁺) by hydrogen peroxide, thereby generating the highly aggressive hydroxyl radical, which exerts its toxicity and modulatory effects on gene expression mainly via the initiation of lipid peroxidation processes (12). Therefore, depletion of iron by chelating agents (desferrioxamine and others) is supposed to completely abrogate the formation of hydroxyl radicals.
to study the role of the iron-driven Fenton reaction in the UVB-dependent up-regulation of matrix-degrading metalloproteinases, confluent fibroblast monolayer cultures were preincubated with the above mentioned different chemical agents in Dulbecco’s modified Eagle’s medium for 18 h, subsequently irradiated with UVB at a dose of 200 J/m² (29), and subjected to Northern blot analysis and densitometry (Fig. 2, A and B). A further increase in intracellular superoxide anions or hydrogen peroxide via the inhibition of copper/zinc superoxide dismutase leading to an increase of intracellular hydrogen peroxide, DDC (an inhibitor of glutathione peroxidase), ATZ (an inhibitor of catalase; both agents leading to an increase in intracellular hydrogen peroxide), DDC or of catalase or glutathione peroxidase by ATZ or BSO, respectively, did not enhance the UVB-dependent MMP-1 response (Fig. 2, A and B); rather, the MMP-1 mRNA levels were slightly, although not significantly, diminished compared with that of the UVB-irradiated control ($p \geq 0.07$). However, the iron-chelating agent DFO alone or in any combination with other compounds significantly reduced the MMP-1 mRNA levels to 85% compared with the level of UVB-irradiated cells, which was set at 100% (Fig. 2A). Similar results were obtained by the iron chelator HBED (data not shown). In a previous paper (59), buthionine sulfoximine at the used concentration extremely reduced total glutathione and, thus, severely impaired the function of the classic glutathione peroxidase in dermal fibroblasts. Depending on the cell type, aminotriazol was demonstrated to significantly abrogate catalase activity by 80–90% (60, 61).

Preincubation of fibroblasts in ascorbate-free cell culture medium with different concentrations of Fe(III) citrate prior to UVB irradiation resulted in a 2.0-fold increase in MMP-1 mRNA levels compared with that of the UVB control (Fig. 2B). Similarly, intracellular increase in iron concentrations by preincubation fibroblast cultures with the iron compound Fe(OH)$_3$-dextrin substantially increased MMP-1 mRNA levels upon UVB irradiation (data not shown), further supporting the role of iron in mediating the UVB response. These data suggest that ferrous/ferric iron represents the limiting step controlling the UVB response. Preincubation of fibroblasts with the different chemical compounds without UVB irradiation only marginally increased MMP-1 mRNA levels compared with the mock-treated cells (data not shown).

Involvement of the Iron-driven Fenton Reaction and Lipid Peroxidation in the UVB Response—To study the involvement of hydroxyl radicals (HO) and lipid peroxides generated by the involvement of Fe$^{2+}$/Fe$^{3+}$ during the UVB-dependent induction of MMP-1 and MMP-3, confluent fibroblast monolayer cultures were preincubated with different nontoxic concentrations of Me$_2$SO, known for its hydroxyl radical scavenging capacity (Figs. 3A and 4A), or Trolox, a water-soluble α-tocopherol (vitamin E) derivative, for 18 h prior to UVB irradiation at a dose of 200 J/m² (Figs. 3B and 4B). Total RNA was isolated 24 h after irradiation and subjected to Northern blot analysis. UVB irradiation resulted in a 4.8-fold increase in MMP-1 mRNA levels (Fig. 3, A and B) and in an up to 3.8-fold increase in MMP-3 mRNA levels (Fig. 4, A and B), while only a low constitutive expression of both MMPs was detected in mock-irradiated cells (Figs. 3 and 4). Preincubation of fibroblasts with dimethyl sulfoxide prior to UVB irradiation led to a significant reduction of MMP-1 mRNA levels to 60–80% (Fig. 3A) and of MMP-3 mRNA levels to 70% (Fig. 4A) of the UVB-irradiated controls. Similar results were obtained by the alternate hydroxy radical scavenger mannitol (data not shown). Preincubation of fibroblasts with Trolox, known for its capacity to reduce lipid peroxidation, prior to UVB irradiation led to a significant reduction of specific MMP-1 mRNA levels ranging from 50 to 60% (Fig. 3B) and MMP-3 mRNA to 75% (Fig. 4B) of the UVB-irradiated controls (two-sided t tests).
the UVB-irradiated controls. Different nontoxic concentrations of Me2SO, mannitol, or Trolox without UVB irradiation did not exert significant effects on specific MMP-1 or MMP-3 mRNA levels (Figs. 3 and 4).

To further dissect the iron-dependent UVB effects on lipid peroxidation, fibroblast monolayer cultures were preincubated with the iron chelator DFO, the hydroxyl radical scavenger dimethyl sulfoxide, or the vitamin E derivative Trolox, characterized by its lipid peroxidation inhibiting potential at the indicated concentrations prior to UVB irradiation. Thereafter, lipid peroxidation end products were determined. As shown previously, UVB irradiation of fibroblast monolayer cultures at doses of 0.2, 0.3, and 1 kJ/m² resulted in a dose-dependent increase in lipid peroxidation end products like MDA and 4-HNE up to 3.5-fold compared with the mock-irradiated control (Fig. 5). Preincubation of fibroblasts with desferrioxamine at a concentration of 1 mM almost completely abrogated the UVB-induced increase in the concentration of MDA and 4-HNE. Although not completely, dimethyl sulfoxide at a concentration of 30 mM and Trolox at 100 μM significantly reduced the UVB-irradiated controls. Different nontoxic concentrations of Me2SO, mannitol, or Trolox without UVB irradiation did not exert significant effects on specific MMP-1 or MMP-3 mRNA levels (Figs. 3 and 4).

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![Figure 3](image3.png)

**Fig. 3.** Effect of the hydroxyl radical scavenger Me2SO (DMSO) and of the lipid peroxidation inhibitor Trolox on the UVB-mediated induction of MMP-1 mRNA levels. Confluent cells were preincubated with Me2SO (DMSO) (A) or Trolox (B) at the indicated concentrations for 18 h and were subsequently irradiated with UVB at a dose of 200 J/m². Total RNA was isolated 24 h after irradiation and subjected to Northern blot analysis. Densitometric data were standardized to 18 S ribosomal RNA and represent -fold increase over control, which was set at 1.0. A representative Northern blot of two independent experiments is shown.

![Figure 4](image4.png)

**Fig. 4.** Modulation of the UVB-mediated induction of MMP-3 mRNA levels by the hydroxyl radical scavenger Me2SO (DMSO) and by the lipid peroxidation inhibitor Trolox. Prior to UVB irradiation at a dose of 200 J/m², confluent cells were preincubated with Me2SO (DMSO) (A) or Trolox (B) at the indicated concentrations for 18 h. Thereafter, total RNA was isolated 24 h after irradiation and subjected to Northern blot analysis. Densitometric data were standardized to 18 S ribosomal RNA and represent -fold increase over control, which was set at 1.0. A representative Northern blot of two independent experiments is shown.

*P < 0.0001 (A and B) compared with UVB-irradiated cells (two-sided t test).
the increase in lipid peroxidation end products (Fig. 5), indirectly suggesting that, in fact, UVB irradiation initiates iron-driven processes like the Fenton reaction and lipid peroxidation.

Central Role of the Iron-driven Fenton Reaction and Lipid Peroxidation in the Regulation of the Signaling Pathway Leading to UVB-induced MMP-1—To address the question of whether specific key steps of the signal transduction pathway leading to the UVB-dependent MMP-1 and MMP-3 induction are affected by hydroxyl radicals or lipid peroxides, JNK2 activity and c-fos and c-jun mRNA levels were studied (Figs. 6 and 7; Table I). A 15-fold increase in JNK2 activity was measured with the maximum at 0.5 h and a subsequent decrease in JNK2 activity 2 h after UVB irradiation (Fig. 6A). Preincubation of cells with desferrioxamine, dimethyl sulfoxide, and Trolox prior to UVB irradiation significantly reduced JNK2 activity 0.5 h after irradiation to 55% compared with UVB-irradiated cells (Fig. 6B).

Time course analysis of c-fos mRNA levels after UVB irradiation of human dermal fibroblasts did not lead to a significant increase in mRNA levels compared with mock-irradiated cells (p > 0.07) (Table I). By contrast, time course analysis of c-jun mRNA levels resulted in a maximal 3.5-fold increase 2 h after UVB irradiation at a dose of 200 J/m² compared with mock-irradiated cells (Fig. 7A). Furthermore, preincubation of the cells with the iron chelator desferrioxamine, the hydroxyl radical scavenger dimethyl sulfoxide, and the lipid peroxidation inhibitor Trolox prior to UVB irradiation resulted in a 40–60% reduction of c-jun mRNA levels 2 h after UVB irradiation compared with UVB-irradiated fibroblasts (Fig. 7B).

**DISCUSSION**

In this study, we focused on the involvement of ferrous/ferric iron on two components of the UVB-mediated downstream signal transduction pathway, JNK2 activity and c-jun transcription, finally leading to an increase in interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) mRNA levels. We found that ferrous/ferric iron plays a central role in mediating the UVB response. Furthermore, the iron-driven generation of hydroxyl radicals (HO·) and lipid peroxides were identified as early events in the downstream signaling pathway after UVB irradiation, finally leading to the induction of MMP-1 on RNA levels.

MMP and reactive oxygen species play a major role in the multistage processes of carcinogenesis and cutaneous photoaging. Accordingly, interstitial collagenase (MMP-1) and stromelysin-1 (MMP-3) are overexpressed during invasion and metastasis in a variety of tumors including nonmelanoma skin cancer (62). Epidermis-specific overexpression of MMP-1 in a transgenic mouse model results in an overall higher tumor susceptibility and appears to be involved in the initiation of tumorigenesis (63). Furthermore, photoaging characterized by severe
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**Fig. 7.** Time course analysis of UVB-mediated induction of c-jun mRNA levels and its modulation by the iron chelator DFO, the hydroxyl radical scavenger MeSO, and Trolox, an inhibitor of lipid peroxidation. After UVB irradiation of confluent human dermal fibroblasts at a dose of 200 J/m² and further incubation for 0.5, 1, 2, 4, and 8 h with medium that prior to UVB irradiation had been on the cells for 7 days, total RNA was isolated and subjected to Northern blot analysis. A, 1, mock-irradiated control. 2, 0.5 h; 3, 1 h; 4, 2 h; 5, 4 h; 6, 8 h after UVB irradiation. *, p = 0.0039 compared with mock-irradiated cells (two-sided Welch test). B, prior to UVB irradiation, fibroblasts were preincubated with DFO, MeSO, and Trolox for 18 h and thereafter irradiated with total UVB at a dose of 200 J/m². After UVB irradiation and a further incubation period of 2 h with conditioned medium as described in A, total RNA was isolated and subjected to Northern blot and densitometric analysis. **, p < 0.03 compared with UVB-irradiated cells (two-sided Welch test). Densitometric data were standardized to 18 S ribosomal RNA and represent fold increase over control, which was set at 1.0. Three independent experiments were performed.

Under physiological conditions, reactive oxygen species are part of normal regulatory circuits, and the cellular redox state is tightly controlled by antioxidants. However, increased concentrations of ROS and loss of cellular redox homeostasis following UVB irradiation can be tumorigenic and promote premature aging (21, 33). We were recently able to establish a causal relationship between increased production of distinct reactive oxygen species and enhanced MMP-1 synthesis (28, 59, 65). In this context, it is interesting that some tumor cells generating high levels of reactive oxygen species reveal a higher invasive potential, most likely due to the ROS-dependent induction of MMPs with subsequent breakdown of the peritumoral connective tissue (26). The major finding of this report is that the iron-driven Fenton reaction with the formation of the highly aggressive hydroxyl radical and lipid peroxidation, via the stress-activated protein kinase JNK2, is responsible for the substantial up-regulation of MMP-1 and MMP-3 mRNA levels after UVB irradiation. These results are of considerable interest, because they imply potential antioxidant approaches for controlling tumor progression and aging. In fact, the UVB-induced iron-dependent signaling events responsible for the up-regulation of MMP-1 were almost completely abrogated by iron chelators such as DFO and HBED. Also, MeSO and mannitol, both scavengers for hydroxyl radicals (66), and Trolox (67), an inhibitor of lipid peroxidation, substantially reduced major steps in this UVB-dependent signaling pathway. In contrast to Trolox, the UVB-mediated increase in MMP-1 mRNA levels could not be prevented by BHT, another inhibitor of lipid peroxidation. BHT and other phenolic antioxidants have been shown to substantially increase c-fos and c-jun mRNAs in quiescent human hepatoma HepG2 cells, leading to an elevated AP-1 DNA binding activity (68). A similar mechanism may also occur in dermal fibroblasts.

The fact that hydroxyl radical scavengers and suppression of lipid peroxidation similarly inhibited the UVB-induced MMP-1 and MMP-3 mRNA levels indirectly suggests a linear sequence of events where it does not matter for the overall response which component of this sequence is antagonized. Therefore, we suggest that UVB-induced superoxide anions (O₂⁻) and hydrogen peroxide (H₂O₂) react with ferrous/ferric iron to generate hydroxyl radicals (HO•), which subsequently initiate lipid peroxidation. This sequence of events has been reported in other experimental settings (16, 17, 69, 70).

Here, we studied the effect of the iron-dependent UVB response on JNK2 activity and c-jun mRNA synthesis. We particularly focused on these steps, because it was shown that UVB-mediated MMP-1 induction is based on the activation of stress-activated protein kinases like JNKs and an increase in c-jun-containing AP-1 complexes leading to increased AP-1 activity, which stimulates the transcription of distinct MMP genes (71). While we, in fact, found a strong UVB-dependent up-regulation of JNK2 activity with subsequently increased c-jun mRNA levels, we did not observe any significant increase in c-fos mRNA levels in fibroblasts upon UVB irradiation, supporting previous data by Fisher et al. (71) and Ariizumi et al. (72). These authors report that UVB irradiation affects neither c-fos mRNA levels nor the activities of extracellular stimulus-responsive kinases (ERK-1, ERK-2) in fibroblasts in vitro and in vivo. Interestingly, a UVB-dependent and reactive oxygen species-dependent ERK-initiated induction of c-fos was observed in tumor cells, primary keratinocytes, and various epidermal cell lines upon UVB irradiation (39, 73). Our find-
ings in conjunction with these data suggest that, depending on the cell type, c-fos is differently regulated on the transcriptional level following UVB irradiation.

Our data are consistent with a model whereby UVB preferentially activates members of the stress-activated protein kinase (JNK) group of mitogen-activated protein kinases (particularly JNK2), which, as confirmed in this paper, rapidly phosphorylate both c-Jun and ATF2 (35, 41). Here, we corroborate and extend these data and provide evidence that iron-dependent reactions like the Fenton reaction and lipid peroxidation are the underlying mechanisms of the UVB-induced JNK2 activity and c-jun transcription. Thus, our results perfectly fit with an earlier published model for the role of increased load of reactive oxygen species in carcinogenesis (21) in that the iron-driven generation of hydroxyl radicals and lipid peroxides activates signal transduction pathways and modulates the activity of genes that regulate effector genes related to tissue degradation. A variety of reports have outlined the regulatory effects of reactive oxygen species on the expression of several genes including c-fos and c-jun (74–76). However, in these studies, a potential role for iron has not been addressed.

The exact biophysical and molecular mechanisms underlying signal transduction induced by reactive oxygen species are as yet unknown. Possible mechanisms include oxidant-macromolecule interaction and alterations in the overall and local cellular redox status as well as calcium signaling (for a review, see Ref. 77). Also, the upstream signaling steps preceding the induction the JNK activity, c-jun and MMP-1 mRNA levels after UVB irradiation, and the potential involvement of cytokine networks in these processes, as shown for the UVA response (78, 79), have not been elucidated. However, there are some indications that activation of AP-1 is mediated by membrane-associated Src tyrosine kinases and Ha-Ras GTP-binding proteins after ultraviolet C irradiation (80).

UVB intensities as used in this study in vitro can be absorbed readily by fibroblasts within the skin in vivo (81). This, in conjunction with data indicating that UVB, in fact, releases ferrous/ferric iron and induces MMPs in the skin (18, 33), points to the relevance of our data particularly in the context of increased UVB fluxes on the earth due to stratospheric ozone depletion (82, 83).

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REFERENCES
1. Hentze, M. W. (1995) Inducible Gene Expression (Baeuerle, P. A., ed) Vol 1, pp. 241–265, Birkhauser, Boston.
2. Jaffrey, S. R., Haie, D. J., Klauser, R. D., and Harford, J. B. (1993) Nucleic Acids Res. 21, 4627–4631.
3. Melefont, O., and Hentze, M. W. (1993) Blood 75, 251–258.
4. Oshiro, S., Nakamura, Y., Ishige, R., Hori, M., Nakajima, H., and Gahl, W. A. (1994) J. Biochem. (Tokyo) 115, 849–852.
5. Burdon, R. H., Gill, V., and Rice-Evans, C. (1989) Free Radical Res. Commun. 7, 149–159.
6. Haq, R. U., Werely, J. P., and Chitambar, C. R. (1995) J. Cell. Biochem. 59, 351–360.
7. Lunt, D. H., Gill, Y., von Zastrow, M., and Zierath, J. (1995) J. Biol. Chem. 270, 4726–4732.
8. Scharffetter-Kochanek, K. (1995) J. Invest. Dermatol. 104, 194–198.
9. Brenneisen, P., Oh, J., Wlaschek, M., Menken, J., Briviba, K., Hennig, M., Hennig, G., Sies, H., and Scharffetter-Kochanek, K. (1996) Photochem. Photobiol. 64, 649–657.
10. Aho, S., Rouda, S., Kennedy, S. H., Qin, H., and Tan, E. M. L. (1997) Eur. J. Biochem. 247, 503–510.
11. Schaich, K. M. (1992) Blood Cells 18, 219–230.
12. Minotti, G., and Aust, S. D. (1992) Lipids 27, 219–226.
13. Lin, F., and Giro, A. W. (1993) Arch. Biochem. Biophys. 300, 714–723.
14. El-Sharkawy, A., and Steiner, M. G. (1990) CR Rend. Seances Soc. Biol. Fil. 184, 199, 455–465.
15. Aramori, H., Asui, T., and Sakurai, H. (1995) Biochem. Biophys. Res. Commun. 206, 474–479.
16. Perez, S., Sergent, O., Morel, P., Chevannne, M., Dubon, M. P., Cillard, P., and Cillard, J. (1995) CR Acad. Sci. Paris 320, 189, 455–465.
17. Bissett, D. L., Chatterjee, R., and Hannon, D. P. (1991) Photochem. Photobiol. 54, 215–223.
18. Masaki, H., Asumi, T., and Sakurai, H. (1995) J. Dermatol. Sci. 10, 25–34.
19. Biemond, P., Swaki, A. J. G., van Eijk, H. G., and Koster, J. F. (1988) Free Radical Biol. Med. 4, 185–198.
20. Cerutti, P. A. (1994) Lancet 344, 862–863.
21. Black, H. S. (1987) Photochem. Photobiol. 46, 213–221.
22. Gilchrist, B. A. (1989) J. Am. Acad. Dermatol. 21, 610–613.
23. Scharffetter-Kochanek, K., Wlaschek, M., Aebi, T., Schottorath, A., Goerz, G., Krieg, T., and Piegiew, G. (1991) Arch. Dermatol. Res. 283, 506–511.
24. Scharffetter-Kochanek, K., Wlaschek, M., Bosen, K., Herrmann, G., Lehmann, P., Goerz, G., Mauch, C., and Piegiew, G. (1992) The Environmental Threat to the Skin (Piegiew, G., and Marks, R., eds) pp. 72–82, Martin Dunitz Publishers, London.
25. Crawford, H. L., and Matrisian, L. M. (1994) Invasion Metastasis 14, 234–245.
26. Herrmann, G., Wlaschek, M., Lange, T. S., Prentzel, K., Goerz, G., and Scharffetter-Kochanek, K. (1993) Exp. Dermatol. 2, 92–97.
27. Wlaschek, M., Briviba, K., Sies, H., and Scharffetter-Kochanek, K. (1995) J. Invest. Dermatol. 105, 1753–1757.
28. Brenneisen, P., Oh, J., Wlaschek, M., Menken, J., Briviba, K., Hennig, M., Hennig, G., Sies, H., and Scharffetter-Kochanek, K. (1996) Photochem. Photobiol. 64, 649–657.
29. Alcantara, O., Obeid, L., Hannun, Y., Ponka, P., and Boldt, D. H. (1994) Blood 84, 3510–3517.
30. Scharffetter-Kochanek, K. (1993) FEBS Lett. 321, 209–218.
31. Masaki, H., Asumi, T., and Sakurai, H. (1995) Biochem. Biophys. Res. Commun. 206, 474–479.
69. Iizawa, O., Kato, T., Tagami, H., Akamatsu, H., and Niwa, Y. (1994) Arch. Dermatol. Res. 286, 47–52
70. Stobs, S. J., and Bagchi, D. (1995) Free Radical Biol. Med. 18, 321–336
71. Fisher, G. J., Talwary, H. S., Wang, Z.-Q., McPhillips, F., Kang, S., and Voorhees, J. (1996) J. Invest. Dermatol. 107, 449
72. Ariizumi, K., Bergstresser, P. R., and Takashima, A. (1996) J. Dermatol. Sci. 12, 147–155
73. Garmyn, M., and Degreef, H. (1997) J. Photochem. Photobiol. B Biol. 37, 125–130
74. Shibanuma, M., Kuroki, T., and Nose, K. (1990) Oncogene 5, 1025–1032
75. Nose, K., Shibanuma, M., Kikuchi, K., Kageyama, H., Sakiyama, S., and Kuroki, T. (1991) Eur. J. Biochem. 201, 99–106
76. Sen, C. K., and Packer, L. (1996) FASEB J. 10, 709–720
77. Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997) Free Radical Biol. Med. 22, 269–285
78. Bender, K., Blattner, C., Knebel, A., Iordanov, M., Herrlich, P., and Rahmsdorf, H. J. (1997) J. Photochem. Photobiol. B Biol. 37, 1–17
79. Wlaschek, M., Heinen, G., Poswig, A., Schwarz, A., Wilmoth, F., Krieg, T., and Scharffetter-Kochanek, K. (1994) Photochem. Photobiol. 59, 550–556
80. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) Cell 71, 1081–1091
81. Bruls, W. A. G., Slaper, H., van der Leun, J. C., and Berrens, L. (1984) Photochem. Photobiol. 40, 485–494
82. Slaper, H., Velders, G. J., Daniel, J. S., de Grujil, F. R., and van der Leun, J. C. (1996) Nature 384, 256–258
83. Crutzen, P. J. (1992) Nature 356, 104–105
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