Low and High Dose UVB Regulation of Transcription Factor NF-E2-Related Factor 2

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Abstract

Transcription factor NF-E2-related factor 2 (Nrf2) regulates antioxidant response element (ARE)–mediated expression and coordinated induction of chemoprotective proteins in response to chemical stress. In this report, we investigated Nrf2 response to low and high dose UVB irradiation. Low dose (7.5 J/m²) UVB exposure of mouse hepatoma, mouse keratinocyte, and human skin fibroblast cells led to the nuclear accumulation of Nrf2 and up-regulation of ARE-mediated gene expression. On the contrary, and intriguingly, high dose (20 J/m²) UVB exposure of cells led to the nuclear exclusion of Nrf2 and down-regulation of chemoprotective gene expression with possible implications in UVB carcinogenesis. We investigated the mechanism by which high dose UVB induced the nuclear exclusion of Nrf2. Prior treatment with nuclear export inhibitor, leptomycin B, abrogated the UVB-induced nuclear exclusion of Nrf2, indicating that the decrease of Nrf2 in the nucleus was due to the nuclear export of Nrf2. High dose UVB increased the phosphorylation of Nrf2Y568 which stimulated the nuclear export of Nrf2. Mutation of Nrf2Y568 to phenylalanine and src kinase inhibitor PP2 abrogated the UVB-induced phosphorylation of Nrf2Y568 and nuclear exclusion of Nrf2. Transfection with src family member Fyn small interfering RNA resulted in the nuclear exclusion of Nrf2 and down-regulation of ARE-mediated chemoprotective gene expression. (Cancer Res 2006; 66(17): 8421-9)

Introduction

Cellular exposure to antioxidants and xenobiotics leads to coordinated induction of a battery of chemoprotective genes including quinone oxidoreductases, NQO1 and NQO2 (1, 2). NQO1 and NQO2 protect cells against oxidative stress and neoplasia (1, 2). Mice lacking the expression of NQO1 or NQO2 were generated (3, 4). Mice deficient in NQO1 and NQO2 showed myelogenous hyperplasia and increased sensitivity to chemical-induced skin carcinogenesis (4–7). A cytosine to thymidine (C → T) polymorphism in exon 6 of the human NQO1 gene produces a proline to serine (P187S) substitution that destabilizes and inactivates the enzyme (1, 8, 9). NQO1 P187S has been associated with greater risk of neutropenia in benzene-exposed adult Chinese workers (8) and is significantly overexpressed in therapy-related and de novo leukemias in adults and infants (9). Recent studies have shown a significant association between NQO1 P187S polymorphism with cigarette smoke–related bladder cancer (10), breast cancer (11), and esophageal cancer (12). The other genes that are coordinately induced with the NQO1 and NQO2 gene include glutathione S-transferases, which conjugate hydrophilic electrophiles and reactive oxygen species with glutathione and γ-glutamylecysteine synthetase, which plays a key role in the regulation of glutathione metabolism (13). In summary, the coordinated induction of these genes provides necessary protection for cells against free radical damage, chemical-induced oxidative stress, and neoplasia.

Antioxidant response element (ARE) present in the promoter of chemoprotective genes and nuclear factor, NF-E2-related factor 2 (Nrf2), regulate the expression and coordinated induction of chemoprotective proteins in response to antioxidants and xenobiotics (13). Nrf2 belongs to the family of basic leucine zipper/cap’n’collar proteins (bZIP; ref. 13). Nrf2−/− mice are viable and live to adulthood, showing that Nrf2 is not required for erythropoiesis, development, or growth (14). A cytosolic inhibitor of Nrf2, I nrf2 (inhibitor of Nrf2) or KEAP1 (Kelch-like ECH-associated protein 1), was reported (15, 16). In nrf2 retains Nrf2 in the cytoplasm. The treatment of cells with antioxidants and xenobiotics leads to the release of Nrf2 from In rnf2. Nrf2 translocates into the nucleus and induces the expression of chemoprotective genes. Reports showed that Nrf2 binding to In rnf2 leads to the degradation of Nrf2 (17–19). The antioxidants and xenobiotics lead to the dissociation of Nrf2 from In rnf2 and stabilization of Nrf2 followed by nuclear translocation. Disruption of In rnf2 in mice leads to postnatal death, probably from malnutrition resulting from hyperkeratosis in the esophagus and forestomach, presumably due to the nuclear accumulation of Nrf2 (20). ARE-mediated gene expression was found to be de-repressed in In rnf2 knockout mice (20).

The signal transduction pathway that leads from antioxidants and xenobiotics to Nrf2, Jun, Fos, and Mafs, the proteins that bind ARE and regulate ARE-mediated expression of antioxidant genes, remains largely unknown. It is believed that superoxides and electrophiles generated during metabolism might act as second messengers, activating ARE-mediated expression of a host of antioxidant genes including NQO1, glutathione S-transferaseYa, γ-GCS, and HO-1 (13). The superoxide and/or electrophilic signal presumably pass through unknown cytosolic factor(s). This factor then catalyzes the modification of In rnf2 and/or Nrf2. As a result, Nrf2 is released from In rnf2. Nrf2 then translocates to the nucleus where it heterodimerizes with unknown heterodimeric partner(s) and induces the expression of NQO1 and other ARE-regulated genes. Several cytosolic kinases that include protein...
kinase C (PKC), mitogen-activated protein kinase, p38, and phosphatidylinositol-3-kinase have been shown to modify Nrf2 and participate in the mechanism of signal transduction from antioxidants and xenobiotics to the ARE (19, 21–30). Among these, two independent studies have shown antioxidant-induced PKC phosphorylation of serine 40 in Nrf2, leading to the dissociation of Nrf2 from IκBα (19, 27). Recently, it was shown that reoxid modulation of cyteine in Nrf2 is capable of releasing Nrf2 from IκBα (31–33). It is possible that this mechanism is redundant to the phosphorylation of Nrf2 by PKC, or that the two mechanisms work in concert. Recently, we identified and characterized a bipartite nuclear localization signal and a leucine-rich nuclear export signal which regulates Nrf2 shuttling in and out of the nucleus (34). The overall distribution of Nrf2 is the result of the balance between these two processes (34).

Skin is constantly exposed to environmental insults including UV irradiation. In particular, UVB irradiation (wavelengths between 280 and 320 nm) is a major risk factor for the development of skin cancer (35). In addition, evidence has been accumulating which shows that cellular responses differ between low and high dose UVB. It is high dose UVB which causes the development of highly aggressive skin squamous cell carcinoma (35). Earlier reports have shown the UV induction of phase II or chemoprotector gene expression (36). Therefore, the role of Nrf2 in cellular responses to UVB is expected but remains unknown.

In the current report, we investigated the Nrf2 response to different doses of UVB irradiation and its effect(s) on the induction of chemoprotective proteins. We observed that low dose UVB exposure led to the nuclear accumulation of Nrf2 and activation of chemoprotective proteins. These results with low dose UVB irradiation were similar to that previously observed with antioxidants. However, intriguingly, we found that high dose UVB exposure of mouse hepatoma, mouse keratinocyte, and human fibroblast cells led to the nuclear exclusion of Nrf2 and the down-regulation of ARE-mediated gene expression. Further experiments showed that high dose UVB induced nuclear translocation of Fyn kinase that phosphorylated Nrf2Y568, leading to the stimulation of nuclear export and the exclusion of Nrf2.

Materials and Methods

Plasmids
The construction of the pcDNA-Nrf2-V5 and pGL2-NQO1 gene ARE-luciferase (NQO1 ARE-Luc) has been previously described (37). The forward primer 5′-GATGAGGATGAAAGCTTTCCATCCTTCCCCATGGA-3′ and reverse primer 5′-AGGCCTTCATCCTCACTACAGTAAACATGCT-3′ containing mutated tyrosine sequences were used in PCR to generate mutant plasmid pcDNA-Nrf2Y568F from template pcDNA-Nrf2-V5. The mutation of tyrosine to phenylalanine was confirmed by sequencing.

Cell Culture
Mouse hepatoma (Hepa-1) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (40 units/mL), and streptomycin (40 μg/mL); Invitrogen, Carlsbad, CA). Human skin fibroblasts from six human subjects were obtained from the Coriell Cell Repository (Camden, NJ). These cells were procured with consent and approval from the Institutional Review Board of Baylor College of Medicine, Houston, TX. The fibroblasts were grown in MEM supplemented with 15% FBS. The Hepa-1 and fibroblast cells were grown in an incubator at 37°C in 95% air and 5% CO2.

Mouse keratinocytes were established from benzo(a)pyrene-induced skin tumors using standard procedures. The tumors were surgically removed, placed in high calcium (HiCa; 200 mM/L) supplemented with antibiotics and antimycotic agents, minced in collagenase, and the contents were removed and incubated at 37°C for 2 hours with agitation. The supernatant was removed after clumps had settled. HiCa (10 mL) was added to the clumps, allowed to settle for 5 minutes before the supernatant was removed. The process of HiCa treatment was repeated. HiCa/keratinocyte growth factor was added to the clumps and plated in the four corner wells of collagen-coated 12-well plates. The medium was replaced with low calcium (50 mM/L)/keratinocyte growth factor with frequent changes of the medium. The growing keratinocyte cells were collected and cultured in DMEM supplemented with 10% FCS and antibiotics. The karyotyping analysis did not reveal loss/gain or translocation of chromosome(s) in established skin tumor cells (data not shown).

UVB Irradiation, Subcellular Localization of Nrf2, and NQO1 Gene Expression
UVB irradiation. Mouse hepatoma (Hepa-1), mouse keratinocyte, and human skin fibroblast cells were grown in six-well plates and Lab-Tek II chamber slides. The cells were grown in a monolayer until 50% confluent. The cells in six-well plates were untransfected or transfected with NQO1 ARE-luciferase (0.2 μg) and renilla luciferase (0.02 μg) plasmids using Effectene Transfection Reagent (Qiagen, Valencia, CA) and the manufacturer’s supplied protocol. The media from untransfected and transfected cells were replaced with PBS (pH 7.4; Invitrogen) and the cells exposed to UVB (0, 50, 7.5, 10, and 20 J/m²). The UVB protocol and facility was availed from the laboratory of Professor Stephen E. Ullrich, Department of Immunology, University of Texas M.D. Anderson Cancer Center, Houston, TX. The source of UVB was generated by using one FS40 sunlamp (National Biological, Twinsburg, OH), which emit 60% of their radiation within the UV range and have a peak emission at 313 nm. A thin layer of PBS-covered cells were placed 20 cm below the UV source. The emitted radiation was checked by IL-700 radiometer (International Light, Newburyport, MA). Immediately after UVB irradiation, the cells were incubated for different time points at 37°C in a humidified atmosphere of 5% CO2 with DMEM containing 10% FBS.

Subcellular fractionation and Western analysis. Cytosolic and nuclear extracts from mouse Hepa-1, keratinocyte, and human skin fibroblast cells were prepared as described previously (34). The protein concentration was determined using the Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). One hundred micrograms of the cell fraction was resolved on a 10% SDS-PAGE, Western blotted, and probed with Nrf2 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by horseradish peroxidase–conjugated secondary antibody. To confirm the purity of subcellular fractionation, the extracts were Western blotted and probed with cyttoplasm-specific anti–lactate dehydrogenase (LDH) antibody (Chemicon International, Temecula, CA) and nuclear specific anti-lamin B antibody (Santa Cruz Biotechnology).

Luciferase assay. Mouse Hepa-1, mouse keratinocyte, and human skin fibroblast cells were grown in six-well plates, transfected with NQO1 gene ARE-luciferase/renilla luciferase plasmids and exposed to UVB and analyzed for luciferase activity by procedures described previously (34). Each set of transfection experiments were repeated thrice.

Immunohistochemistry. The cells grown on Lab-Tek II chamber slides were fixed with 3% formaldehyde (Polysciences, Inc., Warrington, PA) and permeabilized with cold acetone. Slides were blocked with 3% normal rabbit serum, rinsed thrice in PBS for 5 minutes, incubated with secondary-FITC antibodies (1:1,000) for 1 hour at room temperature, rinsed twice in PBS for 20 minutes, and then analyzed. To visualize the nuclei, cells were stained with Hoechst stain (Bio-Rad Laboratories). The fluorescent images were captured using appropriate filters in a Nikon eclipse TE 2000-U fluorescent microscope fitted with a Photometrics CoolSnap CF camera, and images were enhanced using Adobe Photo-Deluxe software.

Subcellular Localization of Nrf2 and Mutant Nrf2Y568F, and Expression of NQO1 and ARE-Luciferase Gene Expression in Response to UVB
Effect of leptomycin B on endogenous Nrf2 localization. Mouse Hepa-1 cells were grown. The cells were untreated or pretreated with...
nuclear export inhibitor leptomycin B (20 ng/mL; EMD Biosciences, Inc., Darmstadt, Germany) for 6 hours. This was followed by exposure to 20 J/m² UVB in the absence or presence of leptomycin B and harvested after 30 minutes of incubation in DMEM + FBS. The cells were homogenized, subcellularly fractionated to generate cytosol and nuclear fractions, and then analyzed for the localization of Nrf2 by SDS-PAGE, Western blotting, and probing with anti-Nrf2 antibody using procedures described above and reported earlier (34).

Effect of nuclear export inhibitor leptomycin B on transfected Nrf2 and mutant Nrf2Y568F localization and NQO1 gene expression. Mouse Hepa-1 cells were grown in six-well plates. The cells were transfected with 0.2 μg of pcDNA-Nrf2-V5 or mutant pcDNA-Nrf2Y568F-V5 in separate experiments using the Effectene transfection reagent from Qiagen. Twenty-four hours after transfection, the cells were treated with leptomycin B and UVB as described before for untransfected cells. The cells were harvested 30 minutes after UVB irradiation and incubation in DMEM supplemented with FBS. The cells were homogenized, fractionated to generate cytosol and nuclear fractions, and analyzed for localization of Nrf2-V5 and Nrf2Y568F-V5 by SDS-PAGE, Western blotting, and probing with anti-V5 antibody using the procedures described above and reported earlier (34). In related experiments, the cells were cotransfected with 0.2 μg of pcDNA-Nrf2-V5 or mutant pcDNA-Nrf2Y568F-V5 and 0.2 μg of NQO1 gene ARE-luciferase plasmids. Renilla luciferase plasmid (0.02 μg) was included in each transfection to normalize transfection efficiency. The cells were treated with leptomycin B and UVB as described before. The cells after leptomycin B and UVB treatment were incubated for 8 hours in DMEM + FBS and analyzed for luciferase and endogenous NQO1 activities by procedures as previously described (19, 34). Each set of transfection experiment was repeated thrice.

Effect of Src Inhibitor, PP2, on Subcellular Localization of Nrf2 and Mutant Nrf2Y568F and NQO1 Gene Expression

Similar experiments as described above were done for src kinase inhibitor PP2. The leptomycin B was replaced with 1 μmol/L of PP2 (EMD Biosciences) and the pretreatment was done for 2 hours. Each set of transfection experiments was repeated thrice.

Immunoprecipitation and Tyrosine Phosphorylation of Nrf2 and Nrf2Y568F in Response to UVB

The cytosolic and nuclear extracts from untransfected, Nrf2-V5, and Nrf2Y568F-V5 transfected control and UVB-exposed Hepa-1 cells were prepared as described in Materials and Methods. The cytosolic and nuclear extracts were incubated with anti-V5 (Invitrogen) or anti-phosphotyrosine (Cell Signaling Technology, Beverly, MA) antibody (20 μL per 500 μL reaction) for 12 hours at 4°C on a rotator, followed by precipitation of the Nrf2-V5 and Nrf2Y568F-V5 complex by incubating with Protein A/G agarose (Santa Cruz Biotechnology) for 40 minutes at 4°C. The mixture was then centrifuged at 10,000 rpm for 10 seconds at 4°C, and the beads washed twice with 1% NP40 buffer without any inhibitors. Washed beads were treated with SDS loading buffer and analyzed by Western blot using anti-V5 and anti-phosphotyrosine antibodies.

Fyn Small Interfering RNA Inhibition and UVB Regulation of Nrf2

Predesigned mouse Fyn small interfering RNA (siRNA) was employed to inhibit Fyn and study its effect on Nrf2 response to UVB. Mouse Fyn siRNA

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**Figure 1.** Differential response of Nrf2 to low and high dose UVB in mouse hepatoma (Hepa-1) cells. A, Nrf2 response to UVB. Hepa-1 cells were irradiated with low and high doses of UVB. The cells were incubated for 30 minutes after exposure to UVB, homogenized, and cytosolic and nuclear fractions prepared by standard procedures. One hundred micrograms of cytosolic and nuclear proteins were separated on SDS-PAGE, Western blotted, and probed with anti-Nrf2, anti-lamin B and anti-LDH antibodies. Nuclear marker lamin B and cytosolic marker LDH were used to show the purity of nuclear and cytosolic fractions. B, Western analysis of endogenous NQO1 gene expression. Hepa-1 cells were exposed to UVB and incubated for 8 hours after UVB exposure. The cells were harvested and cytosolic fractions analyzed for NQO1 protein by SDS-PAGE, Western blotting, and probing with anti-NQO1 antibody. The blot was stripped and probed with β-actin antibodies. C, NQO1 ARE-luciferase expression. Hepa-1 cells were transfected with NQO1 ARE-luciferase, irradiated with UVB, and incubated for 8 hours. The cells were harvested, lysed, and analyzed for luciferase activity. Columns, mean of three independent transfection experiments; bars, ± SE. D, immunohistochemistry. Hepa-1 cells were grown on Lab-Tek II slides, fixed, and probed with anti-Nrf2 followed by FITC-tagged secondary antibody. To visualize the nuclei, the cells were stained with Hoechst stain. The fluorescent images were captured using appropriate filters in a Nikon eclipse TE 2000-U fluorescent microscope fitted with a Photometrics CoolSnap CF camera. Images were enhanced using Adobe Photo-Deluxe software.
irradiation resulted in translocation of Nrf2 from cytosol to the nucleus (Fig. 1A). This was evident from the accumulation of Nrf2 in the nucleus and consequent decrease of Nrf2 in the cytoplasm. Nrf2 accumulation in the nucleus was significantly higher in cells irradiated with 7.5 J/m² UVB as compared with 5 J/m² UVB. Interestingly, the irradiation of Hepa-1 cells with 10 and 20 J/m² showed UVB concentration-dependent nuclear exclusion of Nrf2 and consequent increase in cytosolic Nrf2. The 20 J/m² UVB-induced nuclear exclusion of Nrf2 was significant as compared with 7.5 J/m² UVB and also with Hepa-1 cells unirradiated with UVB.

Immunohistochemistry analysis supported data from Western nuclear. Hepa-1 cells in culture were exposed to 7.5 and 20 J/m² UVB, incubated for 30 minutes in DMEM + FBS. The cells were harvested after incubation, and cytosolic and nuclear extracts were prepared and analyzed for subcellular localization by SDS-PAGE, Western blotting, and probing with anti-Nrf2 and anti-Fyn antibodies.

**UVB Exposure and Fyn Localization**

Hepa-1 cells in culture were exposed to 7.5 and 20 J/m² UVB, incubated for 30 minutes in DMEM + FBS. The cells were harvested after incubation, and cytosolic and nuclear extracts were prepared and analyzed for subcellular localization by SDS-PAGE, Western blotting, and probing with anti-Fyn and anti-Nrf2 antibodies.

**Results**

Exposure of Hepa-1 cells to low doses (5 and 7.5 J/m²) of UVB irradiation resulted in translocation of Nrf2 from cytosol to the nucleus (Fig. 1A). This was evident from the accumulation of Nrf2 in the nucleus and consequent decrease of Nrf2 in the cytoplasm. Nrf2 accumulation in the nucleus was significantly higher in cells irradiated with 7.5 J/m² UVB as compared with 5 J/m² UVB. Interestingly, the irradiation of Hepa-1 cells with 10 and 20 J/m² showed UVB concentration-dependent nuclear exclusion of Nrf2 and consequent increase in cytosolic Nrf2. The 20 J/m² UVB-induced nuclear exclusion of Nrf2 was significant as compared with 7.5 J/m² UVB and also with Hepa-1 cells unirradiated with UVB. Immunohistochemistry analysis supported data from Western

with sense 5'-CCUCGAAUUACGUAGUUt-3' and antisense 5'-CAUACGAAAUUGGAGT-3' oligonucleotides were procured from Ambion-RNA (Austin, TX). The Hepa-1 cells in 60 mm dishes were transfected with varying concentrations of pre-designed mouse Fyn siRNA and NQO1 ARE-luciferase by procedures described previously (38), and then exposed to UVB 24 hours after transfection. The cells were harvested 8 hours after UVB exposure and analyzed for luciferase activity. In related experiments, Hepa-1 cells were transfected with 100 nmol/L of Fyn siRNA, exposed to UVB, and cytosolic and nuclear extracts were prepared. One hundred micrograms of protein was used to analyze the effect of Fyn siRNA on UVB-induced nuclear exclusion of Nrf2. The blot was stripped and probed with nuclear marker lamin B and cytosolic marker LDH.

**Figure 2.** Differential response of Nrf2 to low and high dose UVB in human skin fibroblast and mouse keratinocyte cells. A, Nrf2 response to UVB in human fibroblast cells. Human skin fibroblast cells were cultured and irradiated with low and high doses of UVB. The cells were harvested after 8 hours incubation for SDS-PAGE, Western blotting, and probing with anti-Nrf2, anti-lamin B, and anti-LDH antibodies. Western blot analysis was carried out to show the purity of nuclear and cytoplasmic fractions. Densitometric analysis of Western blots was done to determine fold alteration in nuclear Nrf2 in response to low and high dose UVB (right), columns, mean of three to six independent experiments; bars, ± SE. B, Western analysis of endogenous NQO1 gene expression. Human fibroblast cells were exposed to UVB and incubated for 8 hours after UVB exposure. The cells were harvested and cytosolic fractions analyzed for NQO1 protein by SDS-PAGE, Western blotting, and probing with anti-NQO1 antibody. The blot was stripped and probed with anti-β-actin antibody to show equal loading of proteins among the lanes. C, ARE-luciferase expression. Human fibroblast cells were transfected with NQO1 ARE-luciferase, irradiated with UVB, and incubated for 8 hours. The cells were homogenized, and cytosolic and nuclear fractions prepared. One hundred micrograms of cytosolic and nuclear proteins were separated on SDS-PAGE, Western analysis-Nrf2 response to low and high dose UVB in mouse keratinocytes. Mouse keratinocytes were exposed to UVB, harvested, and nuclear extracts prepared by standard procedure. SDS-PAGE and Western analysis analyzed Nrf2.
analysis and showed nuclear exclusion of Nrf2 in response to 20 J/m² UVB irradiation of cells (Fig. 1D). The data shown in Fig. 1 is representative (mean ± SE) of three independent experiments with similar results.

Irradiation of human skin fibroblast to the various doses of UVB showed similar results as UVB irradiation of Hepa-1 cells (Fig. 2). Nrf2 accumulated in the nucleus in response to 7.5 J/m² UVB irradiation and excluded from the nucleus in response to 20 J/m² UVB irradiation of fibroblast cells (Fig. 2A). Nrf2 regulated NQO1 gene expression and ARE-mediated luciferase gene expression showed a direct correlation with alterations in nuclear Nrf2 in response to the various doses of UVB. Intriguingly, fibroblasts from six different human individuals showed similar differential responses to low and high doses of UVB irradiation. The data shown in Fig. 2 is representative (mean ± SE) of three independent experiments with each of the human skin fibroblast cell lines having similar results.

Interestingly, mouse keratinocytes also showed a similar response to low and high dose UVB as mouse Hepa-1 and human fibroblast cells. The exposure of cells to 7.5 J/m² UVB led to nuclear import and accumulation of Nrf2. In contrast, exposure of mouse keratinocytes to 20 J/m² UVB led to the nuclear exclusion of Nrf2. This resulted in a significant decrease in nuclear Nrf2, as compared with keratinocytes exposed to 7.5 J/m² UVB (P > 0.001) and untreated control cells (P > 0.01).

We focused our studies on the molecular mechanism of the nuclear exclusion of Nrf2 in response to 20 J/m² of UVB irradiation and a significant decrease in Nrf2 downstream gene expression. This is because high dose UVB induction of nuclear exclusion of Nrf2 is expected to have implications in UVB-induced skin carcinogenesis. The mechanistic studies were done with Hepa-1 cells.

To determine if high dose UVB–induced nuclear exclusion of Nrf2 is due to increased nuclear export of Nrf2, we pretreated

Figure 3. Inhibition of high dose UVB-induced nuclear exclusion in the presence of nuclear export inhibitor leptomycin B. A, Western analysis. Hepa-1 cells were pretreated with 20 ng/mL leptomycin B (LMB) for 6 hours followed by irradiation with 20 J/m² UVB. The cells were incubated for 8 hours, harvested and immunoblotted with anti-Nrf2 antibody. The blot was stripped and reprobed with anti-lamin B and anti-LDH antibody. B, Nrf2-V5 response to UVB. Left, Western analysis of Nrf2-V5. Hepa-1 cells were transfected with pcDNA alone or pcDNA-Nrf2-V5, exposed to 20 J/m² UVB in the absence and presence of leptomycin B, harvested, cytosolic and nuclear extracts prepared, and immunoblotted with anti-V5 antibody followed by anti-lamin B and anti-LDH antibodies. Right, Hepa-1 cells were cotransfected with reporter plasmid pGL2-NQO1 ARE-luciferase (NQO1 ARE-Luciferase), exposed to 20 J/m² UVB in the absence and presence of leptomycin B, and analyzed for luciferase activity. C, mutant Nrf2Y568F-V5 response to UVB. Left, Western analysis of mutant Nrf2Y568F-V5. Hepa-1 cells were transfected with pcDNA alone or mutant pcDNA-Nrf2Y568F-V5, exposed to 20 J/m² UVB in the absence and presence of leptomycin B, harvested, cytosolic and nuclear extracts prepared, and immunoblotted with anti-V5 antibody followed by anti-lamin B and anti-LDH antibodies. Right, Hepa-1 cells cotransfected with reporter plasmid pGL2-NQO1 ARE-luciferase (NQO1 ARE-Luciferase) and expression plasmid pcDNA-Nrf2Y568F-V5, exposed to 20 J/m² UVB in the absence and presence of leptomycin B, incubated for 8 hours, and analyzed for luciferase activity. D, endogenous NQO1 activity. Hepa-1 cells were untransfected or transfected with Nrf2-V5 or Nrf2Y568F-V5 and analyzed for cytosolic NQO1 activity. One unit of NQO1 enzyme activity is the amount of NQO1 that catalyzes the reduction of 1 μmol of 2,6-dichlorophenolindophenol/min/mg cytosolic protein. Columns, average of three independent transfection experiments.
Hepa-1 cells with nuclear export inhibitor leptomycin B before irradiation with 20 J/m² UVB and analyzed for subcellular localization of Nrf2 by immunohistochemistry and SDS-PAGE/Western analysis. The results are shown in Fig. 3A. Prior treatment of cells with leptomycin B blocked nuclear exclusion of Nrf2. This was evident from the significant increase in nuclear accumulation of Nrf2 in the presence of leptomycin B in both immunohistochemistry and Western analyses. These results suggested that high dose UVB indeed induced nuclear export of Nrf2.

UVB is known to control nuclear exclusion of net repressor by the activation of src kinases and tyrosine phosphorylation (39). In addition, hydrogen peroxide triggers the nuclear export of telomerase reverse transcriptase via src kinase family–dependent phosphorylation of tyrosine 707 (40). These suggest that tyrosine in Nrf2 and tyrosine kinase might play a role in UVB-induced nuclear exclusion of Nrf2. Nrf2 contains a single conserved tyrosine 568 as a possible tyrosine kinase phosphorylation site. We used site-directed mutagenesis to successfully mutate tyrosine 568 to phenylalanine. The wild-type and mutant Nrf2 tagged with V5 for antibody detection were overexpressed in Hepa-1 cells to investigate the role of tyrosine 568 in UVB-mediated nuclear exclusion of Nrf2 (Fig. 3B–D). Hepa-1 cells overexpressing Nrf2-V5 or mutant Nrf2Y568F-V5 were unexposed or exposed to 20 J/m² UVB in the absence and presence of leptomycin B, and was analyzed for subcellular localization of Nrf2 and Nrf2 regulation of ARE-mediated gene expression. Nrf2-V5 showed a similar response as endogenous Nrf2 to high dose UVB (Fig. 3B; left; compare with Fig. 3A). Nrf2-V5 was localized in the nucleus as well as the cytosol. High dose UVB stimulated nuclear exclusion of Nrf2-V5 whereas leptomycin B treatment blocked the nuclear exclusion of Nrf2-V5.

NQO1 ARE-luciferase expression decreased in response to UVB irradiation and increased in the presence of leptomycin B (Fig. 3B, right). Interestingly, mutant Nrf2Y568F-V5 was predominately localized in the nucleus and UVB treatment failed to induce nuclear exclusion of Nrf2 (Fig. 3C, left). In addition, leptomycin B showed no effect on nuclear localization of mutant Nrf2Y568F-V5 (Fig. 3C, left). The ARE-luciferase gene expression was induced in cells overexpressing mutant Nrf2Y568F-V5 (Fig. 3C, right). However, high dose UVB and leptomycin B treatment more or less did not alter the expression of ARE-mediated luciferase gene expression (Fig. 3C, right). A mutation in NQO1 gene expression in cells expressing endogenous or overexpressing Nrf2-V5 or mutant Nrf2Y568F-V5 showed similar results as ARE-mediated luciferase gene expression in transfected cells. The overexpression of Nrf2-V5, but not mutant Nrf2Y568F-V5, responded to UVB and leptomycin B. The above experiments suggested that UVB-induced phosphorylation of tyrosine 568 might play an important role in the nuclear exclusion of Nrf2.

The next set of experiments investigated if the tyrosine 568 site is phosphorylated and also investigated the effect of UVB on tyrosine 568 phosphorylation (Fig. 4). Hepa-1 cells transfected with pcDNA (control) and Nrf2-V5 or mutant Nrf2Y568F-V5 were immunoprecipitated with IgG or anti-phosphotyrosine (anti-pTyr) antibodies. The immunoprecipitates were run on SDS-PAGE, Western blotted, and probed with anti-V5 antibody. The results indicated phosphorylation of Nrf2-V5 but not mutant Nrf2Y568F-V5 (Fig. 4A). In a similar experiment, Hepa-1 cells were transfected with Nrf2-V5 or Nrf2Y568F-V5, treated with UVB in the presence and absence of leptomycin B and src kinase inhibitor PP2, nuclear extracts were prepared and immunoprecipitated with anti-phosphotyrosine.
antibody and Western blotted with anti-V5 antibody (Fig. 4B). The phosphorylation of Nrf2-V5 was increased in response to UVB in the absence as well as presence of leptomycin B (Fig. 4B, left, compare lanes 1, 2, and 5). The increase of phosphorylation was greater in the presence of leptomycin B because of the blocking of nuclear export of phosphorylated Nrf2. PP2 interfered with UVB-induced phosphorylation of Nrf2-V5 (Fig. 4B, left, compare lanes 6 and 7). In the same experiment, anti-phosphotyrosine antibody failed to immunoprecipitate Nrf2Y568A-V5 (Fig. 4B, right). In related experiments, anti-V5 immunoprecipitated Nrf2-V5 and Nrf2Y568F-V5 and Western blotted with anti-phosphotyrosine antibody (Fig. 4C). The results supported an increase in UVB induction of phosphorylation of Nrf2-V5 (Fig. 4B, left, compare lanes 2, 3, and 5). Nrf2Y568F-V5 was immunoprecipitated with anti-V5 antibody. However, results showed the absence of phosphorylation of Nrf2Y568FV5 (Fig. 4B, right).

The pretreatment of Hepa-1 cells overexpressing Nrf2-V5 or mutant Nrf2Y568F-V5 with PP2 investigated the role of src kinases in UVB induction of Nrf2 phosphorylation and exclusion from the nucleus (Fig. 5). PP2 treatment significantly blocked UVB-induced nuclear exclusion of Nrf2-V5 resulting in nuclear accumulation of Nrf2-V5 (Fig. 5A, compare lanes 4 and 5 with lanes 9 and 10). Interestingly, PP2 also interfered with nuclear export of Nrf2-V5 in the absence of UVB exposure (Fig. 5A, compare lanes 2 and 3 with lanes 7 and 8). In similar experiments, PP2 had no effect on predominant nuclear localization Nrf2Y568F-V5 (Fig. 5B). In a related experiment, PP2 more or less blocked the UVB-induced decrease in NQO1 ARE-mediated luciferase expression in cells transfected with Nrf2-V5 (Fig. 5C). However, PP2 and UVB had more or less no effect on NQO1 ARE-mediated luciferase gene expression in cells overexpressing mutant Nrf2Y568F (Fig. 5C). The small increase in ARE-mediated luciferase gene expression in PP2-treated cells transfected with mutant Nrf2Y568F presumably was due to the effect of PP2 on endogenous Nrf2.

UVB is known to activate src kinase Fyn (41, 42). We used siRNA to study the role of Fyn kinase in UVB-induced nuclear exclusion of Nrf2 (Fig. 6). The transfection of Hepa-1 cells with Fyn-specific siRNA inhibited Fyn, which led to the nuclear accumulation of Nrf2 and induction of ARE-mediated luciferase gene expression in unirradiated and UVB-irradiated cells (Fig. 6A and B). In the same experiment, control siRNA had no effect on Nrf2 and ARE-mediated luciferase gene expression in cells unirradiated or irradiated with 20 J/m² UVB (Fig. 6A and B). In related experiments, subcellular distribution of Fyn in Hepa-1 cells unirradiated and irradiated with 7.5 J/m² UVB was determined (Fig. 6C). Interestingly, the exposure of cells to 20 J/m² but not 7.5 J/m² UVB led to an increase in the nuclear translocation of Fyn (Fig. 6C).

Discussion

The transcription factor, Nrf2, is known to regulate ARE-mediated expression and coordinated induction of a battery of chemoprotective genes (13). The coordinated induction of these genes is a mechanism of critical importance in chemoprevention. Nrf2 is retained in the cytosol by its inhibitor INrf2. Xenobiotics and antioxidants are known to antagonize INrf2/Nrf2 interaction leading to the release and nuclear translocation of Nrf2 and coordinated activation of chemoprotective genes. Therefore, INrf2/Nrf2 serves as cellular sensors of chemical stress. However, information on INrf2/Nrf2 response to UV light and UV-induced skin carcinogenesis is largely absent. Therefore, it is unknown if the INrf2/Nrf2 complex also function as sensors of UV. The present studies have investigated Nrf2 response to UVB.

All three types of cells (mouse hepatic, mouse keratinocyte, and human skin fibroblast cells) responded in the exact similar manner to UVB. However, low doses of UVB response differed from high dose response. Low doses of UVB (7.5 J/m²) induced nuclear localization of Nrf2 leading to the activation of Nrf2 downstream gene expression. On the contrary, high dose UVB (20 J/m²) led to significant nuclear exclusion of Nrf2 and down-regulation of Nrf2-controlled gene expression. Exposure to 5 J/m² UVB caused more or less no change in nuclear Nrf2, as compared with unexposed control cells. It is possible that exposure to 5 J/m² UVB might not be sufficient to initiate signaling for nuclear import of Nrf2, and therefore, more or less no change in nuclear Nrf2. Similarly, the nuclear levels of Nrf2 in cells exposed to 10 J/m² UVB was the same as control levels. This is due to the activation of nuclear exclusion of Nrf2 that exported the increased Nrf2 out of the nucleus. The differential response of Nrf2 to UVB was also clearly evident from Nrf2 downstream NQO1 gene expression and NQO1 gene ARE-mediated luciferase gene expression. The other Nrf2 downstream genes, including glutathione S-transferase, are expected to show similar responses to UVB as observed for NQO1 because these genes are coordinated and regulated by Nrf2 (13).
The observation of low dose UVB–induced nuclear translocation/activation of Nrf2 was similar as reported earlier for UVA irradiation-mediated activation of Nrf2 activation in dermal fibroblasts which protects against UVA-induced apoptosis (43), and chemical treatment led to nuclear translocation/activation of Nrf2 (13). The mechanism of low dose UVB–induced nuclear translocation and up-regulation of ARE-mediated gene expression is unclear but might involve reactive oxygen species, as suspected with chemicals and PKC phosphorylation of Nrf2 serine 40 (19).

The mechanism of the high dose UVB–induced nuclear exclusion and down-regulation of ARE-mediated gene expression was investigated. The results revealed that UVB induced phosphorylation of Nrf2 at tyrosine 568. The phosphorylated Nrf2 was exported out of the nucleus leading to nuclear exclusion of Nrf2 and down-regulation of ARE-mediated gene expression and induction in response to UVB. These conclusions were based on leptomycin B inhibition of UVB-induced nuclear exclusion of Nrf2. UVB induced the phosphorylation of Nrf2Y568 but not mutant Nrf2Y568F, PP2 inhibition of phosphorylation of Nrf2, and measurements of NQO1-ARE-luciferase gene expression.

PP2 is an inhibitor of the Src family of tyrosine kinases (41). PP2 inhibition of phosphorylation and nuclear exclusion of Nrf2 suggested that src kinase(s) mediated the nuclear exclusion of Nrf2 in response to high dose UVB. The src family of tyrosine kinases has four members Fyn, Lyn, Yes, and Src (42). Fyn siRNA inhibited Fyn kinase expression, blocked high dose UVB–induced nuclear exclusion and decrease inARE-mediated gene expression. These results suggested that Fyn kinase mediated the high dose UVB response of nuclear exclusion of Nrf2. This also raised questions regarding the mechanism by which Fyn kinase receives signals from UVB leading to the activation and nuclear export of Nrf2. UVB regulation of Fyn kinase was reported earlier (41). UVB-induced phosphorylation of Fyn led to the nuclear localization of Fyn (41). The current experiments also suggest that Fyn is increased in the nucleus in response to high dose UVB irradiation. The increase in UVB-induced nuclear localization of Fyn phosphorylated Nrf2Y568 leading to nuclear export of Nrf2. It is possible but unknown if other Src kinases including Src, Lyn, and Yes are also capable of phosphorylating tyrosine 568 in Nrf2 in response to high dose UVB.

Experimental evidence suggests that solar UVB and UVA is the most important environmental carcinogen leading to the development of malignant skin melanoma (44–46). The high dose UVB–induced nuclear exclusion of Nrf2 and the loss of downstream gene expression are expected to play significant roles in UVB-induced carcinogenesis and remain to be determined.

Based on the current study, a mechanism of UVB regulation of Nrf2 is suggested. Cellular exposure to low dose UVB activates PKC or yet to be determined kinase(s) that phosphorylate serine 40 in Nrf2 and mutant Nrf2Y568F, leading to the release of Nrf2 and mutant Nrf2Y568F from INrf2. Nrf2 and mutant Nrf2Y568F translocate in the nucleus, bind to ARE, and activate ARE-mediated gene expression. Cellular exposure to high dose UVB phosphorylates Fyn, which translocates into the nucleus as reported earlier (41). Fyn phosphorylates Nrf2 at tyrosine 568. The phosphorylated Nrf2 is exported out of the nucleus, binds to INrf2, and degrades. Some of the phosphorylated Nrf2 might also bind INrf2 in the nucleus and degrade. INrf2 also has nuclear localization for degradation of Nrf2 inside the nucleus and/or carry Nrf2 out of the nucleus to degrade (32, 33). Fyn fails to phosphorylate mutant Nrf2Y568F because of tyrosine mutation to phenylalanine. Mutant Nrf2Y568F binds to INrf2 and degrades.

In conclusion, low and high doses of UVB showed differential response to Nrf2. Low dose UVB stimulated nuclear import of Nrf2...
and up-regulation of ARE-mediated gene expression. High dose UVB–induced nuclear exclusion of Nrf2 and down-regulation of ARE-mediated gene expression. UVB-induced nuclear localization of Fyn led to increased phosphorylation of Nrf2Y568 that led to nuclear export/exclusion of Nrf2 and decrease in ARE-mediated gene expression. High dose UVB response of Nrf2 might contribute to environmental carcinogetic effects of UVB.

References
Low and High Dose UVB Regulation of Transcription Factor NF-E2-Related Factor 2

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