The Role of Constitutive Nitric-oxide Synthase in Ultraviolet B Light-induced Nuclear Factor κB Activity*

Lingying Tong and Shiyong Wu

From the Department of Chemistry and Biochemistry, Edison Biotechnology Institute and Molecular and Cellular Biology Program, Ohio University, Athens, Ohio 45701

Background: Early activation of NF-κB upon UVB irradiation is through a noncanonical eIF2-dependent IκB reduction pathway.

Results: Inhibition of constitutive nitric-oxide synthase inhibited UVB-induced NF-κB activation.

Conclusion: Constitutive nitric-oxide synthase is required for NF-κB activation.

Significance: Learning the regulation of NF-κB upon UVB irradiation is critical for understanding the initiation and development of UVB-induced tumorigenesis.

UV can cause skin damage and result in increased chance of developing skin cancer (1, 2). Upon UV radiation, NF-κB2 is one of the transcription factors that can be activated and regulate the expression of genes that regulate immune and inflammatory responses, as well as genes involved in regulation of cellular growth and apoptosis (3–7). Because of its crucial role in cellular responses to environmental stress, the regulation of NF-κB activation has been widely studied. In canonical NF-κB activation, IκKα phosphorylates IκBα at serine 32 and 36, which leads to its dissociation from NF-κB and being degraded. NF-κB, freed from IκB, then translocates into the nucleus and activates its target genes (8, 9). Although many NF-κB activators go through this pathway, some stimuli such as UV, reactive oxygen species, and hypoxia activate NF-κB through much more complex cellular pathways, which have not been fully elucidated (10–13).

Our previous studies indicated that UVB irradiation quickly induced the activation and uncoupling of constitutive nitric-oxide synthase (cNOS), which led to an imbalance of [NO−]/[ONOO−] (14). The NO− production and [NO−]/[ONOO−] imbalance can induce the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) and inhibit protein synthesis (15). The eIF2α-mediated translation inhibition has been shown to play a critical role in the regulation of NF-κB activation and cell apoptosis post-UV (11, 16, 17). However, the roles of cNOS in regulation of NF-κB activation after UVB radiation are still unclear. The relationship between cNOS and NF-κB in mediating cell survival or death after UVB irradiation is also not known. In this study, we demonstrated that cNOS activity plays important roles in mediating a complex signaling circuit in the regulation of NF-κB activation and cell survival after UVB radiation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human keratinocyte HaCaT cells (kindly provided by Dr. Nihal Ahmad, University of Wisconsin–Madison), HeLa cells, and HEK293 cells were grown in Dulbecco’s minimal essential medium (Cellgro) supplemented with 10% fetal bovine serum and penicillin/streptomycin, at 37 °C with 5% CO2.

UVB Irradiation—UVB was generated from a Bench XX series UV lamp (UVP Inc.) equipped with two 15-watt UVB tubes (UVP Inc.). The intensity of UVB was calibrated using a UVP model UXV digital radiometer (UVP Inc.) after the lamps...
warmed up for 5 min. The dose rate for 8 or 50 ml/cm² of UVB radiation was 0.8 or 3.8 milliwatts/s, respectively. Medium was removed before exposing cells to UVB. After UVB radiation, fresh medium was added to the culture plates with or without drugs, and the cells were continuously incubated at 37 °C with 5% CO₂ until further analysis. BAY11-7085 (5 μM, Sigma) was added to cells to a final concentration of 1 μM at 1 h before UVB irradiation. After irradiation, the cells were either continuously incubated with l-NNAME (1 mM) for 1 h and then replaced with fresh medium (acute treatment) or continuously incubated for the whole period until further analysis (continuous treatment). S-Nitroso-N-acetylpenicillamine (SNAP, Invitrogen) was added to cells to the indicated final concentration at 1 h before UVB irradiation. After irradiation, the cells were continuously incubated with the same concentration of SNAP until further analysis. BAY11-7085 (5 μM, Sigma), JSH-23 (10 μM, Sigma), MG132 (10 μM, Sigma), or Ro106 (10 μM, Sigma) were added to cells immediately after UVB radiation and kept in the medium for the whole period until further analysis.

**Drug Treatments**—l-NNAME (l-NAMe, Sigma) was added to cells to a final concentration of 1 mM at 1 h before UVB irradiation. After irradiation, cells were either continuously incubated with l-NNAME (1 mM) for 1 h and then replaced with fresh medium (acute treatment) or continuously incubated for the whole period until further analysis (continuous treatment). S-Nitroso-N-acetylpenicillamine (SNAP, Invitrogen) was added to cells to the indicated final concentration at 1 h before UVB irradiation. After irradiation, the cells were continuously incubated with the same concentration of SNAP until further analysis. BAY11-7085 (5 μM, Sigma), JSH-23 (10 μM, Sigma), MG132 (10 μM, Sigma), or Ro106 (10 μM, Sigma) were added to cells immediately after UVB radiation and kept in the medium for the whole period until further analysis.

**Western Blot Analysis**—Nonidet P-40 lysis buffer (2% Nonidet P-40, 80 mM NaCl, 100 mM Tris-HCl, pH 8.0, 0.1% SDS) with proteinase inhibitor mixture (Complete™, Roche Applied Science) was used to lyse cells. Cell lysate was incubated on ice for 15 min and then centrifuged at 14,000 rpm at 4 °C for 15 min. Protein concentration was measured by DC protein assay kit (Bio-Rad Laboratories). Equal amounts of protein were subjected on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was then blocked in 5% milk in Tris-buffered saline plus Tween 20 (TBST) for 45 min and probed with anti-NF-κB p65 (sc-8008, Santa Cruz Biotechnology), anti-IκB (sc-371, Santa Cruz Biotechnology), anti-phospho (Ser-32/36)-IκB (9246, Cell Signaling), anti-ELF2α (sc-11386, Santa Cruz Biotechnology), anti-phospho (Ser-52)-ELF2α (44-728G, Invitrogen), anti-IKKα (sc-166231, Santa Cruz Biotechnology), or anti-β-actin (A1978, Sigma) at 4 °C overnight. After washing with TBST, the membrane was incubated with corresponding HRP-conjugated anti-rabbit or anti-mouse antibody for 45 min at room temperature. Membrane was then washed three times in TBST followed by two times in TBS and developed in West Pico SuperSignal chemiluminescent substrate (Pierce).

**ELISA for NF-κB Activity**—Cells were harvested with 0.25% trypsin-EDTA, and nuclear extracts were separated from cytoplasmic extracts by NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) following the manufacturer’s protocol. NF-κB activity in the nuclear extract was detect by the ELISA-based transcription factor assay kits for NF-κB (Promega) using Lipofectamine 2000 (Life Technologies). At 24 h post-transfection, cells were exposed to UVB radiation with or without l-NNAME treatment. Luciferase activity was measured at 6 h post-UVB using Dual-Glo luciferase assay kit (E2920, Promega). The reading of luciferase signal was normalized to the reading of Renilla following the manufacturer’s instructions.

**Immunofluorescence Staining of NF-κB**—Cells were fixed with 3.6% formaldehyde for 10 min at room temperature, rinsed with PBS three times, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then blocked with blocking buffer (2 mg/ml BSA in PBS) for 1 h before incubating with mouse anti-p65 monoclonal antibody (sc-8008, Santa Cruz Biotechnology) for 1 h. After washing three times with PBS, cells were incubated with a fluorescein-conjugated horse anti-mouse antibody (DI-2488, Vector Laboratories) for 1 h, washed with PBS, and mounted with ProLong Gold antifade reagent with DAPI (P36931, Life Technologies). The pictures were taken by NIKON Eclipse E600 with an exposure time of 0.1 s and analyzed with NIS-Elements Basic Research 3.2 imaging software. Three cells of each group were randomly picked for quantification analysis.

**cNOS Silencing Using the RNA Interference Method**—LipoFectamine RNAiMAX reagent (13778030), scrambled siRNA (AM4611), nNOS (human) siRNA (AM16708 siRNA ID 117855), and eNOS (human) siRNA (AM16708 siRNA ID 106158) were purchased from Life Technologies. 2.5 × 10⁶ cells were seeded in a 6-well tissue culture plate in antibiotic-free medium the day before transfection. 4 μl of Lipofectamine RNAiMAX and 2 μl (10 μM) siRNA were prepared separately in 100 μl of DMEM medium (free of FBS and antibiotics) and then mixed and incubated for 5 min at room temperature. The mixture was then added to 1 ml of the medium with cells, and the medium volume was added up to 2 ml at 8 h after transfection. The cells were then incubated at 37 °C with 5% CO₂ for 16 h before UV radiation.

**Real-time PCR**—Total RNA was extracted by the RNeasy Mini Kit (74104, Qiagen). First-strand cDNA was reverse-tran-
cNOS Regulates UVB-induced NF-κB Activity

scribed from 1 μg of RNA using SuperScript III reverse transcriptase (18080-044, Invitrogen) following the manufacturer’s protocol. Briefly, in a 20-μl reaction system, 1 μl of 50 μM oligo(dT)20 and 1 μl of 10 mM dNTP mix were added and heated to 65 °C for 5 min followed by incubation on ice for 2 min. Then 5× first-strand buffer and 0.1 μM DTT and 1 μl of SuperScript III reverse transcriptase were added with 5 min of incubation at 25 °C followed by 60 min at 50 °C and 15 min at 75 °C heating. Quantitative real-time PCR (qPCR) using Maxima SYBR Green/fluorescein quantitative PCR master mix (2×) (K0241, Thermo scientific) was then performed. Primers (synthesized by Invitrogen) of the targeted human genes were as follows: IKKα, 5′-GAGATACAGCGAGCATGAC-3′; 5′-ATGACACCAAACCTCAGCATG-3′; GAPDH, 5′-TGC- ACCA CCCA AACTGTTAGC-3′; 5′-GGCATGGACGTGGTG- CATGAG-3′; and β-actin, 5′-CAGCTTCCAGGCTTCC- CTTCC-3′; 5′-CGGACTGTGCATACTCCTGCT-3′. The real-time PCR was performed on Bio-Rad iCycler with the following protocol: 95 °C for 10 min; 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s for 40 cycles. Data were analyzed by ΔΔCt method.

Cell Survival Analysis—Total cell death was analyzed by determination of the loss of membrane phospholipid symmetry and membrane integrity using a FITC-conjugated-annexin V (ANX5)/propidium iodide (PI) apoptosis detection kit (BD Biosciences) following the manufacturer’s protocol. Briefly, the cells were harvested by 0.25% trypsin digestion, combined with the cells floating in the medium, and washed twice with ice-cold PBS. Cells were then suspended in 200 μl of ANX5 binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). The cell suspension was mixed with 5 μl of ANX5–FITC and 5 μl of PI. The cell mixture was incubated for 15 min in the dark at room temperature, and the ANX5/PI double-stained cells were analyzed by FACSort flow cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences). Total cell number of 1 × 106 was used for each analysis. The number of cells positive for ANX5, PI, and both ANX5 and PI were counted. Cell survival rate (R) was calculated as: R = (1 × 105 – number of positive stained cells)/1 × 105.

Clonogenic Assay—Immediately after treatment, cells were harvested with 0.25% trypsin-EDTA and counted, and then 5 × 103 cells/well were plated in 6-well plates. After 6 days, cells were fixed with cold methanol for 10 min at −20 °C and then stained by 1% crystal violet in 25% methanol for 10 min at room temperature. Cells were then rinsed with distilled water, and colonies with size greater than 0.4 mm were counted by Kodak IS in vivo F system equipped with Kodak Molecular Imaging software (Eastman Kodak).

RESULTS

Both cNOS Activation and NO Front Elevation Mediate UVB-induced 1kB Reduction via Translocation Pathway—The phosphorylation of eIF2α and sequentially the inhibition of protein synthesis play an important role in regulation of UVC-induced 1kB reduction and NF-κB activation (11, 16). Because cNOSs mediate UVB-induced eIF2α phosphorylation via activating eIF2A kinases 3 (EIF2AK3; PERK) and 4 (EIF2AK4; GCN2) (15), we examined whether cNOS also regulates UVB-induced 1kB reduction and NF-κB activation. First, the effect of acute (1 h post-UVB) or continuous (kept until cell collection) treatment of 1-NAME (1 mm), a selective inhibitor of cNOS (18), on 1kB protein expression level was analyzed in UVB-irradiated HaCaT cells. Our data indicated that the acute or continuous treatment of 1-NAME alone did not alter the 1kB level in the cells (Fig. 1A, lanes 2 and 3 versus lane 1) without UVB irradiation. Although the acute treatment had no statistically significant effect on 1kB level (Fig. 1A, lane 5 versus lane 4), the continuous treatment partially protected the reduction of 1kB level at 6 h post-UVB radiation (Fig. 1A, lane 6 versus lane 4). Furthermore, the protective effect of 1-NAME on 1kB at 6 h post-UVB was dependent on its concentration (Fig. 1B). The effect started at 0.1 μM and appeared to be saturated at 1 μM (Fig. 1B).

These results indicated that cNOS activity plays a role in regulation of 1kB after UVB irradiation.

Activated cNOS mediates UVB-induced eIF2α phosphorylation via two pathways. Immediately after UVB radiation, in the first pathway, the coupled cNOS-catalyzed NO/production depletes l-Arg, which leads to the activation of GCN2, and in the second pathway, the uncoupled cNOS-catalyzed O2 production rapidly reacts with NO- to form ONOO− and activates PERK (15). To further determine the mechanism of cNOS-mediated NF-κB activation, we examined the effect of SNAP, a NO donor (19), on the protective effect of 1-NAME on UVB-induced 1kB reduction. Interestingly, the partial protection of 1-NAME on the 1kB (Fig. 1C, lane 13 versus lane 8) was diminished with a low dose (50 μM, Fig. 1C, lane 9 versus lane 13) but not affected by higher doses (100–500 μM, Fig. 1C, lanes 10–12 versus lane 13) of SNAP treatment, indicating that NO- level also plays a role in regulation of 1kB reduction after UVB irradiation. In addition, UVB did not induce a detectable amount of Ser-32/36 phosphorylated 1kB (p-1kB) but induced a high level of Ser-52 phosphorylated eIF2α (p-eIF2α) (Fig. 1D, lane 3). The UVB-induced eIF2α phosphorylation was decreased with 1-NAME treatment (Fig. 1D, lane 4 versus lane 3), suggesting that cNOSs protect 1kB reduction via translation regulation pathway.

Both nNOS and eNOS Are Involved in Regulation of UVB-induced NF-κB Activation—To determine whether the cNOS-mediated 1kB reduction is correlated to NF-κB activation after UVB irradiation, we examined the effect of 1-NAME on the DNA binding activity of NF-κB using an ELISA-based assay (Fig. 2A) and EMSA assay (Fig. 2B). Our data indicated that the acute or continuous treatment of 1-NAME alone had no statistically significant effect on the NF-κB activity (Fig. 2A, lanes 2 and 3 versus lane 1). For the ELISA assay, our data showed that the DNA binding activity of NF-κB was increased to 1.8 ± 0.2-fold at 6 h after UVB irradiation (Fig. 2A, lane 4 versus lane 1). While the acute treatment of 1-NAME did not have a statistically significant effect on NF-κB activity (Fig. 2A, lanes 4 and 5), the continuous treatment of 1-NAME inhibited the UVB-induced NF-κB activity to 0.8 ± 0.1-fold at 6 h post-UVB irradiation (Fig. 2A, lane 6 versus lane 4). Similar results were observed with EMSA assay, which showed that NF-κB activity increased 2.7 ± 0.4-fold with UVB alone, and continuous treatment of 1-NAME reduced the induction to 1.4 ± 0.2-fold, whereas acute treatment of 1-NAME showed no statis-
cNOS Regulates UVB-induced NF-κB Activity

SEPTEMBER 19, 2014 • VOLUME 289 • NUMBER 38

JOURNAL OF BIOLOGICAL CHEMISTRY

26661

A.

| Condition  | Lane  |
|------------|-------|
| UVB (6 h)  | 1     |
| L-NAME (-1 to 1 h) | 2     |
| L-NAME (-1 to 6 h) | 3     |

- IkB
- β-actin

Lane: 1 2 3 4 5 6

Relative density

B.

| Condition  | Lane  |
|------------|-------|
| UVB (6 h)  | 1     |
| L-NAME (μM) | 2     |

- 0.1
- 0.5
- 1
- 5
- 0
- 0.1
- 0.5
- 1
- 10

- IkB
- β-actin

Lane: 1 2 3 4 5 6

C.

| Condition  | Lane  |
|------------|-------|
| UVB (6 h)  | 1     |
| L-NAME (-1 to 6 h) | 2     |
| SNAP (μM)  | 3     |

- 100
- 50
- 200
- 500
- -
- 50
- 100
- 200
- 500

- IkB
- β-actin

Lane: 1 2 3 4 5 6

D.

| Condition  | Lane  |
|------------|-------|
| UVB        | 1     |
| L-NAME     | 2     |
| p-IkB      | 3     |
| T-IkB      | 4     |
| p-eIF2α    | 5     |
| T-eIF2α    | 6     |
| β-actin    | 7     |

Lane: 1 2 3 4 5 6

The effect of cNOS on IkB reduction in the early phase after UVB radiation. HaCaT cells were exposed to 50 mJ/cm² UVB radiation with or without drug treatment as indicated and collected at 6 h post-UVB radiation. The expression levels of indicated proteins were measured by Western blot analysis. A, cells were treated with acute (1 h post-UVB) or continuous (6 h post-UVB) incubation of L-NAME (1 mM), and statistical analysis is shown. *, p < 0.05 versus corresponding control; **, p < 0.05 versus UVB alone. B, dose-dependent treatment of L-NAME and its effect on IkB. C, cells were treated with L-NAME and different dose of SNAP. D, the effect of L-NAME on phosphorylated IkB (p-IkB), phosphorylated eIF2α (p-eIF2α), total IkB (T-IkB), and total eIF2α (T-eIF2α) protein level.

cally significant change (Fig. 2B). To confirm the role of cNOS in regulation of UVB-induced NF-κB, we determined NF-κB activity post-UVB in HeLa and HEK293 cells using an NF-κB-driven luciferase assay. Although HeLa cells express both cNOSs, HEK293 is known to be null for both nNOS and eNOS (20). Our data demonstrated that NF-κB activity was increased to 1.4 ± 0.1-fold at 6 h post-UVB, and the UVB-induced NF-κB activation could be totally inhibited by the continuous treatment of L-NAME in HeLa cells (Fig. 2C). However, the inducibility of NF-κB by UVB was totally diminished in cNOS null
HEK293 cells (Fig. 2D). The result confirms that the early activation of NF-κB upon UVB irradiation is cNOS-dependent. In addition to DNA binding activity of NF-κB, the continuous treatment of l-NAME also had a stronger inhibitory effect on nuclear translocation of NF-κB at 6 h post-UVB than the acute treatment of l-NAME had (Fig. 3). As shown by the semiquantitative analysis, nuclear NF-κB increased from 31 ± 2% to 75 ± 2.5% with UVB alone and decreased to 48 ± 3% with continuous treatment of l-NAME (Fig. 3, bottom panel, bar 1 versus bar 4 versus bar 6). Meanwhile the acute treatment of l-NAME did not statistically affect nuclear translocation of NF-κB (Fig. 3, bottom panel, bar 4 versus bar 5). These results were correlated to the IκB reduction and NF-κB activity with the same treatment (Figs. 1 and 2).

We previously showed that both nNOS and eNOS are expressed in HaCaT cells (14). To determine the contribution of each isoform of cNOS in regulation of UVB-induced reduction of IκB, we analyzed the extent of the effect of nNOS and/or eNOS knockdown on IκB expression after UVB irradiation. Our data showed that treating the cells with nNOS and/or eNOS siRNA partially reduced the expression level of both cNOSs (Fig. 4A). Our data also showed that although it did not alter the background level of IκB (Fig. 4B, lanes 3–5 versus lanes 1 and 2), the siRNA knockdown of nNOS and/or eNOS could
partially protect IκB from UVB-induced reduction (Fig. 4B, lanes 8–10 versus lanes 6 and 7). The increased level of IκB correlated to an increased retention of NF-κB in cytosol post-UVB (Fig. 5), indicating that the activity of both cNOSs contributes to UVB-induced IκB reduction and NF-κB nucleus translocation.

Cross-regulation among cNOS, IκB, NF-κB, and IKKα after UVB Radiation—Previous studies suggested that IκB reduction in the early phase (within 12 h) post-UVC is dependent on the background activity of IKKα, but independent of induced activation of IKKα (3, 11). Previous studies also suggested that UVC-induced NF-κB activation contributes to IκB synthesis (11). To further determine whether UVB-induced cNOS activation is involved in regulation of IκB level via upstream and/or downstream signaling pathways, we compared the effect of l-NAME with two commonly used NF-κB inhibitors, BAY11-7085 and JSH-23 on ubiquitin or proteasomal degradation pathway-mediated IκB degradation. BAY11-7085 inhibits IκB phosphorylation, and JSH-23 interferes with the binding of NF-κB to its target DNA (21, 22). Our data showed that l-NAME and BAY11-7085, but not JSH-23, had the same effect on partially protecting IκB from UVB-induced reduction (Fig. 3).
cNOS Regulates UVB-induced NF-κB Activity

6, lanes 5, 8, and 11 versus lane 2). Interestingly, in combined treatments, the effect of L-NAME and JSH-23, but not BAY11-7085, could be added on top of the effect of a proteasome inhibitor MG132 (10 μM) and with or without combined treatment of Ro106 (10 μM) and MG132 (10 μM). The amount of total IκB was measured by Western blot analysis. The ratio of IκB/β-actin represents the average of 3–6 sets of data. *p < 0.05 versus control; **p < 0.05 versus UVB alone; #p < 0.05 versus corresponding UVB without MG132 and Ro106 treatment (lanes 2, 5, 8, and 11); ± p < 0.05 versus UVB with corresponding MG132 or Ro106 (lanes 3 or 4).

NF-κB Activation Protects Cell Death Upon UVB Radiation—Because both cNOS and NF-κB have dual roles in regulation of apoptosis and cell survival (27–30), we examined the short term (4–6 h) and long term (6 days) effects of L-NAME, BAY11-7085, and JSH-23 on cell survival and recovery after UVB irradiation using apoptotic and clonogenic assays, respectively. For the short term effect of 50 mJ/cm² UVB irradiation, the irradiation alone decreased the cell survival rate to ~85 and 71% at 4 and 6 h post-irradiation, respectively (Fig. 8A, lanes 4 and 7). Although the drug alone did not have statistically significant effect on cell survival (Fig. 8A, lanes 2 and 3 versus lane 1), inhibition of NF-κB activity by BAY11-7085 and JSH-23 further decreased the cell survival rate from 71 to ~66% at 6 h post-UVB (Fig. 8A, lanes 8 and 9). Interestingly, although the continuous treatment of L-NAME increased the cell survival rate from 81 to 85% at 4 h post-UVB but had no statistically significant effect at 6 h post-UVB (Fig. 8B, lane 5 versus lane 3 and lane 8 versus lane 6), the acute treatment of L-NAME increased cell survival rate from approximately 81 to 88% at 4 h post-UVB and from 69 to 74% at 6 h post-UVB (Fig. 8B, lane 4 versus lane 3 and lane 7 versus lane 6). Again, the treatment of drug alone

Figure 5. The effect of cNOS silencing on translocation of NF-κB upon UVB radiation. A, HaCaT cells were transiently transfected with either nNOS or eNOS siRNA or together as cNOS knockdown. A scramble siRNA (Scr) was also transiently transfected into cells as control. At 6 h post-UVB, cells were fixed with formaldehyde and stained with p65 antibody and FITC-conjugated secondary antibody, with mounting reagent containing DAPI. Pictures were taken by NIKON Eclipse E600 and analyzed by NIS-Elements Basic Research 3.2 imaging software, with an exposure time of 1 s, b, quantitative analysis of p65 fluorescent signal. Three cells of each group were randomly picked for the quantitative analysis of the signal intensity of p65 both in the cytoplasm and in the nucleus. Scr siRNA, scramble siRNA. The error bars represent S.D. of three cells. *p < 0.05 versus control group; **p < 0.05 versus UVB.

Figure 6. The ubiquitination and proteasomal effects on UVB-induced IκB reduction. HaCaT cells were treated with L-NAME (1 mM), BAY11-7085 (5 μM), and JSH-23 (10 μM), and with or without combined treatment of Ro106 (10 μM) and MG132 (10 μM). The amount of total IκB was measured by Western blot analysis. The ratio of IκB/β-actin represents the average of 3–6 sets of data. *p < 0.05 versus control; **p < 0.05 versus UVB alone; #p < 0.05 versus corresponding UVB without MG132 and Ro106 treatment (lanes 2, 5, 8, and 11); ± p < 0.05 versus UVB with corresponding MG132 or Ro106 (lanes 3 or 4).
had no significant effect on the cell survival rate (Fig. 8B, lane 2 versus lane 1).

For the long term effect of UVB, a lower dose (8 mJ/cm²) of UVB was used. UVB irradiation alone reduced the colony formation to 30 ± 5% (Fig. 9, A–C). The 6- and 24-h treatment of JSH-23 or L-NAME alone did not show a statistically significant effect on colony formation, but the 144-h (6-day) treatment of either JSH-23 or L-NAME alone reduced colony formation (Fig. 9, A and B). With UVB irradiation, the treatment of JSH-23 further reduced colony formation from 30 to 16–9% depending on the length of drug treatment (Fig. 9A, lanes 6–8 versus lane 5). However, the treatment of L-NAME increased colony formation from 30 to 54–33% depending on the length of drug treatment (Fig. 9B, lanes 6–8 versus lane 5). Further analysis revealed that the double treatment with JSH-23 and L-NAME had no statistically significant effect on colony formation after UVB irradiation (Fig. 9C, lane 5 versus lane 1), indicating that JSH-23 and L-NAME could cancel each other’s effect on UVB-reduced colony formation.

DISCUSSION

Previous studies indicated that UVC-induced eIF2α phosphorylation played an important role in regulation of NF-κB activation in the early phase (within 12 h) of radiation (11, 16, 31, 32). Our previous studies also showed that UVB radiation induced an immediate activation of cNOS, which mediated the activation of eIF2α kinases PERK and GCN2 (15, 33). In this study, we demonstrated that cNOS activation contributed to the activation of NF-κB post-UVB irradiation. Inhibition of cNOS with a continuous, but not acute, treatment of L-NAME led to a partial inhibition of IκB reduction (Fig. 1) and NF-κB activation (Figs. 2 and 3). The role of cNOS in regulation of UVB-induced NF-κB activation was confirmed by an NF-κB luciferase reporter assay, indicating that L-NAME could inhibit the UVB-induced luciferase expression, and the inducibility of luciferase expression by UVB was diminished in cNOS null HEK293 cells (Fig. 2, C and D). In addition, it appears that both eNOS and nNOS are involved in the regulation of UVB-induced NF-κB activation because siRNA knockdown nNOS or eNOS can partially inhibit IκB reduction and NF-κB nuclear translocation post-UVB irradiation (Figs. 4 and 5). However,
cNOS Regulates UVB-induced NF-κB Activity

A.

![Graph](image)

B.

![Graph](image)

C.

![Graph](image)

FIGURE 9. Clonogenic assay of UVB-irradiated HaCaT cells. Cells with or without drug treatment were exposed to 8 mJ/cm² UVB irradiation, and 5 × 10⁴ cells were plated and cultured in 6-well plates for 6 days. Cells were then fixed with cold methanol and stained by 1% crystal violet in 25% methanol. Colonies with a size greater than 0.4 mm were counted by a Kodak IS system equipped with Kodak Molecular Imaging software (Eastman Kodak). The error bars represent S.D. of three sets of independent experiments. A, cells were treated with l-NAME for 1 h before UVB irradiation, and l-NAME was removed at 6, 24, and 144 h after UVB irradiation. B, JSH-23 was added immediately after UVB irradiation and removed at 6, 24, and 144 h after UVB irradiation. C, l-NAME and JSH-23 were added together for 6 h treatment after UVB irradiation. * p < 0.05 versus corresponding control; ** p < 0.05 versus corresponding UVB alone; # p < 0.05 versus UVB plus l-NAME; $ p < 0.05 versus UVB plus JSH-23.

unlike the cNOS null HEK293 cells, the effects of siRNAs on IkB expression and NF-κB activation were limited even with double cNOS/nNOS knockdown, which suggests that cNOS activity might be more critical than its quantity in regulation of NF-κB activation after UVB irradiation and that knockdown of one NOS may lead to the activation of other NOSs as reported previously (34).

The translational inhibition of IkB synthesis as well as ubiquitin and proteasome-mediated IkB degragation coordinately regulate the IkB reduction after UVC irradiation (11, 16). Our data showed that inhibition of the ubiquitin and proteasome pathway by MG132 and Ro106 could restore the IkB level after UVB irradiation (Fig. 6, lanes 3 and 4), indicating that the pathway plays a critical role in regulation of UVB-induced IkB reduction. Our data also showed that the inhibition of IkB phosphorylation by BAY11-7085 or cNOS activity by l-NAME, but not the inhibition of NF-κB activity by JSH-23, increased IkB level after UVB irradiation (Fig. 6, lanes 5, 8, and 11, versus lane 2). Interestingly, the protective effect of l-NAME was additive to the effect of MG132 or Ro106 (Fig. 6, lanes 11–13 versus lanes 5–7). These results agreed with our previous study suggesting that cNOS-mediated IkB reduction after UVB irradiation is independent of ubiquitin and proteasome pathway (15). IKKα phosphorylates IkB and promotes its degradation via ubiquitin and proteasome pathway (23–25, 35). Previous studies suggested that only background activity but not activation of IKKα is required for UVB-induced reduction of IkB (3, 11, 36). Our data indicated that although the protein status of IKKα was not statistically significantly changed after UVB irradiation (Fig. 7A, lane 5 versus lane 1), inhibition of IkB phosphorylation, NF-κB activity, or cNOS could significantly reduce the protein level of IKKα after UVB irradiation (Fig. 7A, lanes 6–9). On the other hand, the mRNA status of IKKα was substantially reduced upon UVB irradiation, and none of the drug treatment resulted in a notable change of the mRNA level of IKKα (Fig. 7B). Because the only common function of the treatments was to inhibit NF-κB activation after UVB irradiation, these results suggest that NF-κB activation post-UVB stabilizes IKKα.

Both cNOS and NF-κB play dual roles in regulation of apoptosis (27, 29, 30, 33, 37–39). To better understand the roles of cNOS and NF-κB in regulation of cell fate after UVB irradiation, we determined the effects of JSH-23 and l-NAME on cell death and recovery after UVB irradiation. Our data indicated that inhibition of NF-κB activity had an opposite effect than the inhibition of cNOS on UVB-induced cell death (Fig. 8A versus Fig. 8B), although l-NAME could inhibit the activation of NF-κB (Figs. 2 and 3). Similar results were observed from clonogenic assays (Fig. 9, A versus B). One possible reason is that l-NAME inhibits the activity of NF-κB via inhibiting cNOS, which contributes to the production of ONOO⁻ after UVB irradiation. Thus when l-NAME inhibited NF-κB, it also reduced the production of ONOO⁻, which promotes cell death (33, 40, 41). An elevation of ONOO⁻ can lead to the oxidation of cholesterol (42), which plays a critical role in regulating UVB-induced apoptosis via induction of lipid rafts clustering and Fas aggregation (43, 44); NF-κB activation can induce iNOS expression (45), and an elevated NO⁻ production inhibits caspase 3 activation in late stage of UVB irradiation (14). Based on these studies and our findings, we propose that early activation of cNOS promotes apoptosis via induction of ONOO⁻ elevation and inhibits apoptosis via activation of NF-κB followed by induced expression of iNOS and escalated NO⁻ pro-
inhibition of IκB could cancel each other's effect on UVB-reduced colony formation (Fig. 9C, lane 5 versus lane 1). 

In summary, we propose a signaling pathway as shown in Fig. 10. UVB irradiation activates cNOS, which leads to the phosphorylation of eIF2α and translational inhibition of 1κB synthesis. With the intact degradation pathway of 1κB, the translation inhibition of 1κB reduced 1κB protein level and thus activated NF-κB, which protects IKKα from UVB-induced reduction. The activated NF-κB also protects cells from UVB-induced apoptosis; however, this anti-apoptotic function can be neutralized by the pro-apoptotic effect of ONOO−.

REFERENCES

1. Dessinioti, C., Antoniou, C., Catsambas, A., and Stratigos, A. J. (2010) Basal cell carcinoma: what’s new under the sun. Photochem. Photobiol. 86, 481–491
2. Maddodi, N., and Setaluri, V. (2008) Role of UV in cutaneous melanoma. Photochem. Photobiol. 84, 528–536
3. Li, N., and Karin, M. (1998) Ionizing radiation and short wavelength UV activate NF-κB through two distinct mechanisms. Proc. Natl. Acad. Sci. U.S.A. 95, 13012–13017
4. Baldwin, A. S. (2001) Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. J. Clin. Invest. 107, 241–246
5. Baldwin, A. S., Jr. (2001) Series introduction: the transcription factor NF-κB and human disease. J. Clin. Invest. 103, 3–6
6. Karin, M. (2006) Nuclear factor-κB in cancer development and progression. Nature 441, 431–436
7. Karin, M., and Lin, A. (2002) NF-κB at the crossroads of life and death. Nat. Immunol. 3, 221–227
8. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) Signal-induced site-specific phosphorylation targets 1κBα to the ubiquitin-proteasome pathway. Genes Dev. 9, 1586–1597
9. Chen, Z. J., Parent, L., and Maniatis, T. (1996) Site-specific phosphorylation of 1κBα by a novel ubiquitination-dependent protein kinase activity. Cell 84, 853–862
10. László, C. F., and Wu, S. (2008) Mechanism of UV-induced 1κBα indepependent activation of NF-κB. Photochem. Photobiol. 84, 1564–1568
11. Wu, S., Tan, M., Hu, Y., Wang, J. L., Scheuner, D., and Kaufman, R. J. (2004) Ultraviolet light activates NFκB through translational inhibition of 1κB synthesis. J. Biol. Chem. 279, 34898–34902
12. Koumenis, C., Naczk, C., Koritzinsky, M., Rastani, S., Diehl, A., Sonenberg, N., Koromilas, A., and Wouters, B. G. (2002) Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2α. Mol. Cell. Biol. 22, 7405–7416
13. Curry, H. A., Clemens, R. A., Shah, S., Braddy, C. M., Botero, A., Goswami, P., and Gius, D. (1999) Heat shock inhibits radiation-induced activation of NF-κB via inhibition of 1κB kinase. J. Biol. Chem. 274, 23061–23067
14. Liu, W., and Wu, S. (2010) Differential roles of nitric oxide synthases in regulation of ultraviolet B light-induced apoptosis. Nitric Oxide 23, 199–205
15. Lu, W., László, C. F., Miao, Z., Chen, H., and Wu, S. (2009) The role of nitric-oxide synthase in the regulation of UVB light-induced phosphorylation of the a subunit of eukaryotic initiation factor 2. J. Biol. Chem. 284, 24281–24288
16. Jiang, H. Y., and Wek, R. C. (2005) GCN2 phosphorylation of eIF2α activates NF-κB in response to UV irradiation. Biochem. J. 385, 371–380
17. Parker, S. H., Parker, T. A., George, K. S., and Wu, S. (2006) The roles of translation initiation regulation in ultraviolet light-induced apoptosis. Mol. Cell. Biochem. 293, 173–181
18. Boer, R., Ulrich, W. R., Klein, T., Mirau, B., Haas, S., and Baur, I. (2000) The inhibitory potency and selectivity of arginine substrate site nitric-oxide synthase inhibitors is solely determined by their affinity toward the different isoenzymes. Mol. Pharmacol. 58, 1026–1034
19. Shaffer, J. E., Han, B. I., Chern, W. H., and Lee, F. W. (1992) Lack of tolerance to a 24-hour infusion of S-nitroso N-acetylpenicillamine (SNAP) in conscious rabbits. J. Pharmacol. Exp. Ther. 260, 286–293
20. Bischof, G., Serwold, T. F., and Machen, T. E. (1997) Does nitric oxide regulate capacitative Ca influx in HEK 293 cells? Cell Calcium 21, 135–142
21. Hansson, A., Marín, Y. E., Suh, J., Rabson, A. B., Chen, S., Huberman, E., Chang, R. L., Conney, A. H., and Zheng, X. (2005) Enhancement of TP53-induced growth inhibition and apoptosis in myeloid leukemia cells by BAY 11–7082, an NF-κB inhibitor. Int. J. Oncol. 27, 941–948
22. Shin, H. M., Kim, M. H., Kim, B. H., Jung, S. H., Kim, Y. S., Park, H. J., Hong, J. T., Min, K. R., and Kim, Y. (2004) Inhibitory action of novel aromatic diaminovinyl compound on lipopolysaccharide-induced nuclear translocation of NF-κB without affecting IκB degradation. FEBS Lett. 571, 50–54
23. Suzuki, H., Chiba, T., Kobayashi, M., Takeuchi, M., Furiuchi, K., and Tanaka, K. (1999) In vivo and in vitro recruitment of an IkBα-ubiquitin ligase to 1κBα phosphorylated by IKK, leading to ubiquitination. Biochem. Biophys. Res. Commun. 256, 121–126
24. Kanarek, N., and Ben-Neriah, Y. (2012) Regulation of NF-κB by ubiquitination and degradation of the IkBs. Immunol. Rev. 246, 77–94
25. Mathes, E., O’Dea, E. L., Hoffmann, A., and Ghosh, G. (2008) NF-κB dictates the degradation pathway of IkBα. EMBO J. 27, 1357–1367
26. Koop, A., Lepenies, I., Baum, O., Davarnia, P., Scherer, G., Fickenscher, H., Kabelitz, D., and Adam-Klages, S. (2011) Novel splice variants of human IKKε negatively regulate IKKε-induced IκBα and NF-κB activation. Eur. J. Immunol. 41, 224–234
27. Yu, L. L., Dai, N., Yu, H. G., Sun, L. M., and Si, J. M. (2010) Akt associates with nuclear factor κB and plays an important role in chemotherapy resistance of gastric cancer cells. Oncol. Rep. 24, 113–119
28. Wu, L. F., Li, G. P., Su, J. D., Pu, Z. J., Feng, J. L., Ye, Y. Q., and Wei, B. L. (2010) Involvement of NF-κB activation in the apoptosis induced by extracellular adenosine in human hepatocellular carcinoma HepG2 cells. Biochem. Cell. Biol. 88, 705–714
29. Chen, W., Wang, X., Bai, L., Liang, X., Zhuang, J., and Lin, Y. (2008) Blockage of NF-κB by IKKβ- or RelA-siRNA rather than the NF-κB super-suppressor IkBα mutant potentiates adriamycin-induced cytotoxicity in lung cancer cells. J. Cell. Biochem. 105, 554–561
30. Wang, C. C., Fang, K. M., Yang, C. S., and Tzeng, S. F. (2009) Reactive oxygen species-induced cell death of rat primary astrocytes through mitochondria-mediated mechanism. J. Cell. Biochem. 107, 933–943
31. Wu, S., Hu, Y., Wang, J. L., Chatterjee, M., Shi, Y., and Kaufman, R. J. (2002) Ultraviolet light inhibits translation through activation of the unfolded protein response kinase PERK in the lumen of the endoplasmic reticulum. J. Biol. Chem. 278, 15861–15868
**cNOS Regulates UVB-induced NF-κB Activity**