Genetic Deletion of NAD(P)H:Quinone Oxidoreductase 1 Abrogates Activation of Nuclear Factor-κB, IκBα Kinase, c-Jun N-terminal Kinase, Akt, p38, and p44/42 Mitogen-activated Protein Kinases and Potentiates Apoptosis

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The NAD(P)H:quinone oxidoreductase 1 (NQO1) is a phase II enzyme that reduces and detoxifies quinones and their derivatives. Although overexpressed in tumor cells, the NQO1 has been linked with the suppression of carcinogenesis, and the effect of NQO1 on tumor necrosis factor (TNF), a cytokine that mediates tumorigenesis through proliferation, invasion, angiogenesis, and metastasis of tumors, is currently unknown. The purpose of our study was to determine the role of NQO1 in TNF cell signaling by using keratinocytes derived from wild-type and NQO1 gene-deleted mice. TNF induced nuclear factor (NF)-κB activation in wild-type but not in NQO1-deleted cells. The treatment of wild-type cells with dicoumarol, a known inhibitor of NQO1, also abolished TNF-induced NF-κB activation. NF-κB activation induced by lipopolysaccharide, phorbol ester, and cigarette smoke, was also abolished in NQO1-deleted cells. The suppression of NF-κB activation was mediated through the inhibition of IκBα kinase activation, IκBα phosphorylation, and IκBα degradation. Further, the deletion of NQO1 abolished TNF-induced c-Jun N-terminal kinase, Akt, p38, and p44/p42 mitogen-activated protein kinase activation. TNF also induced the expression of various NF-κB-regulated gene products involved in cell proliferation, antiapoptosis, and invasion in wild-type NQO1 keratinocytes but not in NQO1-deleted cells. The suppression of these antiapoptotic gene products increased TNF-induced apoptosis in NQO1-deleted cells. We also found that TNF activated NQO1, and NQO1-specific small interfering RNA abolished the TNF-induced NQO1 activity and NF-κB activation. Overall, our results indicate that NQO1 plays a pivotal role in signaling activated by TNF and other inflammatory stimuli and that its suppression is a potential therapeutic strategy to inhibit the proliferation, survival, invasion, and metastasis of tumor cells.

NAD(P)H:quinone oxidoreductase 1 (NQO1), a cytosolic flavoprotein that catalyzes quinone detoxification, is transcriptionally induced in response to various agents, including xenobiotics, oxidants, antioxidants, ultraviolet light, and ionizing radiation (1-4). The deletion of the NQO1 gene in mice has been shown to increase sensitivity to menadione-induced hepatic toxicity (5), increase susceptibility to benzo[a]pyrene-induced and 7,12-dimethylbenz[a]anthracene-induced skin carcinogenesis (6, 7), enhance susceptibility to benzene-induced toxicity (8), and cause myelogenous hyperplasia (9). Additionally, mutations in the NQO1 gene have been identified in patients with myeloid malignancies (10, 11). Further, most tumor cells overexpress the NQO1 protein (12-14), especially those of the lung, ovaries, adrenal gland, thyroid, liver, colon, breast, and pancreas, compared with normal tissues of the same origin (15, 16).

Whereas NQO1 is expressed in the cytoplasm of most normal cells, nuclear expression of NQO1 has been demonstrated in human cancer cells (17). This flavoprotein also plays a major role in apoptosis through the stabilization of p53 (18), and its deletion can promote skin carcinogenesis (19). Further, NQO1 overexpression has been confirmed as a risk factor in lung cancer (20, 21). All these findings indicate that NQO1 has a role in tumorigenesis.

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that has been linked with the proliferation, survival, invasion, angiogenesis, and metastasis of tumors (22). Most of these TNF effects are mediated through the activation of NF-κB, activated

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2 The abbreviations used are: NQO1, NAD(P)H:quinone oxidoreductase 1 enzyme; NF-κB, nuclear factor-κB; IκB, inhibitory subunit of NF-κB; IKK, IκBα kinase; COX-2, cyclooxygenase-2; MMP-9, matrix metalloproteinase-9; TNF, tumor necrosis factor; TNFR, TNF receptor; PMA, phorbol myristate acetate; EMSA, electrophoretic mobility shift assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AP-1, activated protein 1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; SSC, cigarette smoke condensate; LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; siRNA, small interference RNA; MEKK, MAPK/extracellular signal-regulated kinase kinase kinase; IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein; cFLIP, FLICE inhibitory protein.
protein 1 (AP-1), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), p44/p42 MAPK, and Akt (22, 23). For example, the activation of NF-κB and AP-1 regulates the expression of genes involved in tumorigenesis.

It is known that the TNF cell-signaling pathway is regulated by the redox status of the cell (23) and that most stimuli controlling NQO1 expression also regulate NF-κB activation (24). Therefore, our purpose in this study was to determine the role of NQO1 in TNF cell signaling by using cells derived from NQO1 gene-deleted mice. We found that NQO1 is required for the TNF-induced activation of NF-κB, IκBα kinase (IKK), JNK, Akt, p38, and p44/p42 MAPK. We also found that NF-κB-regulated gene products, such as IAP1, IAP2, XIAP, Bcl-2, Bcl-xL, cFLIP, survivin, cyclin D1, cyclooxygenase (COX)-2, and metalloproteaseinase (MMP)-9, were down-regulated by the deletion of NQO1, which resulted in the potentiation of TNF-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Bacteria-derived recombinant murine TNF, purified to homogeneity with a specific activity of 5 × 10^7 units/mg, was kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were obtained from Invitrogen. Cigarette smoke condensate (CSC) was kindly provided by Dr. C. Gary Gariola (University of Kentucky, Lexington, KY). Lipopolysaccharide (LPS) and an anti-β-actin antibody were obtained from Sigma (St. Louis, MO). The antibodies anti-p65, anti-p50, anti-IκBα, anti-cyclin D1, anti-MMP-9, anti-poly(ADP-ribose) polymerase, anti-IAP1, anti-IAP2, anti-Bcl-xL, and anti-Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-COX-2 antibody was obtained from BD Biosciences. The phospho-specific anti-IκBα (Ser-32) antibody was purchased from Cell Signaling (Beverly, MA). Anti-IKK-α, anti-IKK-β, and anti-FLIP antibodies were kindly provided by Imgenex (San Diego, CA). A polyclonal antibody that recognizes the serine 529 phosphorylated form of p65 was obtained from Rockland Laboratories (Gilbertsville, PA).

**Cell Lines**—The mouse keratinocytes from benzo[a]pyrene-induced wild-type and NQO1−/− mouse skin tumors were established by standard procedures. Briefly, the tumors were removed and placed in high calcium (200 mM) supplemented saline containing 1% fetal bovine serum and 0.1% sodium azide. The cells were preincubated with 10% goat serum for 20 min and washed, and then monoclonal rabbit IgG anti-TNFFR1/R2 antibodies were added. Following a 1-h incubation at 4 °C, the cells were washed and incubated for an additional 1 h in fluourescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG monoclonal antibodies. The cells were analyzed with a flow cytometer (FACS Calibur, BD Biosciences) and acquisition and analysis programs (CellQuest, BD Biosciences).

NQO1 Enzyme Activity—Wild-type keratinocytes and human hepatoblastoma (HepG2) cells were grown in monolayers and treated with 1 nM TNFα for different time intervals. The cells were harvested, homogenized in 50 mM Tris, pH 7.4, containing 0.25 mM sucrose and centrifuged at 105,000 × g for 1 h to obtain cytosolic fractions. Dicoumarol-sensitive NQO1 activity was measured in cytosolic fractions by previously described procedure (25). The final reaction mixture contained 25 mM Tris/HCl, pH 7.4, 0.18 mg/ml bovine serum albumin, 5 mM FAD, 0.01% Tween 20, 200 mM NADH, 50 mM 2,6-dichlorophenolindophenol, and 0.2–2.0 mg of cytosolic proteins. The reaction was monitored by measuring the decrease in absorbance due to reduction of 2,6-dichlorophenolindophenol at 600 nm. This gave us total NQO activity. The same experiment was repeated in the presence of 20 mM NQO1-specific inhibitor dicoumarol. The activity obtained in the presence of dicoumarol was subtracted from the total activity to obtain the dicoumarol-inhibitable NQO1 activity. The experiments were independently repeated three times.

**siRNA Inhibition of NQO1 Activity and Protein**—HCT116 cells were grown in monolayer culture. Pre-designed NQO1 siRNA was purchased from Ambion, Austin, TX (siRNA ID# 8953). The HCT116 cells were transfected with scrambled or NQO1-specific siRNA using the HiPerFect transfection kit from Qiagen (Chatsworth, CA) by using the procedure as suggested in the manufacturer’s protocol. The cells were harvested and homogenized, and nuclear and cytosolic fractions were prepared using a kit and instruction manual from Active Motif, Carlsbad, CA. The cytosolic fractions were analyzed for NQO1 activity by using a previously described procedure, and NQO1 protein was analyzed by Western blotting and probing with anti-NQO1 antibody. The nuclear fractions were analyzed for NF-κB binding in electrophoretic mobility shift assay (EMSA) experiments.

EMSA—To assess NF-κB activation, we performed EMSAs as previously described (26). Briefly, nuclear extracts prepared from TNF-treated cells (1 × 10^6/ml) were incubated with a 32P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long-terminal repeat, 5’-TTGGTACCAAGGGACATTTCGGCTGGGAGATTCCAGGGAGCCG-TGG-3’ (boldface indicates NF-κB-binding sites) for 30 min at 37 °C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide (5’-TTGGTACCAACTCATTTCGCCGCTGGCATTTCAGGGAGGCGGTTG-3’) was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, some(s) in established skin tumor cells (data not shown).

**Cell-surface Expression of TNF Receptors**—For analysis of cell-surface expression of TNF receptors (TNFRs), cells were harvested and suspended in Dulbecco’s phosphate-buffered saline containing 1% fetal bovine serum and 0.1% sodium azide. For example, the activation of NF-κB and AP-1 regulates the expression of genes involved in tumorigenesis. The anti-COX-2 antibody was obtained from BD Biosciences. The anti-NQO1 antibody was obtained from Active Motif, Carlsbad, CA. The cytosolic fractions were analyzed for NQO1 activity by using a previously described procedure, and NQO1 protein was analyzed by Western blotting and probing with anti-NQO1 antibody. The nuclear fractions were analyzed for NF-κB binding in electrophoretic mobility shift assay (EMSA) experiments.
FIGURE 1. A, results of a Western blot analysis of the NQO1 protein. Whole cell extracts were prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which we performed a Western blot analysis with an anti-NQO1 antibody (Ab). B, cell-surface expression of TNFRs in NQO1 wild-type and NQO1-deleted cells. Cells were harvested and labeled with anti-TNFR1 or anti-TNFR2 antibodies and then with a FITC-conjugated goat anti-rabbit IgG antibody and analyzed by flow cytometry. C, time-dependent effects of NF-κB activation on NQO1-deleted keratinocytes treated with TNF. One million cells were treated with 0.1 nM TNF for the indicated times, after which nuclear extracts were prepared; NF-κB activation was then analyzed by EMSA. D, effect of dicoumarol on TNF-induced NF-κB activation. One million cells from wild-type NQO1 were pretreated with dicoumarol for 4 h and then treated with 0.1 nM TNF for 30 min, after which nuclear extracts were prepared; NF-κB activation was then analyzed by EMSA. The results shown are representative of three independent experiments.
nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either p50 or p65 from NF-kB for 30 min at 37 °C before the complex was analyzed by EMSA. Antibodies against cyclin D1 and preimmune serum were included as negative controls. The dried gels were visualized, and radioactive bands were quantitated with a PhosphorImager (Amersham Biosciences) and ImageQuaNT software (Molecular Dynamics).

Western Blot Analysis—To determine the levels of protein expression in the cytoplasm or the nucleus, we prepared extracts of TNF-treated cells from each and fractionated them by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blotted with each antibody, and detected by electrochemiluminescence reagents (Amersham Biosciences). The bands were quantitated with densitometry (Personal Densitometer Scan version 1.30) and ImageQuaNT software version 3.3 (both from Molecular Dynamics).

IKK Assay—The IKK assay was performed by a method described previously (27). Briefly, IKK complexes from whole cell extracts were precipitated with an antibody against IKK-α, after which they were treated with protein A/G-Sepharose beads (Pierce). After a 2-h incubation, the beads were washed with lysis buffer and then assayed in a kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl2, 2 mM dithiothreitol, 20 μCi of [γ-32P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate glutathione S-transferase-IκBα (amino acids 1–54). After incubation at 30 °C for 30 min, the reaction was terminated by boiling the beads with an SDS sample buffer for 5 min. Finally, the proteins were resolved by 10% SDS-PAGE; the gel was dried, and the radioactive bands were visualized by the PhosphorImager. To determine the total amounts of IKK-α and IKK-β in each sample, 50 μg of the whole cell protein was resolved by 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and blotted with either an anti-IKK-α or an anti-IKK-β antibody.

AP-1 Activation Assay—To assay AP-1 activation with EMSA, we incubated 6 μg of nuclear extract protein with 16 fmol of the 32P-end-labeled AP-1 consensus oligonucleotide 5′-CGCTTGATGACTCAGCCGAA-3′ (boldface indicates the AP-1 binding site) for 15 min at 37 °C, after which the DNA-protein complexes formed were resolved from free oligonucleotide on 6%
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native polyacrylamide gels. The specificity of binding was examined by a competition assay with unlabeled oligonucleotide. The radioactive bands were visualized and quantified as indicated above.

**NF-κB-dependent Reporter Gene Expression Assay**—To examine TNF-induced reporter gene expression, we placed cells (3 \( \times \) 10^5) in 6-well plates and transiently transfected them by using the calcium phosphate method with 0.5 \( \mu \)g of pNF-κB-secretory alkaline phosphatase (Clontech Laboratories, Inc., Mountain View, CA) and the control plasmid pCMVFLAG1 DNA (2 \( \mu \)g). After 24 h, cells were washed, exposed to 1 nM TNF for 24 h, harvested from the cell culture medium, and then analyzed for secretory alkaline phosphatase activity, essentially according to the protocol described by the manufacturer, with a 96-well fluorescence plate reader (Fluoroscan II, Labsystems, Chicago, IL) with its excitation set at 360 nm and emission at 460 nm.

**MTT Assay**—The cell growth effects of TNF were determined by the MTT uptake method as described. Briefly, 2 \( \times \) 10^3 cells were seeded in triplicate in 96-well plates and then treated with various concentrations of TNF for 48 h at 37 °C. Thereafter, MTT solution was added to each well. After a 2-h incubation at 37 °C, an extraction buffer (20% SDS and 50% dimethylformamide) was added; the cells were then incubated overnight at 37 °C, and the absorbance was measured at 570 nm by using a 96-well multispanner (MRX Revelation, Dynex Technologies, Chantilly, VA).

**Thymidine Incorporation Assay**—The cell-proliferative effects of TNF were also monitored by the thymidine incorporation method. Briefly, 1000 cells in 100 \( \mu \)l of medium were cultured in triplicate in 96-well plates with various concentrations of TNF for 48 h at 37 °C. Six hours before the completion of the experiment, cells were pulsed with 0.5 \( \mu \)Ci (0.0185 mBq) of [\(^{3}H\)]thymidine, and the uptake of [\(^{3}H\)]thymidine was monitored with a Matrix-9600 \( \beta \)-counter (Packard Instrument Co., Downers Grove, IL).

**Annexin V Assay**—An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of annexin V. Therefore, to identify apoptosis, we used the annexin V antibody, which was conjugated with FITC fluorescence dye. Briefly, 1 \( \times \) 10^5 cells were pretreated with 1 \( \mu \)g/ml cycloheximide, treated with 1 nM TNF for 16 h at 37 °C, and then subjected to annexin V staining. Cells were washed in phosphate-buffered saline, resuspended in 100 \( \mu \)l of a binding buffer containing FITC-conjugated anti-annexin V antibody, and then analyzed with flow cytometry (FACSCalibur, BD Biosciences).

**RESULTS**

We examined the expression of the NQO1 protein in both wild-type (NQO1-WT) and NQO1-deleted murine keratinocytes (NQO1-KO). As expected, NQO1 was expressed in NQO1-WT cells but not in NQO1-deleted cells (Fig. 1A). Further, we examined the expression of TNF receptor (TNFR)-1 and TNFR2 with flow cytometry. No difference was found in the expression of the two types of receptors between the two cell types (Fig. 1B).

**NQO1 Is Required for TNF-dependent NF-κB Activation**—We investigated the effect of NQO1 deletion on TNF-induced NF-κB activation. We treated the cells with TNF for different times, prepared the nuclear extracts, and then analyzed them for NF-κB activation by the EMSA method. As shown in Fig. 1C, TNF stimulated NF-κB activation in a time-dependent manner in NQO1-WT keratinocytes but abrogated its activation in NQO1-KO keratinocytes.

**Dicoumarol (NQO1-specific Inhibitor) Suppresses TNF-induced NF-κB Activation**—Dicoumarol has been shown to be a specific inhibitor of NQO1 (28), so whether dicoumarol inhibition of NQO1 in WT cells also suppresses TNF-induced NF-κB activation was examined. As shown in Fig. 1D, dicoumarol suppressed TNF-induced NF-κB activation, thus indicating that the activity of NQO1 is required for the suppression of NF-κB.

**NQO1 Wild-type Cells Increase TNF-induced NF-κB Activation in a Dose-dependent Manner**—Because the activation of NF-κB by TNF is more robust at higher concentrations (29), we evaluated the effect of NQO1 deletion on the NF-κB activation
induced by higher concentrations of TNF. The activation of NF-κB by TNF was strongly evident in NQO1-WT cells but not in NQO1-KO cells, even at a concentration of 10 nM TNF (Fig. 2A). These results indicate that NQO1 is required for TNF-induced NF-κB activation.

**NQO1 Is Required for NF-κB Activation Induced by LPS, PMA, or CSC**—Besides TNF, NF-κB is activated by various carcinogens and inflammatory stimuli through a mechanism that may differ from that of TNF (30–32). We examined the role of NQO1 in NF-κB activation induced by LPS, PMA, or CSC. As shown in Fig. 2B, LPS, PMA, and CSC all stimulated NF-κB, and NQO1 deletion suppressed NF-κB activation in every case. These results indicated that NQO1 acts at a step in the NF-κB activation pathway that is common to all these agents.

Various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in the DNA (33). Antibodies to both the p50 (NF-κB1) and the p65 (RelA) subunits of NF-κB shifted the major retarded band visualized by EMSA to a higher molecular mass (Fig. 2C), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. The addition of preimmune serum had no effect, and excess unlabeled NF-κB (100-fold) caused complete disappearance of the band; however, a mutant oligonucleotide of NF-κB did not affect NF-κB binding activity.

**NQO1 Is Required for Expression of TNF-induced NF-κB-dependent Reporter Gene Expression**—DNA binding does not always correlate with NF-κB-dependent gene transcription (34). NF-κB-regulated reporter gene expression was activated by TNF in a dose-dependent manner in wild-type keratinocytes, but minimal activation was detected in NQO1-deleted keratinocytes (Fig. 2D). These results suggested that NQO1 is needed not only for p65 binding to DNA but also for NF-κB-regulated reporter gene expression.

**NQO1 Is Required for TNF-dependent IκBα Degradation**—Translocation of NF-κB to the nucleus is preceded by proteolytic degradation of IκBα (33). TNF induced IκBα degradation, and it reached maximum at 15 min after TNF stimulation in wild-type keratinocytes (Fig. 3A). In NQO1-deleted keratinocytes, however, TNF had no effect on IκBα degradation (Fig. 3A, upper panel). Thus, NQO1 is required for degradation of IκBα.

**NQO1 Is Required for TNF-dependent IκBα Phosphorylation**—The proteolytic degradation of IκBα is known to require phosphorylation at serines 32 and 36 (33). TNF stimulated IκBα
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phosphorylation in wild-type keratinocytes, but in NQO1-KO cells, the IκBα phosphorylation induced by TNF was almost completely suppressed (Fig. 3A, middle panel).

NQO1 Deletion Inhibits TNF-induced IKK Activation—Given that IKK is required for TNF-induced NF-κB activation (33), we investigated the effect of NQO1 deletion on TNF-induced IKK activation. NQO1 deletion completely repressed TNF-induced activation of IKK without any effect on the expression of IKK-α or IKK-β (Fig. 3B). These results suggested that NQO1 is required for TNF-induced IKK activation.

NQO1 Is Required for TNF-induced Phosphorylation and Nuclear Translocation of p65—Because the degradation of IκBα leads to nuclear translocation of the p65 subunit of NF-κB, we also analyzed the effect of the NQO1 deletion on TNF-induced nuclear translocation of p65 with Western blot analysis. TNF induced nuclear translocation of p65 in a time-dependent manner as early as 5 min after TNF stimulation in NQO1 wild-type keratinocytes (Fig. 3C). In NQO1-deleted keratinocytes, TNF failed to induce nuclear translocation of p65.

TNF induces the phosphorylation of p65, which is required for its transcriptional activity (35). As shown in Fig. 3C, TNF also induced phosphorylation of p65 in a time-dependent manner as early as 5 min after TNF stimulation in NQO1 wild-type keratinocytes (Fig. 3C). In NQO1-deleted keratinocytes, TNF failed to induce phosphorylation of p65.

NQO1 Is Required for TNF-induced Activation of JNK—We investigated the effect of NQO1 on other signals transduced by TNF. Activation of JNK is one of the earliest events induced by TNF (22). To evaluate the specific role of NQO1 on TNF-induced JNK activation, we stimulated cells with TNF for varying time intervals, prepared whole cell extracts, and analyzed them for JNK activity with an immune-complex kinase assay. TNF induced the time-dependent activation of JNK in wild-type but not in NQO1-deleted keratinocytes (Fig. 4A). These results indicated that NQO1 is required for TNF-induced JNK activation.

NQO1 Is Required for TNF-induced Activation of Akt—Activation of Akt is also one of the earliest events induced by TNF (36). TNF induced time-dependent activation of Akt in wild-type but not in NQO1-deleted keratinocytes (Fig. 4B). These results indicated that NQO1 is needed for TNF-induced Akt activation.

NQO1 Is Required for TNF-induced Activation of p38—Activation of p38 has been linked with TNF-induced IκB phosphorylation and NF-κB activation (37). TNF induced the time-dependent activation of p38 in wild-type but not NQO1-deleted keratinocytes (Fig. 4B). These results indicated that NQO1 is needed for TNF-induced p38 activation.

NQO1 Is Required for TNF-induced Activation of p44/p42 MAPK—TNF has been shown to activate p44/p42 MAPK through the Ras/Raf/MAPK kinase cascade (22). TNF induced the time-dependent phosphorylation of p44/p42 MAPK in wild-type keratinocytes but abolished p44/p42 MAPK activation in NQO1-deleted keratinocytes (Fig. 4B). These results suggested that NQO1 is needed for TNF-induced p44/p42 MAPK activation.

NQO1 Is Required for TNF-induced Activation of AP-1—Because JNK is needed for TNF-induced AP-1 activation, we also investigated the role of NQO1 in TNF-induced AP-1 activation. Cells were treated with TNF, and then nuclear extracts were prepared and assayed for AP-1 activation by EMSA. As shown in Fig. 4C, NQO1 deletion completely repressed TNF-induced AP-1 activation, suggesting that NQO1 is required for TNF-induced AP-1 activation.

NQO1 Is Required for Expression of TNF-induced, NF-κB-dependent Cyclin D1, COX-2, and MMP-9 Proteins—Given that TNF has been shown to induce cyclin D1, COX-2, and MMP-9 (38–40), we examined whether NQO1 is needed for the induction of these gene products. Cyclin D1, COX-2, and MMP-9 expressions were induced by TNF in a time-dependent manner in wild-type but not in NQO1-deleted keratinocytes (Fig. 5A).

NQO1 Is Required for Expression of TNF-induced NF-κB-dependent Antiapoptotic Proteins—Because NF-κB also regulates the expression of various antiapoptotic proteins, including survivin, IAP1/2, Bcl-xL, TNFR-associated factor 2, Bcl-2, XIAP, cFLIP, and β-actin. The results shown are representative of three independent experiments.
type but not NQO1-deleted keratinocytes (Fig. 5B). These results indicated that NQO1 is required for TNF-induced NF-κB-regulated gene products.

Deletion of NQO1 Inhibits TNF-induced Proliferation and Enhances TNF-induced Apoptosis—Whereas some cell types undergo apoptosis in response to TNF, others undergo proliferation (44). Activation of NF-κB has been shown to inhibit apoptosis and promote proliferation through the expression of the previously described gene products (45–47). Thus, deletion of NQO1 might suppress the proliferation induced by TNF. Whether suppression of NF-κB by deletion of NQO1 affects

TNF-induced proliferation was therefore investigated by using the MTT and [3H]thymidine incorporation methods (Fig. 6, A and B). Both the MTT and [3H]thymidine incorporation methods indicated that TNF induced the proliferation of WT cells but that this proliferation was attenuated in NQO1-deleted cells, suggesting that the deletion of NQO1 suppressed TNF-induced cell proliferation.

The results of annexin V staining to determine whether this suppressed cell proliferation was due to apoptosis showed that TNF induced apoptosis in 10.5% of WT cells and 75% of NQO1-deleted cells (Fig. 6C). These results suggested that NQO1 deletion potentiates TNF-induced apoptosis.

TNF Increases NQO1 Enzyme Activity in Wild-type Keratinocytes and HepG2 Cells—The results so far suggest that NQO1 plays a major role in TNF signaling. Whether TNF activates NQO1 activity was investigated. NQO1 enzyme activity measurements were done in NQO1-WT keratinocytes (Fig. 7A). Treatment of these cells with TNF resulted in a time-dependent increase in the NQO1 enzyme activity, which is in agreement with our earlier results. Whether TNF activates NQO1 in cells other than keratinocytes was also examined. We found that TNF also caused a time-dependent induction of NQO1 activity in human hepatoma HepG2 cells (Fig. 7B).

Above we showed that dicoumarol inhibits TNF-induced NF-κB activation in keratinocytes. Whether it also inhibits TNF-induced NF-κB activation in other cell types was investigated. We found that dicoumarol abolished the TNF-induced NF-κB activation in human myeloid KBM-5 cells also (Fig. 7C). Thus the role of NQO1 in TNF signaling is not cell type-specific.

Abrogation of NQO1 Expression by siRNA Inhibited TNF-induced NF-κB Activation—Thus far all studies of the role of NQO1 in TNF signaling were carried out by using either genetically deleted NQO1 cells or by using a pharmacological inhibitor of NQO1. Whether the suppression of NQO1 expression by siRNA abrogates TNF signaling was also investigated. NQO1 siRNA was used to knock-down NQO1 protein expression in HCT116 colon carcinoma cells. NQO1 expression was effectively abolished in the cells treated with NQO1 siRNA but not in those treated with the scrambled siRNA, as observed both by Western blotting (Fig. 7D) and by NQO1 activity (Fig. 7E). TNF also failed to induce the NQO1

**FIGURE 6.** A, effect of NQO1 deletion on TNF-induced cell growth. Cells (2000) were seeded in triplicate in 96-well plates and then treated with the indicated concentrations of TNF for 48 h. Thereafter, cell growth was analyzed with the MTT method described under “Experimental Procedures.” The results are shown as the mean (± S.D.) from triplicate cultures. B, effect of NQO1 deletion on TNF-induced cell proliferation. Cells (1000) were seeded in triplicate in 96-well plates and then treated with the indicated concentrations of TNF for 48 h. Cell proliferation assays were performed as described under “Experimental Procedures.” Results are shown as the mean (± S.D.) percent increase in [3H]thymidine incorporation from triplicate cultures compared with the untreated control. C, cells (1 × 10⁵ cells/well) were pretreated with 1 μg/ml cycloheximide for 1 h, incubated with 1 nM TNF for 12 h, and then subjected to annexin V staining. Cells were washed, incubated with a FITC-conjugated anti-annexin V antibody, and then analyzed with flow cytometry.
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activity in the cells treated with NQO1 siRNA (Fig. 7E), and this correlated with inhibition of TNF-induced NF-κB activation (Fig. 7F). Thus these results with siRNA also demonstrate the critical role of NQO1 in TNF signaling.

**DISCUSSION**

We found that TNF induced NF-κB activation, IKK activation, IκBα phosphorylation, IκBα degradation, and NF-κB reporter gene transcription in NQO1-WT but not in NQO1-deleted keratinocytes. Further, the NF-κB activation induced by LPS, PMA, or CSC was completely abolished in NQO1-deleted keratinocytes, and the activation of TNF-induced JNK, p38 MAPK, p44/p42 MAPK, and Akt was also abrogated in NQO1-deleted keratinocytes. TNF induced the expression of NF-κB-dependent gene products such as cyclin D1, COX-2, MMP-9, survivin, IAP1, IAP2, Bcl-2, Bcl-xL, XIAP, and cFLIP in WT cells, but all were down-regulated in NQO1-deleted cells, and this correlated with the inhibition of cell proliferation and the potentiation of the apoptosis induced by TNF (Fig. 8).

Our results further indicate that TNF-induced NF-κB activation is abrogated not only in NQO1-deleted cells but also after treatment of cells with dicoumarol, the pharmacological inhibitor of NQO1. Although not investigated in detail, dicoumarol has been shown to inhibit NF-κB through alteration of the cellular redox status of the cell (48, 49). Like dicoumarol, NQO1 is also inhibited by another quinone analogue capsaicin (50, 51). We also found that TNF activated NQO1 activity in different cell types. NQO1-specific siRNA abolished the expression of NQO1 protein and abrogated TNF-induced NQO1 activity and NF-κB activation. Our laboratory previously showed that capsaicin also suppresses TNF-induced NF-κB activation (52). Similarly, curcumin has been shown to be a potent inhibitor of both NQO1 (53) and NF-κB activation (54). How NQO1 suppresses TNF-induced NF-κB activation is uncertain, but the alteration of the redox status of the cell is the most likely mechanism. This is in agreement with earlier observations from our laboratory that overexpression of the antioxidant enzymes superoxide dismutase (55) and γ-glutamyl cysteine synthetase (56) can abolish TNF-induced cell signaling, including NF-κB activation.

Our results in this study indicate that deletion of NQO1 abolished TNF-induced IκBα phosphorylation and degradation through inhibition of IKK activation. How the deletion of NQO1 leads to suppression of IKK activation, however, is not yet clear, partly because the kinase that activates IKK is unknown. NF-κB-inducing kinase, MEKK1, MEKK3, Akt, transforming growth factor-β-activated kinase 1, glycogen synthase kinase 3β, and various other kinases have been implicated in the IKK activation induced by TNF (57–60). We found that TNF-induced AKT activation was indeed abrogated by deletion of the NQO1 gene. We found that, besides NF-κB, NQO1 deletion also abol-
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FIGURE 8. Proposed model of the role of NQO1 in TNF-induced activation.
The model suggests the role of NQO1 in TNF-induced activation of NF-κB, JNK, p38, p42/p44 MAPK, and apoptosis.

ished TNF-induced activation of MAPKs, including JNK. These results are in agreement with Cross et al. (49) who reported that inhibitors of NQO1 suppressed JNK activation. Because JNK activation is needed for AP-1 activation, we found that NQO1 deletion also abolished AP-1 activation.

Our results also demonstrate for the first time that the deletion of NQO1 abolishes the expression of various NF-κB-dependent gene products, including COX-2, cyclin D1, MMP-9, survivin, IAP1, IAP2, Bcl-2, Bcl-x<sub>L</sub>, XIAP, and cFLIP. The down-regulation of these gene products suggests that deletion of NQO1 suppresses TNF-induced proliferation and enhances apoptosis. Indeed, we found that TNF-induced cell proliferation was inhibited and apoptosis significantly increased by NQO1 deletion. Similar to our study, several reports suggest that inhibition of NQO1 enhances apoptosis (48, 49, 62). In contrast, Siemankowski et al. (61) found that transfection of NQO1-deleted mice.

Therefore, the suppression of NF-κB and cell signaling activated by TNF and other inflammatory stimuli, as described herein.

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Thus, suppression of NF-κB activation by deletion of NQO1 appears to be paradoxical. However, this may not be the case, because most agents mediate opposite pathways, depending on other factors such as cell type. For instance, although TNF induces apoptosis in some cells, it induces the proliferation of others (22). Similarly, whereas NQO1 inhibits carcinogenesis by catalyzing the two-electron reduction of quinone compounds, thus preventing the formation of reactive oxygen species, it also mediates cytotoxicity and carcinogenesis by catalyzing the reductive activation of quinoid chemotherapeutic agents and of environmental carcinogens, such as nitrosamines, heterocyclic amines, and cigarette smoke (63, 64). Thus, although numerous studies suggest that activation of NF-κB leads to carcinogenesis, others have suggested that inhibition of NF-κB can mediate skin carcinogenesis (65). Therefore, the suppression of NF-κB activation might have contributed to increased skin carcinogenesis observed in NQO1-deleted mice.

Because NQO1 has been shown to be abundantly expressed in tumor cells (10–21, 66), its suppression has potential as a therapeutic target. Although it is known that both NF-κB and NQO1 are regulated by pro-oxidants, it is less clear whether the expression of NQO1 requires NF-κB activation. However, given that TNF and NF-κB are known to regulate several disease states such as cancer, inflammation, diabetes, and neurodegenerative disorders (43, 67), the potential of suppressing the TNF cell-signaling pathway by using NQO1 inhibitors exists. Interestingly, several chemopreventive agents have already been shown to inhibit NQO1 (51, 53, 68). Thus, NQO1 suppression is a novel target for chemoprevention, possibly through the suppression of NF-κB and cell signaling activated by TNF and other inflammatory stimuli, as described herein.
