Deficiency of NRH:Quinone Oxidoreductase 2 Differentially Regulates TNF Signaling in Keratinocytes: Up-regulation of Apoptosis Correlates with Down-regulation of Cell Survival Kinases

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Abstract

NRH:quinone oxidoreductase 2 (NQO2) is a cytosolic flavoprotein that catalyzes the two-electron reduction of quinones and quinoid compounds to hydroquinones. Although the role of a homologue, NAD(P)H:quinone oxidoreductase 1 (NQO1), is well defined in oxidative stress, neoplasia, and carcinogenesis, little is known about the mechanism of actions of NQO2 in these cellular responses. Whether NQO2 has any role in tumor necrosis factor (TNF) signaling was investigated using keratinocytes derived from wild-type and *NQO2* knockout (*NQO2*$^{-/-}$) mice. Although exposure of wild-type cells to TNF led to activation of nuclear factor-κB (NF-κB) and IκBα kinase, IκBα degradation, p65 phosphorylation, and p65 nuclear translocation, this cytokine had no effect on *NQO2*$^{-/-}$ cells. Deletion of *NQO2* also abolished TNF-induced c-Jun NH_2-terminal kinase, Akt, p38, and p44/p42 mitogen-activated protein kinase activation. The induction of various antiapoptotic gene products (MMP-9, cyclin D1, COX-2, IAP1, IAP2, Bcl-2, cFLIP, and XIAP) by TNF was also abolished in NQO2$^{-/-}$ cells. This correlated with potentiation of TNF-induced apoptosis as indicated by cell viability, Annexin V staining, and caspase activation. In agreement with this, we also found that TNF activated NQO2, and *NQO2*-specific small interfering RNA abrogated the TNF-induced NQO2 activity and NF-κB activation. Overall, our results indicate that deletion of *NQO2* plays a differential role in TNF signaling pathway: by suppressing cell survival signals and potentiating TNF-induced apoptosis. [Cancer Res 2007;67(20):10004–11]

Introduction

NRH:quinone oxidoreductase 2 (NQO2) is a flavoprotein that catalyzes metabolic reduction of quinones (1–3). It is a homologue of the well-characterized NAD(P)H:quinone oxidoreductase 1 (NQO1). The two proteins possess ~49% amino acid sequence identity and have similar substrate specificities, but differ in affinity for electron-donating cofactors. Although NQO2 is resistant to typical inhibitors of NQO1, such as dicumarol, NQO2 is inhibited by flavones such as quercetin and benzo(*a*)pyrene (1–5). The cDNA and gene encoding NQO2 were identified from human liver (4, 5).

NQO2$^{-/-}$ mice have been shown to develop normally, indicating that NQO2 is not associated with mouse development (6), but deletion of the gene has been reported to cause myelogenous hyperplasia of bone marrow, decrease sensitivity to menadione-induced hepatic toxicity (6), and increase susceptibility to benzo(*a*)pyrene-induced and 7,12-dimethylbenz(*a*)anthracene–induced skin carcinogenesis (7), suggesting that NQO2 plays an important role in protection against myelogenous hyperplasia and in chemoprevention. *NQO1*$^{-/-}$ mice, on the other hand, showed altered intracellular redox status and altered metabolism of carbohydrates, fatty acids, and nucleotides and reduced accumulation of abdominal fat with age (8). *NQO1*$^{-/-}$ mice, in contrast to NQO2$^{-/-}$ mice, showed increased sensitivity to menadione-induced hepatic damage (6). Like *NQO2*$^{-/-}$ mice, *NQO1*$^{-/-}$ mice also developed myelogenous hyperplasia of bone marrow and increased sensitivity to skin carcinogenesis in response to benzo(*a*)pyrene and dimethylbenz(*a*)anthracene (9).

Although NQO1 protein is expressed in most tumor cells (10–13), information about expression of NQO2 protein in tumor tissues is still limited in spite of many studies (5, 14–16). Our previous report has been shown that the deletion of *NQO1* gene potentiates tumor necrosis factor (TNF)–induced apoptosis through down-modulation of nuclear factor-κB (NF-κB)–regulated gene products (17). It is evident that there are more similarities than dissimilarities between NQO1 and NQO2, and it is plausible to assume that the NQO2 too has a role in tumorigenesis.

TNF is one of the most potent proinflammatory cytokines. It is required for maintenance of immune system in normal conditions, but if it is dysregulated, it is associated with the proliferation, survival, invasion, angiogenesis, and metastasis of tumors (18). Most of these TNF effects occur through the activation of NF-κB, activated protein 1 (AP-1), c-Jun NH_2-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), p44/p42 MAPK, and Akt (18, 19).

Therefore, our purpose in this study was to determine the role of NQO2 in TNF cell signaling by using cells derived from NQO2$^{-/-}$ mice. We found that NQO2 was 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, cFLIP, cyclin D1, cyclooxygenase (COX)-2, and metalloproteinase (MMP)-9, were down-regulated by the deletion of *NQO2*, which potentiated apoptosis.

Materials and Methods

Reagents. Bacteria-derived recombinant murine TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech. Penicillin, streptomycin, DMEM, and fetal bovine

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serum were obtained from Invitrogen. Anti–β-actin antibody was obtained from Sigma. The antibodies anti-NQO2 (N-15), anti-NQO1, anti-p65, anti-IκBα, anti-Akt, anti-p44/p42, anti-p38, anti–cyclin D1, anti–MMP-9, anti–poly(ADP-ribose) polymerase (PARP), anti-IAP1, anti-IAP2, and anti–Bcl-2 were obtained from Santa Cruz Biotechnology. The anti–COX-2 and XIAP antibodies were obtained from BD Biosciences. The phospho-specific anti-p65 (Ser^{536}), phosphospecific anti-Akt (Ser^{473}), phosphospecific anti-p44/p42 (Thr^{202}/Tyr^{203}; Thr^{185}/Try^{187}), and phosphospecific anti-p38 (Thr^{180}/Tyr^{182}) antibodies were purchased from Cell Signaling. Anti–IKK-α, anti–IKK-β, and anti-cFLIP antibodies were kindly provided by Imgenex.

Cell lines. Keratinocytes derived from benzo(*a*)pyrene-induced tumors in wild-type and *NQO2*-null mice were grown in monolayer by procedures previously described (17). Briefly, the tumors were removed and placed in high calcium (200 mmol/L) supplemented with antibiotics and antimitotic agents. The tumors were exposed to collagenase at 37°C for 2 h with agitation. The settled clumps were gently removed from the supernatant, washed twice with high calcium buffer, exposed to high calcium/keratinocyte growth factor, and plated in the four corner wells of collagen (Invitrogen)–coated 12-well plates. The medium was replaced with low calcium (50 mmol/L)/keratinocyte growth factor and replaced every 2 to 3 days. The growing keratinocyte cells were collected and cultured in DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The karyotyping analysis did not reveal loss and/or gain or translocation of chromosome (1) in established skin tumor cells (data not shown).

NQO2 enzyme activity. NQO2 activity was determined as described previously (6). In brief, NRH was synthesized by adding 1,000 units of calf intestinal alkaline phosphatase (Sigma) to 500 μL of 10 mmol/L nicotinamide mononucleotide (Sigma) in PBS. The reaction was allowed to proceed for 15 min at room temperature. Ten microliters of the NRH were added to 50 mmol/L Tris (pH 7.4), 100 μmol/L dichlorophenolindophenol, and cytosolic extract in a 1-mL standard cuvette. Absorbance was monitored at 600 nm for 1 min with a Beckman DU640 spectrophotometer. Cytosolic extract concentrations were used that produced a 0.08 to 0.15 A change/min. This gave us total NQO activity. The same experiment was repeated in the presence of 10 μmol/L NQO2-specific inhibitor benzopyrene. The activity obtained in the presence of benzopyrene was subtracted from the total activity to obtain the benzopyrene-inhibitable NQO2 activity. The specific activity of NQO2 was calculated from the change in absorbance per microgram of protein. The experiments were independently repeated thrice.

Small interfering RNA inhibition of NQO2 activity and protein. Human hepatoblastoma (HepG2) cells were grown in monolayer cultures in six-well plates in MEM-a supplemented with 10% fetal bovine serum. Lamin A/C and NQO2 small interfering RNA (siRNA) was purchased from Dharmacon. The HepG2 cells were transfected with *Lamin A/C* or *NQO2*-specific siRNA using LipofectAMINE RNAiMAX from Invitrogen 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. The cytosolic fractions were analyzed for NQO2 activity by using a previously described procedure, and NQO2 protein was analyzed by Western blotting and probing with anti-NQO2 antibody. The nuclear fractions were analyzed for NF-κB binding in electrophoretic mobility shift assay (EMSA) experiments.

Electrophoretic mobility shift assays. To assess NF-κB activation, we did EMSA as previously described (20). The dried gels were visualized and radioactive bands quantitated with a PhosphorImager (Amersham Biosciences) and ImageQuant software (Molecular Dynamics).

Western blot analysis. To determine the levels of protein expression in the cytoplasm and 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 v1.30) and ImageQuant software version 3.3 (both from Molecular Dynamics; data not shown).

IKK assay. The IKK assay was done by a method described previously (17).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cell growth effects of TNF were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as described. Briefly, 2×10^4 cells were seeded in triplicate in 96-well plates, pretreated with cycloheximide for 1 h, and then treated with various concentrations of TNF for 24 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 multiscanner (MRX Revelation; Dynex Technologies).

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

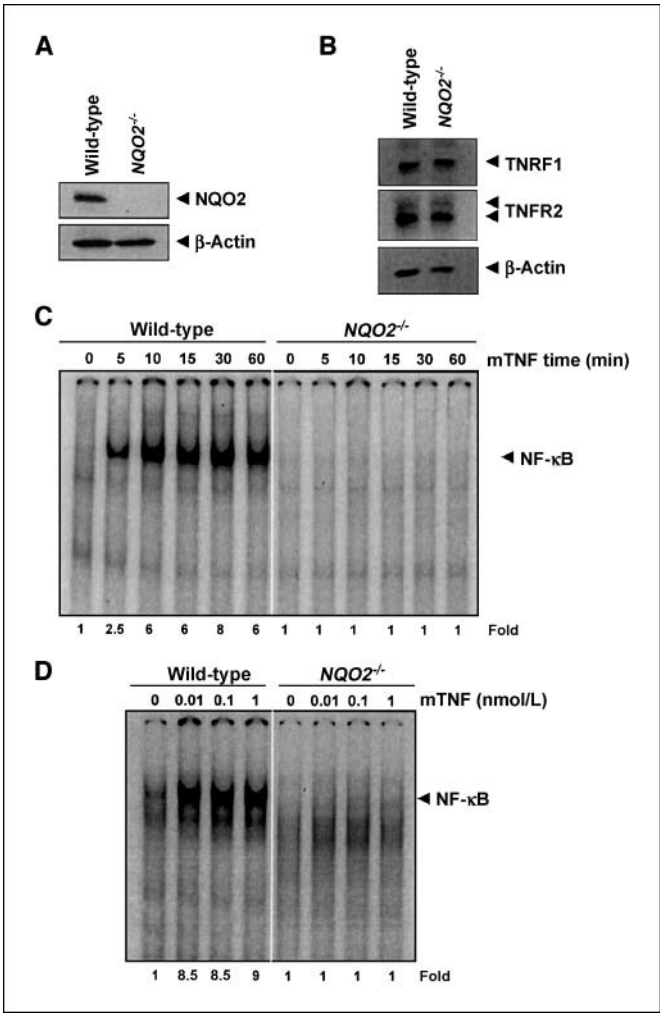

Figure 1. *A,* Western blot analysis of the NQO2 protein. Whole-cell extracts were prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which we did a Western blot analysis with an anti-NQO2 antibody. *B,* cell surface expression of TNFRs in *NQO2*-WT and $NQO2^{-/-}$ cells. Cells were harvested and resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which we did a Western blot analysis with anti-TNFR1 and anti-TNFR2 antibodies. *C* and *D,* time-dependent effects of NF-κB activation on $NQO2^{-/-}$ cells treated with TNF. One million cells were treated with 0.1 nmol/L TNF for the indicated times, after which nuclear extracts were prepared; NF-κB activation was then analyzed by EMSA. *E,* dose-dependent effect of NF-κB activation in $NQO2^{-/-}$ cells treated with TNF. One million cells were treated with the indicated concentrations of TNF for 30 min, after which nuclear extracts were prepared; NF-κB activity was then analyzed by EMSA.

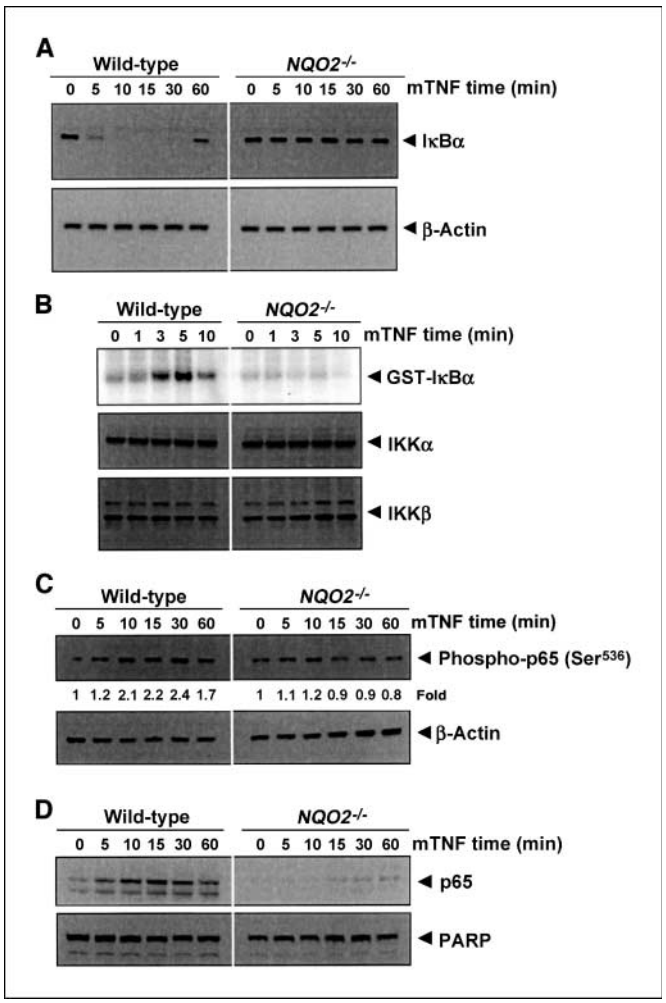

Figure 2. *A,* effects of *NQO2* deletion on TNF-induced degradation of IκBα. One million cells were treated with 0.1 nmol/L TNF for the indicated times. Cytoplasmic extract was prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which Western blot analysis with an anti-IκBα antibody was done. *B,* effects of *NQO2* deletion on TNF-induced activation of IKK. Four million cells were stimulated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were incubated with anti–IKK-α antibody for 2 h, immunoprecipitated with protein A/G-Sepharose beads overnight at 4°C, and then analyzed with an immunocomplex kinase assay using glutathione *S*-transferase-IκB (*GST-IκB*) as substrate. To examine the level of expression of IKK proteins, the same whole-cell extracts were resolved by SDS-PAGE, after which we did a Western blot analysis by using anti–IKK-α and anti–IKK-β antibodies. *C,* effects of *NQO2* deletion on TNF-induced phosphorylation and nuclear translocation of the p65 subunit of NF-κB in cytoplasm. One million cells were treated with 0.1 nmol/L TNF for the indicated times; a cytosol extract was prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which a Western blot analysis was done by using anti-phosphospecific p65 antibody. The results shown are representative of three independent experiments. *D,* effects of *NQO2* deletion on TNF-induced nuclear translocation of the p65 subunit of NF-κB in nucleus. One million cells were treated with 0.1 nmol/L TNF for the indicated times; a nuclear extract was prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which a Western blot analysis was done by using an anti-p65 antibody. The results shown are representative of three independent experiments.

properties of Annexin V. Therefore, to identify apoptosis, we used the Annexin V antibody, which was conjugated with FITC fluorescence dye. Briefly, 1×10^5 cells were pretreated with 1 μg/mL cycloheximide, treated with 1 nmol/L TNF for 16 h at 37°C, and then subjected to Annexin V staining. Cells were washed in PBS, resuspended in 100 μL 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 NQO2 protein in both wild-type (*NQO2*-WT) and *NQO2*-null ($NQO2^{-/-}$) murine keratinocytes. As expected, *NQO2* was expressed in NQO2-WT cells but not in $NQO2^{-/-}$ cells (Fig. 1*A*). We found no difference in the expression of TNF receptor (TNFR)-1 and TNFR2 with Western blot analysis in the two cell types (Fig. 1*B*).

NQO2 is required for TNF-dependent NF-κB activation. We first investigated the effect of *NQO2* deletion on TNF-induced NF-κB activation. Cells were stimulated with 0.1 nmol/L TNF for indicated times; the nuclear extracts were prepared and were then analyzed for NF-κB activation by EMSA. As shown in Fig. 1*C*, TNF stimulated NF-κB activation in a time-dependent manner in *NQO2*-WT, but its activation was completely abrogated in $NQO2^{-/-}$ cells.

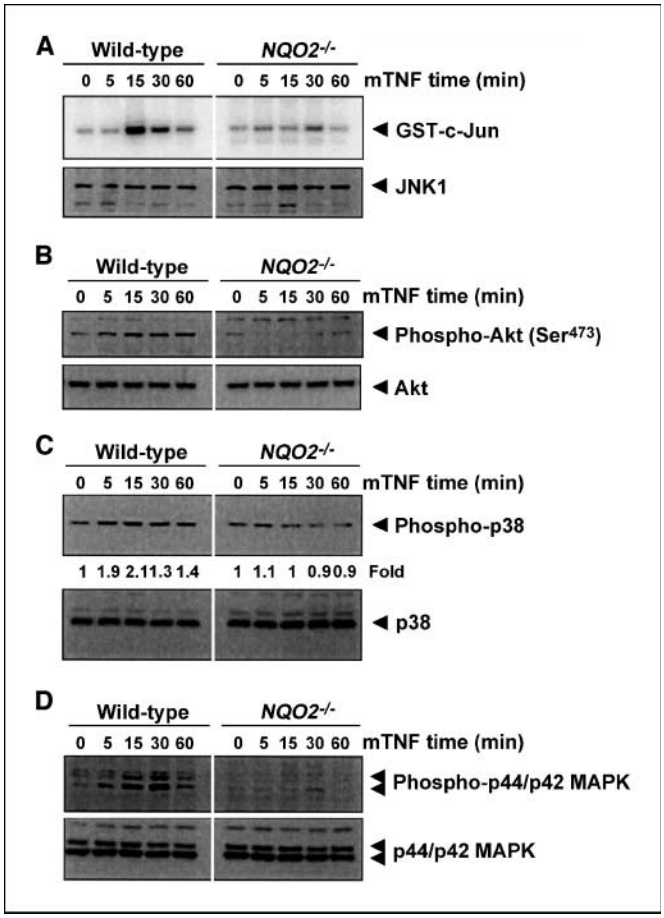

Figure 3. *A,* effects of *NQO2* deletion on TNF-induced activation of JNK. Four million cells were treated with 1 nmol/L TNF for the indicated times, after which whole-cell extracts were prepared, incubated with anti-JNK1 antibody for 2 h, and then immunoprecipitated with protein A/G-Sepharose beads overnight at 4°C. The beads were washed and subjected to a kinase assay as described in Materials and Methods. The same protein extracts were resolved by SDS-PAGE and electrotransferred to a nitrocellulose membrane, after which we did a Western blot analysis by using an anti-JNK1 antibody. *B* to *D,* effects of *NQO2* deletion on TNF-induced activation of Akt, p38 MAPK, and p42/44 MAPK. One million cells were treated with 1 nmol/L TNF for the indicated times; whole-cell extracts were prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which Western blot analyses using phosphospecific anti-Akt, phosphospecific anti-p38 MAPK, and phosphospecific anti-p42/44 MAPK antibodies were done. The same membranes were reblotted with anti-Akt, anti-p38 MAPK, and p42/44 MAPK antibodies. The results shown are representative of three independent experiments.

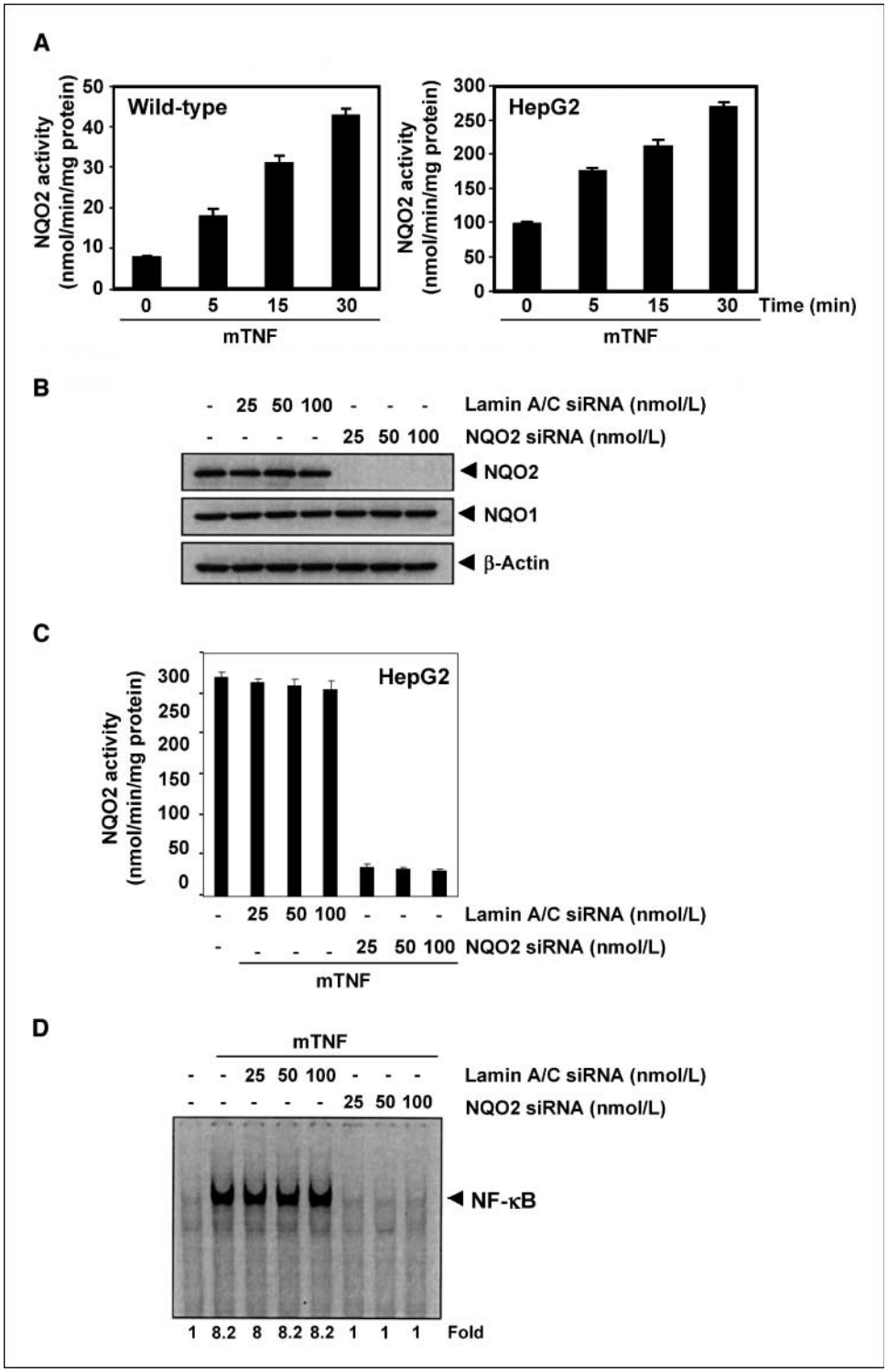

Figure 4. *A, NQO2*-WT (*left*) and HepG2 (*right*) cells were treated with TNF for the indicated times. Cytosolic fractions were prepared and analyzed for benzopyrene-inhibitable NQO2 enzyme activity as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SD. *B* and *C,* siRNA-mediated knockdown of *NQO2*. HepG2 cells were grown in six-well plates and transfected with either scrambled or *NQO2*-specific siRNA (25, 50, and 100 nmol/L). After 48 h, cells were treated with TNF, and cytosolic fractions were subjected to Western blotting using anti-NQO2 antibody and to NQO2 activity following the procedures as described earlier. *D,* effect of *NQO2* knockdown on NF-κB activity. HepG2 cells were grown in six-well plates and transfected with either scrambled or *NQO2*-specific siRNA (25, 50, and 100 nmol/L). After 48 h, cells were treated with 1 nmol/L TNF for 30 min, and nuclear fractions were subjected to EMSA to measure NF-κB activation.

Because the activation of NF-κB by TNF is more robust at higher concentrations (21), we evaluated the effect of *NQO2* deletion on NF-κB activation induced by varying concentrations of TNF. The activation of NF-κB by TNF in the 0.01 to 1 nmol/L range was strongly evident in *NQO2*-WT cells but not in $NQO2^{-/-}$ cells (Fig. 1*D*). These results indicate that NQO2 is required for TNF-induced NF-κB activation.

NQO2 is required for TNF-dependent IκBα degradation. Translocation of NF-κB to the nucleus is preceded by proteolytic degradation of IκBα (22). To determine whether inhibition of TNF-induced NF-κB activation in $NQO2^{-/-}$ cells was due to inhibition of IκBα degradation, we exposed cells to TNF for various intervals and assayed degradation of IκBα by Western blot analysis. TNF induced IκBα degradation, reaching maximum at 10 to 30 min

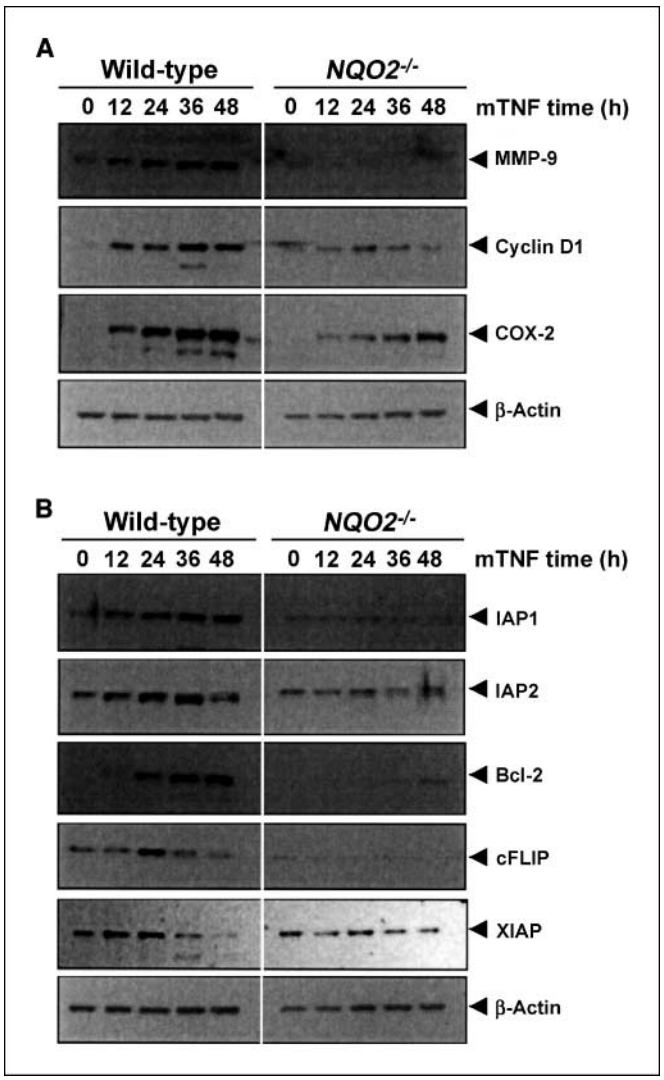

Figure 5. *A,* effects of *NQO2* deletion on expression of NF-κB–regulated gene products. Cells (5×10^5 per well) were treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blot analysis with antibodies against COX-2, MMP-9, cyclin D1, and β-actin. *B,* effects of *NQO2* deletion on expression of NF-κB–regulated antiapoptotic proteins. Cells (5×10^5 per well) were incubated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blot analysis with antibodies against IAP1, IAP2, Bcl-2, cFLIP, XIAP, and β-actin. The results shown are representative of three independent experiments.

after TNF stimulation in *NQO2*-WT cells (Fig. 2*A, left*). In $NQO2^{-/-}$ cells, however, TNF had no effect on IκBα degradation (Fig. 2*A, right*), indicating that NQO2 is required for degradation of IκBα.

***NQO2* deletion inhibits TNF-induced IKK activation.** Given that IKK is required for TNF-induced NF-κB activation (22), we investigated the effect of *NQO2* deletion on TNF-induced IKK activation. $NQO2^{-/-}$ cells completely lacked TNF-induced activation of IKK, whereas the expression of IKK-α and IKK-β proteins in both wild-type and null cells was equivalent (Fig. 2*B*). These results suggested that NQO2 is required for TNF-induced IKK activation.

NQO2 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 *NQO2* deletion on TNF-induced nuclear translocation of p65 with Western blot analysis. In the cytoplasm, TNF induced phosphorylation of p65 as early as 10 min in *NQO2*-WT cells. After 30 min, the fold of activation was 2.4-fold. In $NQO2^{-/-}$ cells, TNF did not induce the phosphorylation of p65 compared with control (Fig. 2*C, top*).

As shown in Fig. 2*D*, TNF also induced translocation of p65 in a time-dependent manner as early as 10 min after TNF stimulation in *NQO2*-WT cells. In $NQO2^{-/-}$ cells, TNF failed to induce phosphorylation of p65 in nuclei.

NQO2 is required for TNF-induced activation of JNK. We investigated the role of NQO2 on other signals transduced by TNF. To examine the specific role of NQO2 on TNF-induced JNK activation, one of the earliest events induced by TNF (18), we stimulated cells with TNF for various time intervals, prepared whole-cell extracts, and analyzed them for JNK activity with an immune-complex kinase assay. TNF induced the maximum activation (4.3-fold) of JNK at 15 min in *NQO2*-WT but in $NQO2^{-/-}$ cells, almost no activation was observed (Fig. 3*A*). These results indicated that NQO2 is required for TNF-induced JNK activation.

NQO2 is required for TNF-induced activation of Akt. To examine the specific role of NQO2 on TNF-induced Akt activation, another early event induced by TNF (23), we exposed cells with TNF for indicated time intervals, prepared whole-cell extracts, and assayed them for phosphorylation of Akt by Western blot analysis. TNF induced activation of Akt in *NQO2*-WT but not in $NQO2^{-/-}$ cells (Fig. 3*B*). These results indicated that NQO2 is needed for TNF-induced Akt activation.

NQO2 is required for TNF-induced activation of p38. The activation of p38 has been associated with TNF-induced IκB phosphorylation and NF-κB activation (24). TNF induced activation of p38 in *NQO2*-WT (~2-fold) but in $NQO2^{-/-}$ cells, it remained same as to the control (Fig. 3*C*). These results indicated that NQO2 is needed for TNF-induced p38 activation.

NQO2 is required for TNF-induced activation of p44/p42 MAPK. TNF has been reported to activate p44/p42 MAPK through the Ras/Raf/MAPK kinase cascade (18). TNF induced the time-dependent phosphorylation of p44/p42 MAPK in *NQO2*-WT but p44/p42 MAPK activation was abolished in $NQO2^{-/-}$ cells (Fig. 3*D*). These results suggested that NQO2 is also needed for TNF-induced p44/p42 MAPK activation.

TNF increases NQO2 enzyme activity in wild-type keratinocytes and HepG2 cells. Our results thus far suggest that NQO2 plays a major role in TNF signaling. Next, we determined whether TNF activates NQO2 activity. NQO2 enzyme activity was measured in *NQO2*-WT keratinocytes (Fig. 4*A, left*). Treatment of these cells with TNF resulted in a time-dependent increase in the NQO2 enzyme activity. Whether TNF activates NQO2 in cells other than keratinocytes was also examined. We found that TNF also caused a time-dependent induction of NQO2 activity in human hepatoblastoma HepG2 cells (Fig. 4*A, right*).

Abrogation of *NQO2* expression by siRNA inhibits TNF-induced NF-κB activation. All studies of the role of NQO2 in TNF signaling up to this point used genetically deleted *NQO2* cells. Whether the suppression of *NQO2* expression by siRNA would also abrogate TNF signaling was investigated next. *NQO2* siRNA was used to knock down NQO2 protein expression in human hepatoblastoma HepG2 cells. *NQO2* expression was effectively abolished in the cells treated with *NQO2* siRNA but not in those treated with the scrambled siRNA, as observed by Western blot analysis (Fig. 4*B*). TNF failed to induce the NQO2 activity in cells transfected with *NQO2* siRNA (Fig. 4*C*), and this correlated with inhibition of TNF-induced NF-κB activation (Fig. 4*D*). Thus, these

results with *NQO2* siRNA also show the critical role of NQO2 in TNF signaling.

NQO2 is required for expression of TNF-induced, NF-κB–dependent MMP-9, cyclin D1, and COX-2 proteins. Given that TNF induces cyclin D1, COX-2, and MMP-9 (25–27) expression, we examined whether NQO2 is needed for the induction of these gene products. Cells were treated with TNF for different intervals, whole-cell extracts were prepared, and the extracts analyzed by Western blot analysis for the expression of MMP-9, cyclin D1, and COX-2 (Fig. 5*A*). All these were induced by TNF in a time-dependent manner in *NQO2*-WT but not in *NQO2*$^{-/-}$ cells.

NQO2 is required for expression of TNF-induced NF-κB–dependent antiapoptotic proteins. Because NF-κB also regulates the expression of various antiapoptotic proteins, including IAP1/2, Bcl-2, XIAP, and cFLIP (28–30), we investigated the effect of NQO2 on the TNF-induced expression of these antiapoptotic gene products. TNF induced the expression of IAP1/2, Bcl-2, XIAP, and cFLIP in a time-dependent manner in *NQO2*-WT but not in *NQO2*$^{-/-}$ cells (Fig. 5*B*). These results indicated that NQO2 is required for TNF-induced NF-κB–regulated survival gene products.

Deletion of NQO2 potentiates TNF-induced apoptosis. Activation of NF-κB has been shown to inhibit TNF-induced apoptosis and promote proliferation through the expression of the previously mentioned gene products (31–33). Our results suggest that deletion of *NQO2* might enhance apoptosis induced by TNF through suppression of NF-κB–regulated antiapoptotic gene products. Whether suppression of NF-κB by *NQO2* deletion affects

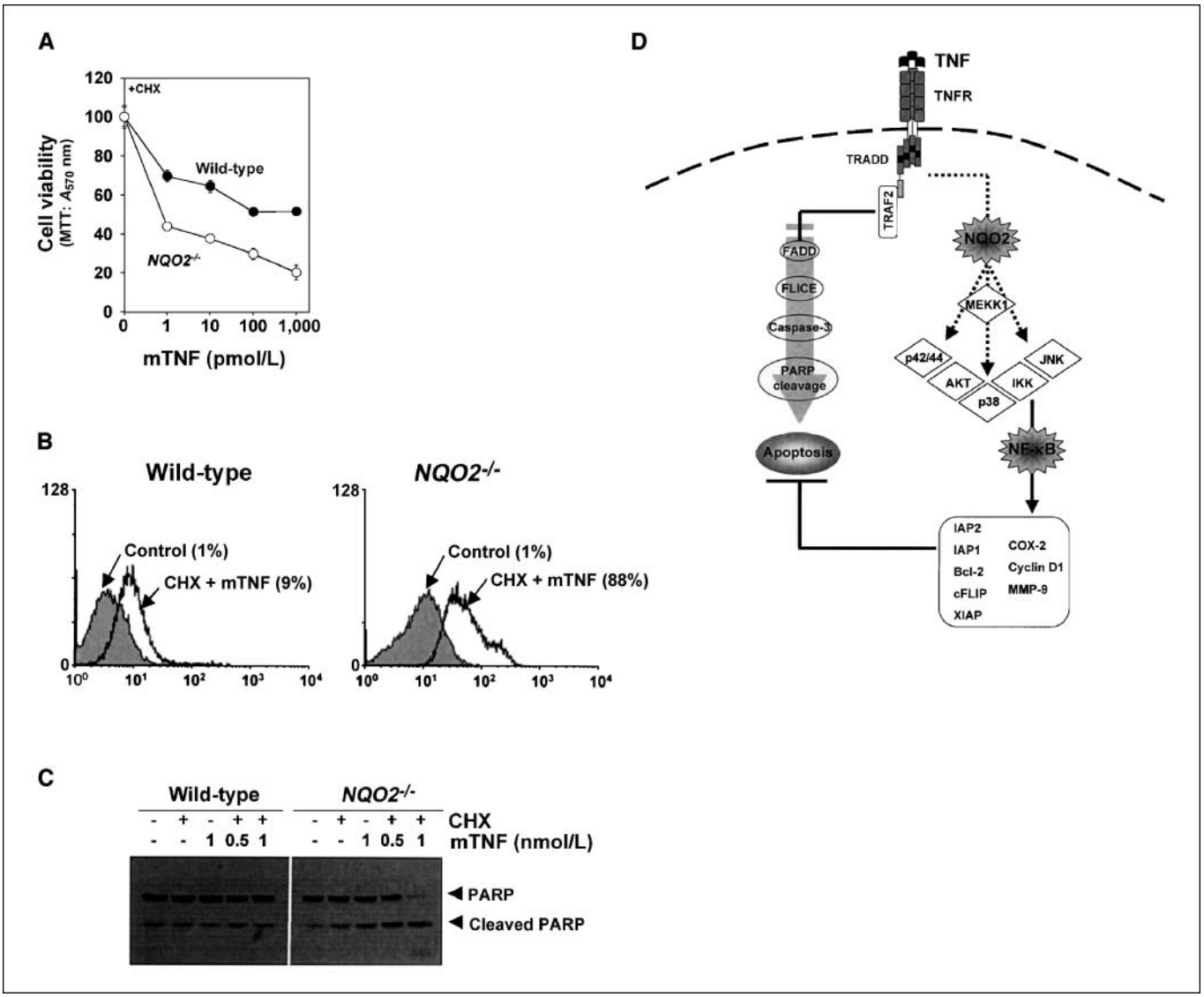

Figure 6. *A,* effect of *NQO2* deletion on TNF-induced cell growth. Cells (2×10^4 per well) were seeded in triplicate in 96-well plates, pretreated with cycloheximide (*CHX*; 1 μg/mL) for 1 h, and then exposed to the indicated concentrations of TNF for 24 h. Thereafter, cell growth was analyzed by the MTT assay as described in Materials and Methods. *Points,* mean from triplicate cultures; *bars,* SD. *B,* cells (1×10^5 per well) were pretreated with 1 μg/mL cycloheximide for 1 h, incubated with 1 nmol/L 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 by flow cytometry. *C,* cells (1×10^6 per well) were pretreated with 1 μg/mL cycloheximide for 1 h, and incubated with indicated concentrations of TNF for 16 h. Whole-cell extracts were prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, after which we did a Western blot analysis with an anti-PARP antibody. *D,* the role of NQO2 in TNF signaling. The model suggests the role of NQO2 in TNF-induced activation of NF-κB, JNK, p38, p42/p44 MAPK, Akt, and apoptosis.

TNF-induced apoptosis was therefore investigated. MTT assay showed that TNF was cytotoxic to cells and that *NQO2* deletion enhanced that cytotoxicity (Fig. 6*A*).

The results of Annexin V staining to determine whether this suppressed cell proliferation was due to apoptosis showed that TNF-induced apoptosis in 9% of *NQO2*-WT cells and 88% of $NQO2^{-/-}$ cells (Fig. 6*B*). The PARP cleavage assay showed that NQO2 deletion potentiated TNF-induced caspase activity (Fig. 6*C*). All these assay results suggested that *NQO2* deletion potentiates TNF-induced apoptosis.

Discussion

This study was designed to investigate the role of NQO2 on TNF-induced cell signaling. We found that TNF induced NF-κB activation, IKK activation, IκBα degradation, p65 phosphorylation, and p65 translocation in *NQO2*-WT but not in $NQO2^{-/-}$ keratinocytes. Further, the activation of TNF-induced JNK, p38 MAPK, p44/p42 MAPK, and Akt was also abrogated in $NQO2^{-/-}$ cells. TNF induced the expression of NF-κB–dependent gene products cyclin D1, COX-2, MMP-9, IAP1, IAP2, Bcl-2, XIAP, and cFLIP in *NQO2*-WT cells, but all were down-regulated in $NQO2^{-/-}$ cells, and this correlated with the inhibition of cell proliferation and the potentiation of apoptosis induced by TNF (Fig. 6*D*).

Our results are the first to show that deletion of the *NQO2* gene abolished TNF-induced NF-κB activation. Iskander et al. (34) found that lipopolysaccharide-induced NF-κB activation was suppressed in $NQO2^{-/-}$ mice compared with wild-type mice and indicated that the suppression was caused by the loss of NF-κB expression (34). However, we found that $NQO2^{-/-}$ clearly suppressed TNF-induced NF-κB activation compared with *NQO2*-WT cells without the loss of NF-κB expression, suggesting that NQO2 is involved in TNF-induced cell signaling. We also found that TNF activated NQO2 activity in different cell types. *NQO2*-specific siRNA abolished the expression of NQO2 protein and abrogated TNF-induced NQO2 activity and NF-κB activation.

Although how NQO2 plays a role in TNF-induced NF-κB activation is not clear, the alteration of the redox status of the cell by NQO2 presumably regulates activation of redox-sensitive transcription factor(s), such as NF-κB (35). This is in agreement with earlier observations from our laboratory that overexpression of the antioxidant enzymes superoxide dismutase (36) and γ-glutamyl cysteine synthetase (37) can abolish TNF-induced cell signaling, including NF-κB activation. Thus, our results suggest that the deletion of *NQO2* leads to altered intracellular redox status that results in the suppression of NF-κB activation. Intriguingly, deletion of the *NQO1* gene leads to a similar phenotype, suggesting a nonredundant role for NQO1 and NQO2 in TNF-induced activation of NF-κB (17).

Our results indicate that deletion of *NQO2* abolished TNF-induced IκBα degradation and phosphorylation through inhibition of IKK activation. How the deletion of *NQO2* leads to suppression of IKK activation, however, is unclear, partly because the kinase that activates IKK is unknown. NIK, MEKK1, MEKK3, Akt, transforming growth factor-β–activated kinase 1, glycogen synthase kinase 3β (GSK-3β), NQO1, MKK4, and PKR have been shown to be involved in the IKK activation induced by TNF (17, 38–42). Sarbassov et al. (43) recently reported that rictor kinase phosphorylates Akt, which in turn can activate IKK, leading to IκBα phosphorylation and NF-κB activation. We found that TNF-induced Akt activation was indeed abrogated by deletion of the *NQO2* gene.

Resveratrol, an active constituent found in cranberry, red grapes, and nuts, has been shown to modulate NQO2 activity in two different ways. First, resveratrol suppresses melanoma cell proliferation through the induction of NQO2 protein and activity (44). Second, resveratrol is also a potent inhibitor of NQO2 activity *in vitro* with a dissociation constant of 35 nmol/L (45, 46). Our group has already shown that resveratrol suppresses TNF-induced activation of NF-κB and AP-1 through down-modulation of reactive oxygen intermediate generation and lipid peroxidation (47). Here, we also found that besides NF-κB activation, TNF-induced JNK activation was also abolished in $NQO2^{-/-}$ cells. These results are in agreement with Manna et al. (47), who showed that resveratrol, a specific NQO2 inhibitor, significantly suppressed TNF-induced AP-1 activation because JNK activation causes the activation AP-1. Moreover, we established for the first time that $NQO2^{-/-}$ also abolishes TNF-induced activation of Akt, p38, and p44/p42 MAPK. These results parallel those of our previous reports, in which we showed that $NQO2^{-/-}$ and GSK-3β abolished TNF-induced activation of Akt and MAPK factors (17, 41). Although the role of these genes differ from each other in response to stimuli, it is possible that these genes have similar TNF-induced responses.

Our results also show for the first time that the expression of various NF-κB–dependent gene products, including COX-2, cyclin D1, MMP-9, IAP1, IAP2, Bcl-2, XIAP, and cFLIP is abolished in $NQO2^{-/-}$ cells. The down-regulation of these gene products suggests that deletion of *NQO2* suppresses TNF-induced proliferation and enhances apoptosis. Indeed, we found that in $NQO2^{-/-}$ cells, TNF-induced cell proliferation was inhibited and apoptosis increased significantly. Other studies indicate that the deletion of *NQO2* increases sensitivity to skin carcinogenesis and suppresses apoptosis of bone marrow cells (6, 7, 34). NF-κB activation, however, has been linked with suppression of apoptosis, cellular proliferation, invasion, angiogenesis, and metastasis (30). Thus, suppression of NF-κB activation by deletion of *NQO2* seems to be paradoxical. However, 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 (18).

Recently, it has been reported that NQO2 activity of ovarian tissues is higher than in bladder samples, indicating that a potential role of the gene in the treatment of tumor (48). Although various pro-oxidants can modulate both NF-κB and NQO2 activity, it is less clear whether the expression of NQO2 requires NF-κB activation. However, given that TNF and NF-κB are involved in the several disease states, including cancer, inflammation, diabetes, and neurodegenerative disorders (30, 49), the potential of suppressing the TNF cell-signaling pathway by using NQO2 inhibitors exists (47). Thus, our results show that NQO2 can regulate TNF-induced apoptosis through the NF-κB–regulated gene products, indicating that NQO2 plays a pivotal role in TNF-induced signaling pathway and that its suppression is a potential therapeutic approach in tumor cells.

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Deficiency of NRH:Quinone Oxidoreductase 2 Differentially Regulates TNF Signaling in Keratinocytes: Up-regulation of Apoptosis Correlates with Down-regulation of Cell Survival Kinases

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