Nitric Oxide Disrupts H₂O₂-dependent Activation of Nuclear Factor κB

ROLE IN SENSITIZATION OF HUMAN TUMOR CELLS TO TUMOR NECROSIS FACTOR-α-INDUCED CYTOTOXICITY

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Tumor necrosis factor α (TNF-α) exerts its effect by two distinct signaling pathways. It can trigger cytokoty

icity in sensitive target cells. TNF-α can also promote nuclear factor κB (NF-κB) activity and regulate the ex-

pression of genes that interfere with apoptosis and thus conferring resistance to several apoptotic stimuli. We

have observed that interferon-γ (IFN-γ) sensitizes human ovarian carcinoma cell lines to TNF-α-mediated ap-

optosis and further, IFN-γ induces the expression of the inducible nitric-oxide synthase (iNOS) and the gen-

eration of nitric oxide (NO). This study examines the role of NO in the sensitization of the ovarian carcinoma

cell line AD10 to TNF-α-mediated cytotoxicity. Treatment of AD10 cells with the NOS inhibitor L-NMA

blocked the IFN-γ-dependent sensitization whereas NO donors (S-nitroso-N-acetylpenicillamine) sensitized

these cells to TNF-α cytotoxicity. Analysis of the activation status of NF-κB upon treatment with NO donors

confirmed the inhibitory role of NO on both the NF-κB DNA-binding property and its activation. Moreover, the

inhibition of NF-κB nuclear translocation by NO donors directly correlated with the intracellular concentra-

tion of H₂O₂ and was reversed by the addition of exogenous H₂O₂. These findings show that NO might interfere

with TNF-α-dependent NF-κB activation by interacting with O₂⁻ and reducing the generation of H₂O₂, a potent

NF-κB activator. Therefore, NO-mediated disruption of NF-κB activation results in the removal of anti-apoptotic/res-

istance signals and sensitizes tumor cells to cytotoxic cytokines like TNF-α.

The development of resistance to either the immune system or chemo-immunotherapeutic strategies remains a disad-

vantage in the therapy of cancer, particularly in cases where recurrences and/or relapses occurred. Apoptosis has been ac-

cepted as a distinct pathological mechanism in tumors responding to anticancer therapies. Further, resistance to apo-

ptosis in tumor cells has been recognized as a common pathway to multiple drug resistance (1, 2). Multiple lines of

evidence have implicated the activation of the transcription factor NF-κB as one of the primary signals in the onset of

resistance to many apoptotic stimuli, particularly TNF-α (3–5).

TNF-α is a proinflammatory cytokine that exerts a broad spectrum of biological effects by its interaction with two dis-

tant cell surface receptors, TNFR1 and TNFR2 (6). Most cyto-

toxic effects of TNF-α are mediated by the TNFR1. It has been demonstrated that, upon interaction with TNF-α, trimeri-

zation of TNFR1 takes place and results in cellular signaling leading to the recruitment of the TNFR1-associated death domain protein and the receptor-interacting protein to the receptor complex (7). The TNFR1-associated death domain protein interacts with the Fas-associated death domain to initiate the death pathway and engages several proteins such as the TNFR-associated factor-1, the TNFR-associated factor-2, and receptor-interacting protein to initiate the TNF signaling pathways such as the activation of NF-κB (8).

Reactive oxygen species (ROS) have also been implicated in the signaling pathways initiated by TNF-α. Stimulation of mammalian cells with TNF-α triggers the generation of various ROS (9, 10). Hence, the use of antioxidants results in the inhibition of various TNF-α-related effects such as the activation of transcription factors, gene expression, and cytotoxicity. In addition, the use of exogenous ROS mimics the biological activity of TNF-α (11). These data support the hypothesis that ROS function as second messengers for TNF-α-mediated sig-

naling. In biological systems the most important ROS gener-

ated upon TNF-α stimulation are the result of enzymatic par-

tial reduction of oxygen yielding superoxide (O₂⁻), which is either immediately reduced by superoxide dismutase to hydro-

gen peroxide (H₂O₂) or alternatively reacts rapidly with nitric

oxide (NO) to generate ONOO⁻ (12–14). However, the regulat-
y role of NO in TNF-α signaling via the disruption of ROS-

dependent activation of NF-κB has not been established.

Several lines of evidence showed that resistant tumors could be sensitized to TNF-α-mediated cytotoxicity by various cyto-

kines or pharmacological treatments (15–20). Recently, we have reported that IFN-γ induced the sensitization of the hu-

man ovarian carcinoma AD10 cell line to Fas-mediated apo-

ptosis and the sensitization was due in part to the generation of

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1 The abbreviations used are: NF-κB, nuclear factor κB; IFN-γ, inter-

feron γ; iNOS, inducible nitric-oxide synthase; SNAP, S-nitroso-N-

acetylpenicillamine; L-NMA, N⁵-monomethyl-L-arginine; TNF, tumor

necrosis factor; LDH, lactate dehydrogenase; RT, reverse transcription;

PCR, polymerase chain reaction; PDTC, pyrrolidine dithiocarbamate;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electro-

phoretic mobility shift assay; TNFR, tumor necrosis factor receptor;

FBS, fetal bovine serum; ROS, reactive oxygen species; H₂DCFDA,

2′,7′-dichlorofluorescein diacetate.
nitric oxide by the induction of iNOS in these cells (21). NO has been identified as a potential second messenger based on its ability to chemically interact with a broad range of regulatory proteins. Furthermore, NO can interact with metal cluster- and thiol-containing proteins (for review, see Ref. 22) resulting in the modification of both the structures and functions of these proteins. Although NO has been shown to react very rapidly with O$_2^-$, the only biological effect to this chemical reaction has been assigned to the generation of ONOO$^-$, a proposed cytotoxic derivative (23, 24).

Herein, we hypothesize that NO is interfering with the TNF-α-mediated signaling by chemically reacting with O$_2^-$ Since can serve as a precursor to H$_2$O$_2$, which is a proposed activator of the anti-apoptotic transcription factor NF-κB, the reaction of O$_2^-$ with NO will interfere with the activation of NF-κB and will result in the removal of anti-apoptotic signals and sensitization of the tumor cells to TNF-α cytotoxicity. This study has been designed to test this hypothesis, and the following has been examined: (a) the molecular mechanism by which IFN-γ sensitizes the human ovarian carcinoma cell line to TNF-α-induced cytotoxicity, (b) the specific role of NO in the disruption of TNF-α-mediated generation of H$_2$O$_2$, and, subsequently, (c) the mechanism by which NO can disrupt the TNF-α-dependent NF-κB activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The AD10 cell line is an adriamycin-resistant, MDR phenotype-expressing subline derived from the human ovarian carcinoma cell line A2780 and was obtained from Dr. Ozols (Fox Chase Cancer Center, Philadelphia, PA). The PC-3 cell line is a metastatic bone-derived human prostatic adenocarcinoma, CRL-1435, obtained from ATCC (American Type Culture Collection, Manassas, VA). Cell cultures were maintained as monolayers on plastic dishes in RPMI 1640 medium (MediaTech, Inc., Herndon, VA), supplemented with 10% heat-inactivated FBS (Gemini Bio-Products, Inc., Calabasas, CA), 1% l-glutamine (Life Technologies, Inc.), 1% pyruvate (Life Technologies, Inc.), 1% nonessential amino acids (Life Technologies, Inc.), and incubated at 37 °C and 5% CO$_2$. For every experimental condition, the cells were cultured in 1% FBS 24 h prior to treatments. In cases where SNAP (kindly provided and synthesized by Dr. Jon Fukuto, UCLA, Los Angeles, CA) was used, 500 μM photo-activated SNAP 2 h prior to stimulation with TNF-α was treated with 500 μM nonidet P-40, 0.1 mM EDTA and was added to the top of the washed cells and incubated on ice for 5 min. Lysed cells were collected by gentle pipetting three to four times and transferred to a microcentrifuge tube and centrifuged at 1200 rpm. Nuclear pellets for each experimental condition were generated by two consecutive centrifugation and washing steps at 12000 rpm. Nuclear pellets were lysed in buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl$_2$, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). Total nuclear protein concentrations were determined using the method of Bradford (26).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear protein extracts (2 μg) were assayed for DNA interaction by EMSA as described previously with modifications (27). The double-stranded NF-κB consensus binding sequence (5′-AGT TCA GGG GAT TCC AGG C-3′) oligonucleotide was radiolabeled with [γ-32P]ATP (ICN Pharmaceuticals, Inc. Costa Mesa, CA) by incubation with 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and further purified by QIAquick nucleotide removal kit (Qiagen, Valencia, CA). After the DNA-binding reaction, the samples were resolved on 4–15% Tris-HCl-polyacrylamide minigels (Bio-Rad) and the gels were dried and autoradiographed. Specificity of the DNA-binding reaction was determined by competition assays performed with 100-fold excess of unlabeled NF-κB or unrelated oligonucleotide (i.e. AP-1: 5′-GAT CCA TGC TCC TCT TGC-3′). The relative concentrations of specific NF-κB-shifted bands were assessed by densitometric analysis of the digitized autoradiographic images using the NIH Image program described above.

Determination of Intracellular H$_2$O$_2$ Generation—1 × 10$^6$ cells were cultured in a six-well plate for 18 h in culture medium supplemented with 1% FBS. In some instances, the minimal serum-cultured cells were treated with 1 or 100 μM phenylephrine and SNAP 2 h prior to treatment with 10 or 100 units/ml TNF-α. Intracellular H$_2$O$_2$ levels were evaluated using the fluorescent cell permeable probe, 2',7'-dichlorofluorescein diacetate (H$_2$DCFDA) (Molecular Probes, Inc., Eugene, OR). Then, the culture medium was replaced with Dulbecco’s phosphate-buffered saline, pH 7.4, containing 5 μM H$_2$DCFDA. Fluorescence intensity was analyzed on an EPICS™ XL-MCL flow cytometer (Beckman Coulter Inc., Fullerton, CA).

Transfections and Reporter Gene System—The intracellular activation of NF-κB was determined by transient transfection of AD10 cells with the pNF-κB-d2EGFP reporter vector (CLONTECH, Palo Alto, CA). 7 × 10$^4$ cultured cells were transfected with 10 μg of DNA using 60 μl of Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer’s recommendations. Transfected cells were then distributed onto a six-well culture plate and incubated under different experimental conditions. The relative fluorescence intensity was analyzed on an EPICS™ XL-MCL flow cytometer.

Statistical Analysis—The experimental values were expressed as the means ± standard error of the mean (S.E.) for the number of separate experiments indicated in each case. One-way analysis of variance was performed to compare multiple groups and Student’s t tests were used to establish the homogeneity of variance on the basis of the differences among standard deviations (S.D.). Whenever necessary, post hoc unequal multiple comparison tests (Bonferroni’s test) and Student’s t test were used for comparison between two groups. Significant differences were considered for those probabilities < 5% (p < 0.05).
the sensitization was blocked by the addition of L-NMA (1 mM) to AD10 cells were pretreated with 100 units/ml IFN-γ for 18 h in the presence or absence of 1 mM NOS inhibitor l-NMA. AD10 cells were treated with the NO donor SNAP (0, 10, and 100 μM) 18 h prior to exposure to TNF-α. **, p < 0.005; ***, p < 0.001.

**RESULTS**

**IFN-γ-mediated Sensitization of the Human Ovarian Carcinoma AD10 to TNF-α-induced Cytotoxicity**—To investigate the role of nitric oxide on the sensitization of the human ovarian carcinoma AD10 cell line to TNF-α-mediated cytotoxicity, we first stimulated quiescent AD10 cells with IFN-γ in the presence or absence of 1 mM potent NOS inhibitor l-NMA. The sensitivity of AD10 cells to the cytotoxic effect of increasing concentrations of TNF-α (0.01, 0.1, and 1 ng/ml) was evaluated by the release of LDH into the culture medium after 24 h of incubation. Exposure of AD10 cells to IFN-γ (100 units/ml) for 18 h sensitized the tumor cells to TNF-α-mediated cytotoxicity and the degree of sensitization increased with increasing concentrations of TNF-α. Sensitization by IFN-γ was significantly decreased in the presence of 1 mM NOS inhibitor l-NMA (Fig. 1A).

To confirm the specific role of nitric oxide in the sensitization of AD10 cells, we assessed the cytotoxic effect of TNF-α in the presence of 18 h with different concentrations of SNAP. We observed a significant increase in the sensitivity of AD10 cells to TNF-α-mediated cytotoxicity in a 24-h assay that directly correlated with the concentrations of SNAP (Fig. 1B).

Similarly, we have found that IFN-γ (100 units/ml) sensitized the prostatic adenocarcinoma cell line PC3 to TNF-α-mediated cytotoxicity (1 ng/ml) from 5 ± 1.9% to 37 ± 1.2% and the sensitization was blocked by the addition of l-NMA (1 mM) to 20 ± 2.1%. Like IFN-γ, the use of the NO donor SNAP (100 μM) sensitized PC-3 cells to TNF-α (1 ng/ml) cytotoxicity from 1.9 ± 3% to 58 ± 5%.

**Nitric Oxide and Pyrrolidine Dithiocarbamate (PDTC) Inhibit TNF-α-induced Expression of Endogenous TNF-α mRNA in AD10 Cells**—The transcription factor NF-κB has been demonstrated to tightly regulate the gene expression of TNF-α, establishing a self-regulatory loop in tumor cells that secrete TNF-α that in turn activates NF-κB (28). Furthermore, PDTC has been shown to inhibit TNF-α-mediated activation of NF-κB in several cell types and in macrophages (29). To demonstrate the specific effect of nitric oxide on the NF-κB-mediated expression of TNF-α, we incubated AD10 cells with 1, 10, 100, and 500 μM SNAP for 18 h and then stimulated the cells with 100 units/ml TNF-α for 4 h. The relative levels of endogenously generated TNF-α were assessed by amplification of the specific TNF-α cDNA using RT-PCR. The constitutive expression of TNF-α by AD10 cells was demonstrated and a significant increased level was observed upon treatment with exogenous TNF-α. Moreover, this increased level of TNF-α was blocked following treatment of the cells with SNAP (500 μM nitric oxide donor) up to the complete disappearance of the amplified TNF-α mRNA (Fig. 2A). These findings suggest that NO inhibits NF-κB and consequently down-regulates TNF-α mRNA expression. Similar results to those observed with AD10 cells were obtained with the human prostatic adenocarcinoma cell line PC-3. The expression of TNF-α messenger RNA in PC-3 was decreased approximately 4–5-fold upon treatment with 500 μM SNAP, suggesting the role of nitric oxide in the NF-κB-dependent expression of TNF-α.

To confirm the control of NF-κB on TNF-α expression, we examined the relative levels of expression of endogenous

**FIG. 1.** Effect of nitric oxide on the sensitization of AD10 cells to TNF-α-induced cytotoxicity. The cytotoxic effect of increasing concentrations of TNF-α (0, 0.01, 0.1, and 1 ng/ml) in a 24-h incubation assay was assessed by the LDH release into the culture medium. A, AD10 cells were pretreated with 100 units/ml IFN-γ for 18 h in the presence or absence of 1 mM NOS inhibitor l-NMA. B, AD10 cells were treated with the NO donor SNAP (0, 10, and 100 μM) 18 h prior to exposure to TNF-α. **, p < 0.005; ***, p < 0.001.

**FIG. 2.** Role of NO and PDTC on endogenous TNF-α gene expression. The relative expression of TNF-α mRNA was assessed by RT-PCR. A, AD10 cells were pretreated in the presence or absence of increasing concentrations of the NO donor SNAP (1, 10, 100, and 500 μM) for 18 h and then stimulated with 100 units/ml TNF-α for 4 h. B, AD10 cells were pretreated in the presence or absence of increasing concentrations of PDTC (1, 10, 100, and 500 μM) for 18 h and then stimulated with 100 units/ml TNF-α for 4 h. Amplification of G3-PDH (G3-PDH) mRNA was used as internal standard control of gene expression for relative comparison.
TNF-α mRNA after treatment of AD10 cells with 1, 10, 100, and 500 μM PDTC for 18 h followed with TNF-α (100 units/ml) stimulation for 4 h. Endogenous TNF-α gene expression of TNF-α-stimulated cells decreased in the presence of PDTC but was never completely blocked as was observed above following treatment with SNAP (Fig. 2B). These results confirm the role of ROS in the activation of the transcription factor NF-kB and the subsequent expression of TNF-α.

Nitric Oxide Disrupts the H_2O_2-dependent Activation of NF-kB in AD10 Cells—To determine whether nitric oxide could interfere with the TNF-α-mediated activation of NF-kB, we examined the NF-kB DNA-binding activity by EMSA. As shown in Fig. 3, nuclear extracts from TNF-α-stimulated AD10 cells exhibited an increased binding activity specific for the NF-kB heterodimer p65-p50. H_2O_2 also induced specific NF-kB binding activity in AD10 cells after 30 min of incubation. Further, NF-kB binding activity was significantly inhibited by the incubation of AD10 cells with 500 μM SNAP for 2 h prior to stimulation with TNF-α for 30 min. The impaired NF-kB binding activity by SNAP was restored by the addition of H_2O_2 to similar levels as those detected in the H_2O_2-stimulated AD10 cells. Thus, these results suggest that the step at which nitric oxide interferes preceded the step at which H_2O_2 is generated after stimulation of AD10 cells with TNF-α.

Noteworthy, AD10 cells exhibit a constitutive level of NF-kB binding activity that is not affected by nitric oxide (Fig. 3, lanes 1 and 10), whereas in TNF-α-stimulated cells the NF-kB binding activity decreased below the basal levels in the presence of nitric oxide (Fig. 3, lane 6).

Nitric Oxide Decreases TNF-α-dependent Generation of H_2O_2—To examine whether nitric oxide affects the generation of H_2O_2 in AD10 cells stimulated with TNF-α, we determined the intracellular generation of H_2O_2 using the fluorescent cell-permeable probe, H_2DCFDA. AD10 cells were incubated in the presence or absence of 500 μM SNAP and then incubated with 10 and 100 units/ml TNF-α, respectively, for 15 min. Fluorescence cytometric analysis of these experimental groups revealed a significant increase in H_2O_2 levels generated by the TNF-α treatment. Incubation of the TNF-α-stimulated cells in the presence of SNAP significantly reduced the relative amount of H_2O_2 generated by these cells (Fig. 4). These data suggest that nitric oxide is affecting the intracellular biogenesis of H_2O_2 by superoxide dismutase via its chemical interaction with TNF-α-induced O_2-.

Exogenous H_2O_2 Restored the Nitric Oxide-mediated Blocking of the TNF-α-dependent Activation of NF-kB—Nitric oxide has been shown to directly affect the structure of NF-kB and decrease its DNA-binding ability due to thiol modification of critical amino acid residues (30). To determine the direct effect of nitric oxide on the activation of NF-kB, we used an enhanced green fluorescent protein-based reporter system driven by four tandem-repeated kB responsive elements linked to the thymidine kinase minimal promoter (pNF-kB-d2EGFP). We transiently transfected AD10 cells with the pNF-kB-d2EGFP reporter vector and then stimulated the cells in the presence or absence of 500 μM SNAP. Cytofluorometric analysis of these cells revealed a significant activation of the reporter gene by TNF-α and H_2O_2, and the extent of activation was a function of the concentrations used. The TNF-α-induced activation of the NF-kB-dependent reporter gene was significantly decreased in the presence of 500 μM SNAP (Fig. 5), corroborating the findings obtained in the NF-kB binding assay. The inhibitory activity of SNAP on the TNF-α-induced activation of the NF-kB-dependent reporter gene was significantly rescued by stimulation with 200 μM exogenous H_2O_2 (Fig. 5). These data confirm the inhibitory effect of nitric oxide on the H_2O_2-dependent activation of NF-kB in TNF-α-treated AD10 cells. We also noticed that untreated AD10 cells were able to maintain basal levels of NF-kB activation that were not inhibited by treatment with nitric oxide, corroborating the findings observed in the binding assay in Fig. 3.

**DISCUSSION**

The activation of the transcription factor NF-kB by TNF-α and many other stimuli has been implicated in the development of resistance of tumor cells to a variety of cytotoxic molecules including TNF-α (3, 5). NF-kB is an oxidative stress-responsive transcription factor that has been shown to respond to small concentrations of exogenous H_2O_2 or to reactive oxygen species endogenously generated as part of the signaling cascade triggered by many molecules such as TNF-α (31–33).

We have reported that the IFN-γ-induced sensitization of the human ovarian carcinoma AD10 cell line to Fas-mediated apoptosis is due in part to the generation of nitric oxide, or its reaction products, by iNOS in these cells (21). In the present study, evidence is presented for the first time that demonstrates that NO also sensitizes tumor cells to TNF-α-mediated cytotoxicity. Further, we describe a novel molecular mechanism by which nitric oxide disrupts the H_2O_2-dependent activation of NF-kB resulting in sensitization of the AD10 cells to TNF-α cytotoxicity.

The specific role of nitric oxide in tumor biology is not estab-
trolling TNF-α gene expression in many cells, either as a major activator or synergistically in association with other transcription factors (28). Thus, the significant basal activation of the NF-κB in AD10 cells might explain the presence of a constitutive expression of TNF-α by these cells (Fig. 2, A and B, last lanes). Moreover, TNF-α has been implicated as a survival cytokine used by tumor cells either to control anti-apoptotic mechanisms or promoting cellular proliferation (40–42). Therefore, the maintenance of a self-regulated loop in which the expression of TNF-α is perpetuated by the TNF-α-mediated basal activation of NF-κB could play a major role in the survival and/or proliferation of tumor cells. PDTC has been shown to be a potent and specific inhibitor of the NF-κB-mediated expression of TNF-α (29, 43). Untreated AD10 cells exhibited a basal expression of TNF-α, which was enhanced by stimulation with exogenous TNF-α and subsequently inhibited by PDTC (Fig. 2B). Similarly, using the nitric oxide donor SNAP, we were able to completely abrogate the expression of endogenous TNF-α (Fig. 2A). In contrast, nitric oxide was unable to block the basal expression of endogenous TNF-α in the absence of exogenous stimulation. These results strongly suggest the inhibitory role of nitric oxide on TNF-α-induced activation of NF-κB and consequently resulting in the disruption of TNF-α gene expression.

TNF-α induces the generation of ROS that may serve as second messengers in the activation of divergent pathways related to the cell death processes (44–46). Stimulation of many cell types with TNF-α results in the generation of intracellular superoxide (O₂) (10). In biological systems, O₂ is immediately reduced by superoxide dismutase to H₂O₂ or rapidly reacts with NO, generating ONOO⁻ (13). Therefore, decreased amounts of TNF-α-generated O₂ will result in a reduced generation of total H₂O₂. This could subsequently affect the H₂O₂-dependent activation of NF-κB (47). Examining the endogenous generation of H₂O₂ in TNF-α-stimulated AD10 cells, we have found a significant reduction in the total amount of H₂O₂ being generated in the presence of nitric oxide (Fig. 4). These results strongly suggest the scavenging effect of NO on the O₂⁻ being generated upon TNF-α treatment. Alternatively, NO can inhibit O₂⁻ production by the modification of the activity of NADPH oxidase, the main enzyme that generates O₂⁻ within the cell (48, 49).

Further, we have found that the addition of NO donors to TNF-α-stimulated AD10 cells inhibited either the DNA binding activity of NF-κB (Fig. 3) or its activation (Fig. 5). This inhibition was restored to the normal H₂O₂-stimulated level by treatment with exogenous H₂O₂. In contrast, nitric oxide did not affect the NF-κB activation in untreated AD10 cells, confirming the previous observation with TNF-α gene expression. These results suggest the presence of at least two pathways in the activation of NF-κB in AD10 cells that may differ in their sensitivity to H₂O₂ and the selectivity of nitric oxide to affect just one of these two pathways. The inactivation of NF-κB upon NO treatment was not mediated by guanylate cyclase activation since the cGMP analogue 8-bromo-cGMP had no effect on NF-κB and we could not block the inhibitory effect of NO on NF-κB activation by the use of the guanylate cyclase blocker 1H-(1,2,4)oxadiazolo-[4,3-e]quinoxalin-1-one (data not shown).

Previous reports have implicated the role of nitric oxide on the activation of NF-κB. NO has been shown to increase the expression of the NF-κB inhibitory subunit IκB or affects its cellular stability by inhibiting protein degradation (50). Due to the rapid generation of H₂O₂ upon TNF-α treatment and the immediate activation NF-κB (less than 15 min) in AD10 cells, it is highly unlikely that secondary regulatory factors like the induction of IκB interfered with the rapid NF-κB activation. It
is likely that, in the long run, a combination of both mechanisms may account for the total inhibitory role of nitric oxide on the TNF-α-induced activation of NF-κB.

An alternative proposed mechanism implicated in the inhibition of the NF-κB activity by NO is via the alteration of critical thiol groups, resulting in the disruption of the NF-κB structure and subsequently affecting its DNA-binding ability (30). However, the in vivo situation may be much more complex due to the high concentrations of glutathione and other redox-active proteins within the cell, which may prevent the modification of thiol groups.

In conclusion, our findings suggest that the mechanism by which NO sensitizes the human ovarian carcinoma cell line to TNF-α-mediated apoptosis is due to the specific disruption of the TNF-α-generated H₂O₂ and the subsequent inhibition of the NF-κB-dependent expression of anti-apoptotic genes. These results can be extended to other solid tumor cells, as observed with the human prostatic adenocarcinoma cell line PC-3. As shown in Fig. 6, the survival autocrine-paracrine loop involving the NF-κB-dependent expression of TNF-α can be interrupted by the inhibitory activity that nitric oxide exerts on the TNF-α-induced activation of NF-κB. Furthermore, in an in vivo situation, the exposure of tumor cells to pro-inflammatory cytokines such as IFN-γ will promote the induction of iNOS by the tumor cells or neighboring lymphocytes and which in turn will result in the generation of nitric oxide. Hence, the endogenously generated or the exogenously provided NO would scavenge the TNF-α-generated O₂⁻ and decrease the H₂O₂-dependent activation of NF-κB.

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REFERENCES
1. Dive, C., and Hickman, J. A. (1991) Br. J. Cancer 64, 192–196
2. Eastman, A. (1990) Cancer Cells 2, 275–280
3. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) Science 274, 784–787
4. Wang, C. Y., Cusack, J. C., Jr., Liu, K., and Baldwin, A. S., Jr. (1999) Nat. Med. 5, 412–417
5. Beg, A. A., and Baltimore, D. (1996) Science 274, 782–784
6. Tartaglia, L. A., and Goeddel, D. V. (1992) Immunol. Today 13, 151–153
7. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
8. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) Science 281, 1680–1683
9. Garcia-Ruiz, C., Coelli, A., Mari, M., Morales, A., and Fernandez-Checa, J. C. (1997) J. Biol. Chem. 272, 11369–11377
10. Hennet, T., Richter, C., and Peterhans, E. (1993) Biochem. J. 289, 587–592
11. Shimizu, Y., Forman, H. J., and Sevanian, A. (1997) Free Radiol. Biol. Med. 22, 269–285
12. Huie, R. E., and Padmaja, S. (1993) Free Radiol. Res. Commun. 18, 195–199
13. Szabo, C., and Oshshima, H. (1997) Nitric Oxide 1, 573–385
14. Fukuto, J. M. (1995) Adv. Pharmacol. 34, 1–15
15. Han, S. Y., Chong, S. Y., Paik, I. S., Kang, H. J., Choi, Y. H., Kim, S. J., and Lee, M. O. (2000) Biol. Pharm. Bull. 23, 420–426
16. Mizutani, Y., Bonavida, B., No, Y., and Yoshida, O. (1994) J. Urol. 151, 1697–1702
17. Saffrit, J. T., Berek, J. S., and Bonavida, B. (1993) Gynecol. Oncol. 48, 214–220
18. Saffrit, J. T., Beldregur, A., and Bonavida, B. (1993) J. Urol. 149, 1292–1298
19. Morimoto, H., Yonehara, S., and Bonavida, B. (1993) Cancer Res. 53, 2591–2596
20. Frost, P. J., Beldregur, A., and Bonavida, B. (1999) Prostate 41, 20–30
21. Garban, H. J., and Bonavida, B. (1999) Gynecol. Oncol. 73, 257–264
22. Stamler, J. S. (1995)Curr. Top Microbiol. Immunol. 196, 19–36
23. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624
24. Beckman, J. S. (1991) J. Dev. Physiol. 15, 53–59
25. Decker, T., and Lohmann-Matthes, M. L. (1988) J. Immunol. Methods 113, 61–69
26. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
27. Lenardo, M. J., and Baltimore, D. (1989) Cell 58, 227–229
28. Liu, H., Sidipiroulos, P., Song, G., Pagliari, L. J., Birrer, M. J., Stein, B., Anrather, J., and Pepe, R. M. (2000) J. Immunol. 164, 4727–4735
29. Ziegler-Heitbrock, H. W., Stermsdorf, T., Lise, J., Belohradsky, B., Weber, C., Wedel, A., Schreck, R., Bauerle, P., and Strobel, M. (1995) J. Immunol. 151, 2696–2699
30. Park, S. K., Lin, H. L., and Murphy, S. (1997) Biochem. J. 322, 609–613
31. Hong, Y. H., Peng, H. B., La Fata, V., and Liao, K. J. (1997) J. Immunol. 159, 2418–2423
32. Schreck, R., Albermann, K., and Bauuerle, P. A. (1992) Free Radiol. Res. Commun. 17, 221–237
33. Schreck, R., Rieber, P., and Bauerle, P. A. (1991) EMBO J. 10, 2247–2258
34. Moronu, S., Palmer, R. M., and Higgs, E. A. (1991) Pharmacol. Rev. 43, 109–142
35. Jenkins, D. C., Charles, I. G., Thomsen, L. L., Moss, D. W., Holmes, L. S., Bayly, S. A., Rhodes, B. P., Westmore, K., Eason, P. C., and Moncada, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4392–4396
36. Liu, L., and Stamler, J. S. (1999) Cell Death Differ. 6, 937–942
37. Li, J., and Billiar, T. R. (2000) Semin. Perinatol. 24, 46–50
38. Shi, Q., Xiong, Q., Wang, R., Le, X. K., Huang, N. A., and Xie, K. (2000) Cancer Res. 60, 2579–2583
39. Taylor, R. S., Alarcon, L. H., and Billiar, T. R. (1998) Biochemistry (Moscow) 63, 766–781
40. Khwaja, A., and Tatton, L. (1999) J. Biol. Chem. 274, 5867–5863
41. Sawyer, S. T., and Jacobs-Heiber, S. M. (2000) J. Hematother. Stem Cell Res. 9, 21–29
42. Selinsky, C. L., and Howell, M. D. (2000) Cell Immunol. 200, 81–87
43. Nemeth, Z. H., Hasko, G., and Vizi, E. E. (1998) Shock 10, 49–53
44. Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyeart, R., Jacob, W. A., and Fiers, W. (1996) J. Biol. Chem. 271, 17103–17107
45. Schulze-Osthoff, K., Beyeart, R., Vandervoorde, V., Haegeman, G., and Fiers, W. (1993) EMBO J. 12, 3095–3104
46. Morrone-Manzano, V., Ishikawa, Y., Luizzi-Cazana, J., and Kitamura, M. (2000) J. Biol. Chem. 275, 12684–12691
47. Bauerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
48. Clancy, R. M., Leszczynska-Trzeciak, J., and Abramson, S. B. (1992) J. Clin. Invest. 90, 1116–1121
49. Fujii, H., Ichimori, K., Hoshiai, K., and Nakazawa, H. (1997) J. Biol. Chem. 272, 32773–32778
50. Peng, H. B., Libby, P., and Liao, J. K. (1995) J. Biol. Chem. 270, 14214–14219