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Superoxide Attenuates Macrophage Apoptosis by NF-κB and AP-1 Activation That Promotes Cyclooxygenase-2 Expression

Andreas von Knethen, Dagmar Callsen, and Bernhard Brüne

Macrophages are a major source of cytokines and proinflammatory radicals such as superoxide. These mediators can be both produced and utilized by macrophages in autocrine-regulatory pathways. Therefore, we studied the potential role of oxygen radical-regulatory mechanisms in reprogramming macrophage apoptosis. Preactivation of RAW 264.7 cells with a nontoxic dose of the redox cycle 2,3-dimethoxy-1,4-naphtoquinone (5 μM) for 15 h attenuated S-nitrosoglutathione (1 mM)-initiated apoptotic cell death and averted accumulation of the tumor suppressor p53, which is indicative for macrophage apoptosis. Preactivation with superoxide promoted cyclooxygenase-2 induction that was NF-κB and AP-1 mediated. NF-κB activation was confirmed by p50/p65-heterodimer formation, IκB-α degradation, and stimulation of a NF-κB luciferase reporter construct. Furthermore, a NF-κB decoy approach abrogated cyclooxygenase-2 (Cox-2) expression as well as inducible protection. The importance of AP-1 for superoxide-mediated Cox-2 expression and cell protection was substantiated by using the extracellular signal-regulated kinase-inhibitor PD98059 and the p38-inhibitor SB203580, which blocked Cox-2 expression. In corroboration, Cox-2 expression was hindered by a dominant-negative c-Jun mutant (TAM67). Protection from apoptosis was verified in human macrophages with the notion that superoxide promoted Cox-2 expression, which in turn attenuated nitric oxide-evoked caspase activation. We conclude that the sublethal generation of oxygen radicals reprograms macrophages by NF-κB and AP-1 activation. The resulting hyporesponsiveness reveals an attenuated apoptotic program in association with Cox-2 expression. The Journal of Immunology, 1999, 163: 2858–2866.
The striking possibility to reprogram the apoptotic behavior of macrophages by using sublethal concentrations of superoxide prompted us to explore the molecular mechanisms in detail. We established NF-κB and AP-1 activation by the O$_2^-$-generator DMNQ in close association with Cox-2 expression. For murine and human macrophages, we envision how ROS circumvent cell death, i.e., apoptosis, supporting the notion that expression of Cox-2 attenuates programmed cell death.

**Materials and Methods**

**Materials**

Diphenylamine and LPS (*Escherichia coli* serotype 0127:B8) were purchased from Sigma (Deisenhofen, Germany). The Cox-2 Ab was bought from Transduction Laboratories (Lexington, KY). The p50- and p65-supershift Abs as well as the IκB-α Ab were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Murine rIFN-γ was provided by Dr. Nicotera, University of Konstanz (Konstanz, Germany). PD98059, provided by Eurogentec (Seraing, Belgium). DMNQ was kindly provided by Dr. P. Angel, Deutsches Krebsforschungszentrum, Heidelberg, Germany). Blood was diluted 1/2 with PBS and added as indicated. PD98059 and SB203580 were dissolved in 100% ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 1 mM MgCl$_2$, 2 mM MgCl$_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM Na$_2$EDTA, 0.5 mM PMSF, pH 7.5 on ice; 50 μl ice on ice for 30 min at 4°C, vortex mixed, and centrifuged at 15,000 × g for 30 s. Pelleted nuclei were gently resuspended in 50 μl ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortex mixed, and centrifuged at 15,000 × g for 5 min at 4°C. Aliquots of the supernatant that contain nuclear proteins were frozen in liquid nitrogen and stored at −70°C. Protein was determined using a Bio-Rad II Kit (Richmond, CA).

**Fluorogenic caspase-3-like activity determination**

Cells (2 × 10$^6$) were incubated as indicated, recovered from cultured plates, and centrifuged (1,200 × g, 4°C, 5 min). Cell pellets were resuspended in lysis buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM EDTA) and left on ice for 30 min. Following sonication (Branson sonifier, 10 s, duty cycle 100%, output control 100%), centrifugation (10,000 × g, 10 min, 4°C), protein was determined with the DC Protein Assay. Cell supernatants (30 μg protein) were incubated in 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 1 mM PMSF, and 10 μg/ml leupeptin at 30°C with 12 μM of the caspase-3-like substrate Ac-DEVD-AMC. Substrate cleavage and accumulation of AMC were followed fluorometrically with excitation at 360 nm and emission at 460 nm during a 120-min incubation period. Substrate cleavage during the linear phase of the reaction was quantitated by internal AMC standards. Enzyme activity was expressed as nM AMC per minute per milligram protein (nM/min × mg).

**Electrophoretic mobility shift assays**

An established EMSA method, with slight modifications, was used (33). Nuclear protein (5 μg) was incubated for 20 min at room temperature with 20 μg BSA, 2 μg poly(dI-dC) from Pharmacia (Piscataway, NJ), 2 μl buffer D (20 mM HEPES/KOH, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM DTT, 0.5 mM PMSF, pH 7.9), 4 μl buffer F (20% Ficoll-400, 100 mM HEPES/KOH, 300 mM KCl, 10 mM DTT, 0.5 mM PMSF, pH 7.9), and 20,000 cpm of a 32P-labeled oligonucleotide in a final volume of 20 μl. Supershift Abs (2 μg) were added as indicated. DNA-protein complexes were resolved at 180 V for 4 h in a 4% native polyacrylamide gel (4% for supershifts), dried, and visualized (autoradiography using a Fuji x-ray film). Oligonucleotide probes were labeled by a filling reaction using the Klenow fragment (Boehringer Mannheim, Mannheim, Germany). Oligonucleotide (1 pmol) was labeled with 50 μCi of [α-32P]dCTP (3000 Ci/mmol; Amersham, Braunschweig, Germany), cold nucleotides (dATP, dCTP, dGTP from Life Technologies, Egggenstein, Germany), purified on a CHROMA SPIN-10 column (Clontech, Heidelberg, Germany), and stored at −20°C until use. The following oligonucleotide sequences were used: the NF-κB site from the mouse Cox-2 promoter (34), 5′-GAG GTG AGG GGA TTC CCT TAG-3′ and 3′-AC TCC CCT AAG GGA GTA AATC-5′, and a mutated NF-κB site, 5′-GAG GTG AGG GCC TTC CCT TAG-3′ and 3′-AC TCC GGA AAG GGA GTA AATC-5′, the AP-1 site from the human collagenase gene (35), 5′-AGC TAA AGG ATG AGT CAC ACA GGA TTC-3′ and 3′-TT TCG TAC TCA GTG CTC GAC ATG GA-5′ (the oligonucleotide was kindly provided by Dr. P. Angel, Deutsches Krebsforschungszentrum, Heidelberg, Germany); and the specific p53 binding site (36), 5′-GGG CCT GAG GCC GTA TCC-3′ and 3′-GTA CAC GCC GTG ACA AGG-5′.

**Immunoblot analysis**

Cell lysates was prepared with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, pH 8) and sonication (Branson sonifier; 20 s, duty cycle 100%, output control 60%). Following centrifugation (14,000 × g, 5 min), protein was determined. Proteins (100 μg) were resolved on 10% polyacrylamide gels and blotted onto nitrocellulose. Equal loading was confirmed by Ponceau S staining. Filters were incubated overnight at 4°C with the Cox-2 Ab (1:250; Dianova, Hamburg, Germany), nNOS (hybridoma supernatant, clone PAb122, 1:5; kindly provided by Dr. H. Stahl, Hamburg/ Saar, Germany), or the IκB-α Ab (1:500). Proteins were detected by a HRP-conjugated polyclonal Ab (1:10,000) using the ECL method (Amersham, Braunschweig, Germany).
Quantitative DNA fragmentation analysis

DNA fragmentation was measured with the diphenylamine assay, as reported elsewhere (37). Briefly, following incubations, cells were scraped off the culture plates; resuspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8 (TE buffer); and incubated with additional 250 μl lysis buffer (5 mM Tris, 20 mM EDTA, pH 8, 0.5% Triton X-100) for 30 min at 4°C. After lysis, intact nuclei (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13,000 × g. Pellets were resuspended in 500 μl TE buffer, and samples were precipitated overnight by adding 500 μl 10% TCA at 4°C. DNA was pelleted by centrifugation (4,000 × g, 10 min) and the supernatant was removed. After addition of 300 μl 5% TCA, samples were boiled for 15 min. DNA contents were quantitated using the diphenylamine reagent (38). The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Transient transfection of a dominant-negative c-jun mutant (TAM67)

To target transcription factor activation by transient transfection of upstream signaling components requires high transfection efficiency and/or selection of cells expressing the mutant protein. One day before transfection, cells were seeded at a density of 1 × 10^5 cells/ml into 10-cm non-cell culture petri dishes. RAW 264.7 macrophages were transiently transfected with 15 μg of the expression vector (TAM67) that contains the sequence of a dominant-negative c-jun mutant (kindly provided by Dr. E. Gulbins, Tübingen, Germany) (39, 40). For positive selection of TAM67-positive cells, the vector pMAC4s, designed to express a truncated human CD4 molecule, was cotransfected (ratio 1:10 compared with the TAM-67 expression vector). Transfection was achieved using a Pro Gentor II electroporator ( Hoefer Scientific Instruments, San Francisco, CA). A total of 3 × 10^7 cells was resuspended in 400 μl complete medium, transferred to a cuvette, and pulsed (260 V, 1080 μF, 26 ms). Transfected cells were pooled and seeded in 10 ml complete medium into a 10-cm non-cell culture petri dish. Overnight (15-h) cultured cells were harvested and CD4-positive clones were selected. Cell selection was unnecessary pending on the dose and the duration of exposure.

Luciferase plasmid expression containing the NF-κB site of the mouse Cox-2 promoter

NF-κB reporter constructs, cloned into the pGL3-enhancer plasmid (Promega), contained four copies of the NF-κB element taken from the murine Cox-2 promoter (NF-κB-sense) or its mutated form (NF-κB-mut) (see EMSA). Corresponding sequences were verified by DNA sequencing. RAW 264.7 macrophages were transiently transfected using the DEAE-dextran method, as previously described (41). Cell selection was unnecessary because the synthesis of two macrophage-unrelated proteins was analyzed. Briefly, one day before transfection, cells were seeded in suspension at a density of 1 × 10^5 cells/ml. A total of 1 × 10^6 cells was harvested, washed twice with PBS, and incubated for 3 h at 37°C in 1 ml RPMI 1640 supplemented with 50 mM Tris-HCl (pH 7.3), 400 μg DEAE-dextran, 20 μg luciferase-reporter construct (NF-κB-sense or NF-κB-mut), and 5 μg CMV-β-gal plasmid as an internal control. To discard the DNA/DEAE-dextran mixture, cells were washed twice with PBS and seeded at a density of 1 × 10^5 cells/ml and cultured for 24 h. Afterward, cells were stimulated with 1 mM GSNO or vehicle, incubations went on for an additional 4-h period, as indicated. Data are means ± SD of three individual experiments (*, p ≤ 0.005 vs GSNO-treated samples).

FIGURE 1. A, Inverse expression of Cox-2 and p53 in RAW 264.7 macrophages. Western blot analysis of Cox-2 and p53 in RAW 264.7 macrophages. Cells were stimulated with 5 μM DMNQ for 15 h. Following the addition of 1 mM GSNO or vehicle, incubations went on for an additional 4-h period (total incubation period of 19 h). GSNO-mediated p53 accumulation (lane 3) was measured after 4 h. Details are described under Materials and Methods. The blot is representative of three similar experiments. B, NS398 restored p53 accumulation. Western blot analysis of Cox-2 and p53 in RAW 264.7 macrophages. Cells were stimulated with 10 μM NS398 or simultaneously with 5 μM DMNQ and 10 μM NS398 for 15 h. Following stimulation, 1 mM GSNO was added where indicated, and incubations went on for an additional 4-h period (total incubation period of 19 h). Details are described under Materials and Methods. The blot is representative of three similar experiments. C, DMNQ protected against NO-mediated apoptosis. DNA fragmentation was assessed by the diphenylamine method, as described under Materials and Methods. Cells were preincubated with 5 μM DMNQ, 10 μM NS398, or a combination of both for 15 h. Following preincubation, 1 mM GSNO was added for an additional 8-h incubation period, as indicated. Data are means ± SD of three individual experiments (*, p ≤ 0.005 vs GSNO-treated samples).

Statistical analysis

Each experiment was performed at least three times, and statistical analysis was performed using the two-tailed Student’s t test. Otherwise, representative data are shown.

Results

Cox-2 and p53 accumulation are inversely related in RAW 264.7 macrophages

Incubation of macrophages with a high dose of the NO donor GSNO (1 mM) led to accumulation of the tumor suppressor p53 after 4 h and promoted 30–35% DNA fragmentation after 8 h. This is in corroboration with other studies and substantiates NO-initiated apoptotic cell death (42, 43). Assuming a gene-regulatory potency of superoxide (O_{2}^{-}), we analyzed, in a first set of experiments, the influence of a nontoxic dose of the superoxide-generating agent DMNQ on subsequent NO-mediated DNA fragmentation and p53 accumulation. GSNO was used as the NO donor because high doses were shown to generate high amounts of NO leading to apoptosis in macrophages (42). DMNQ was described to continuously generate O_{2}^{-} through redox cycling (44), thereby stimulating growth, triggering apoptosis, or causing necrosis depending on the dose and the duration of exposure.
Previous studies (5, 45) pointed to Cox-2 induction and subsequent cAMP-evoked gene activation as the underlying mechanisms to confer resistance against high dose NO-mediated toxicity. As a result of these examinations, we incubated RAW 264.7 macrophages with 5 μM DMNQ for 15 h. Prestimulation promoted Cox-2 expression (Fig. 1A, lane 2) and attenuated the p53 response following GSNO addition (Fig. 1A, lane 4 compared with lane 3). DNA fragmentation detected by the diphenylamine assay was significantly increased in response to GSNO, but preactivation with 5 μM DMNQ for 15 h reduced GSNO-elicited DNA fragmentation to control values (Fig. 1C, lanes 2 and 4). The impact of Cox-2 in promoting protection was assured by using the Cox-2-specific inhibitor NS398 (Fig. 1B). NS398 has been described to be a specific competitive inhibitor for Cox-2 (4). NS398 did not affect DMNQ-elicited Cox-2 expression, but restored the NO-evoked p53 response, thus pointing to an active Cox-2 in conveying cell protection. In analogy, inhibition of Cox-2 by NS398 restored DNA fragmentation in response to 1 mM GSNO (Fig. 1C, last three columns) despite DMNQ pretreatment. This is in line with our previous notion that enforced Cox-2 overexpression protected macrophages from apoptosis (5). Our results point to an inverse expression of Cox-2 and p53 that obviously is closely correlated to initiation of apoptosis or its inhibition. Superoxide-evoked protection encouraged us to identify molecular mechanisms leading to Cox-2 expression.

**NF-κB activation by low dose DMNQ**

With the notion that Cox-2 expression is regulated at least in part by NF-κB (14, 34), we analyzed NF-κB activation in response to the O₂⁻-generating compound DMNQ. Based on gel-shift analysis, we proved dose-dependent NF-κB activation by DMNQ (Fig. 2A). Activation was minor in response to 0.5 μM DMNQ, was stronger following the addition of 1 μM DMNQ, and was strongest with 5 μM of the O₂⁻ generator.

Active NF-κB was absent in unstimulated cells, but was noticed following the addition of LPS/IFN-γ, well-established NF-κB-activating agents (Fig. 2A, second lane). The response to 5 μM DMNQ and LPS/IFN-γ was comparable. Higher concentrations of DMNQ have not been analyzed because of their proapoptotic and/or necrotic behavior (data not shown). Gel-shift examinations with an oligonucleotide that contained a mutated NF-κB sequence (replacement of two bases) demonstrated no shift and therefore referred to the specificity of the NF-κB/oligonucleotide binding (data not shown).

The identity of the NF-κB complex was further analyzed by supershift experiments. Addition of either an anti-p50 or an anti-

![FIGURE 2](attachment://figure2.png)

**FIGURE 2**. A, Activation of NF-κB by DMNQ. Activation of NF-κB was analyzed by EMSA using a specific NF-κB oligonucleotide, derived from the mouse Cox-2 promoter, as described in Materials and Methods. Macrophages were stimulated with a combination of LPS (10 μg/ml) and IFN-γ (100 U/ml), or DMNQ at the indicated concentrations for 4 h, or remained unstimulated. B, NF-κB supershift analysis. Supershift analysis of the active NF-κB complex was performed as described in Materials and Methods. Macrophages were stimulated with 5 μM DMNQ for 4 h. For supershift analysis, a p50 Ab or a p65 Ab was included. Gel-shift analysis without Ab addition is used as a control (lane 1). Data are representative of three similar experiments. C, IκB-α degradation following macrophage stimulation. IκB-α Western blot analysis in RAW 264.7 macrophages following stimulation with 5 μM DMNQ for 4 h. Details are described under Materials and Methods. The blot is representative of three similar experiments. D, NF-κB-driven luciferase activity. RAW 264.7 macrophages were cotransfected with NF-κB-luciferase plasmid constructs and a plasmid encoding β-gal. Luciferase and β-gal expression were analyzed, after both activities were normalized, as described under Materials and Methods. Cells were stimulated with 5 μM DMNQ with or without the addition of NF-κB decoy oligonucleotides or vehicle (control). Data are means ± SD of three individual experiments. E, NF-κB decoy oligonucleotides abrogated DMNQ-induced Cox-2 expression. Western blot analysis of Cox-2 in RAW 264.7 macrophages. Cells were prestimulated with 5 μM DMNQ for 15 h in the presence or absence of NF-κB decoy oligonucleotides (3 μM). Details are described under Materials and Methods. The blot is representative of three similar experiments.
Transient TAM67-transfected cells or controls were prestimulated with indicated or remained unstimulated (C). Western blot analysis revealed a time-dependent decrease of IκB-α expression, leading to almost complete disappearance of the inhibitor after a 4-h treatment with 5 μM DMNQ. These results substantiate O₂⁻-mediated NF-κB activation.

In additional experiments, we paid attention to the time course of NF-κB activation by following the degradation/disappearance of the NF-κB-specific inhibitor IκB-α (Fig. 2C). Western blot analysis revealed a 3-fold induction of luciferase activity in response to 5 μM DMNQ. Corresponding plasmids that contained a mutated NF-κB site showed no luciferase transactivation. Luciferase activity that indicated NF-κB activation in response to DMNQ was attenuated by the addition of NF-κB decoy oligonucleotides. Decoy oligonucleotides, by competing with promoter regions in target genes for the activated transcription factor, revealed effective suppression of NF-κB-mediated gene activation. This was verified by abrogating DMNQ-induced Cox-2 expression following NF-κB decoy-oligonucleotide addition (Fig. 2E).

Our studies substantiate superoxide-mediated NF-κB activation leading to an enhanced expression of Cox-2, a NF-κB-responsive gene.

**AP-1 activation by low dose DMNQ**

In another set of experiments, we wished to study activation of the redox active transcription factor AP-1, which is composed of c-Jun homodimers or c-Jun/c-Fos heterodimers. Initial examinations considered two MAPK-specific inhibitors such as PD98059 (MEK-specific kinase inhibitor) and SB203580 (p38-kinase inhibitor) on DMNQ-elicited Cox-2 expression (Fig. 3A). Western blot analysis revealed a dramatically decreased DMNQ-stimulated Cox-2 expression in the presence of PD98059 (20 μM). SB203580 (5 μM) showed a less potent, albeit significant inhibition (70 ± 8% SD vs control). PD98059 and SB203580 could not be used at higher concentration because of toxic side effects, as judged by trypan blue uptake. The used inhibitors completely abrogated ERK-specific kinase and p38-kinase activity (data not shown).

Cox-2 expression vanished completely when both MAPK inhibitors were combined (Fig. 3A, lane 5). These results encouraged us to explore expression of c-Jun, which resembles one component of AP-1, in response to low level of the O₂⁻-generator DMNQ (Fig. 3B). Western blot analysis showed up-regulation of c-Jun in response to DMNQ. Expression was low at 1 h, increased after 2 h, and revealed highest expression after a 4-h incubation period. Additional experiments such as electrophoretic mobility shift analysis were undertaken to demonstrate a transcriptionally active AP-1 in response to DMNQ (Fig. 3C). Activation was low in response to 0.5 μM DMNQ, became stronger after the addition of 1 μM p65 Ab shifted the existing NF-κB complex to a higher m.w. or diminished binding completely (Fig. 2B), which is in accordance with previous studies (46). Therefore, it can be concluded that NF-κB consists of the p50 and p65 (Rel A) subunits. This complex has been shown to include a DNA binding domain (p50) as well as transactivation domain (p65), therefore enhancing or promoting gene induction.

GSNO was added for an additional 4-h incubation period (total incubation period of 19 h). GSNO-mediated p53-binding activity (lane 3) was measured after 4 h. Details are described under Materials and Methods. The Western blot and the EMSA are representative of three similar experiments.
DMNQ, and showed maximal activation after a 5 μM challenge. With respect to the time dependency, AP-1 activation in response to 5 μM DMNQ peaked at 4 h, with a lower gel-shift response at 2 and 6 h, respectively (data not shown).

To correlate AP-1 activation to the inhibitory action of MAPK inhibitors on Cox-2 expression, we sought to explore the potency of PD98059 and SB203580 in AP-1 gel shifts (Fig. 3D). Addition of PD98059 completely attenuated AP-1 activation in response to DMNQ. SB203580 showed a minor, but still significant inhibitory action, whereas AP-1 activation was attenuated in TAM67-transfected cells. Apoptosis was assessed by the diphenylamine method. Data are means ± SD of three individual experiments (*, p ≤ 0.005 vs inhibited controls).

Whereas AP-1 activation was attenuated in TAM67-transfected cells, apoptosis was assessed by the diphenylamine assay. For control reasons, a mutated decoy oligonucleotide was used, and TAM67-unrelated transfections (control transfections) were conducted in parallel.

Protection from GSNO (1 mM)-elicited apoptosis resulted from preincubation with DMNQ, which was unaffected by the mutated NF-κB decoy-oligonucleotide approach. However, eliminating NF-κB by the use of specific decoy oligonucleotides abrogated DMNQ-evoked protection. Also, protection from apoptosis was eliminated in TAM67-transfected cells, whereas protective principles remained intact when control transfections were performed.

Conclusively, our data imply that low level superoxide confers protection from apoptosis via AP-1 and NF-κB activation, thus leading to Cox-2 induction.

Cox-2 expression in primary human macrophages attenuated NO-mediated apoptosis

To verify our results obtained with the murine macrophage-like cell line RAW264.7 in primary macrophages, we isolated human monocytes, followed by their differentiation to macrophages. In a first set of experiments, we explored expression of Cox-2 in response to DMNQ (5 μM). Semiquantitative PCR showed low expression at 1 h, increased levels after 2 h, and revealed highest mRNA amounts after a 4- or 6-h incubation period (Fig. 5A). Relative appearance of Cox-2 fragments was verified in proportion to the occurrence of GAPDH PCR fragments (data not shown). As a positive control known to affect Cox-2 transcription, we analyzed
LPS-evoked Cox-2 mRNA accumulation during a 2- to 6-h incubation period.

Exposure of human macrophages to GSNO resulted in caspase activation, which is indicative for apoptosis (Fig. 5B). Premutation of human macrophages for 15 h with the O$_2^-$ -generating compound DMNQ (5 μM) blocked NO-initiated caspase activation (Fig. 5B), while the further addition of the Cox-2-specific inhibitor NS398 completely restored caspase activation. We conclude that preactivation of murine or human macrophages for an extended time with DMNQ attenuated NO-induced apoptosis by a pathway demanding an active Cox-2 enzyme.

**Discussion**

In the present study, we provide experimental evidence for the induction of macrophage tolerance toward initiation of apoptosis by preactivating murine and human cells with a subtoxic dose of the superoxide-generating agent DMNQ. Macrophage unresponsiveness is controlled by activation of the redox-sensitive transcription factors NF-κB and AP-1, which both contribute to induction of Cox-2. The active enzyme has been shown to convey resistance to apoptosis in different cell types (5, 47, 48). This is achieved by the production of prostaglandins (PGE$_2$) that led to an increase of intracellular cAMP, which in turn promoted cAMP-evoked gene induction. We further provide evidence for an inverse expression of Cox-2 and p53, which reflect protection from cell death and apoptosis-related pathways, respectively. Our data support the notion that ROS are efficient regulators of gene activation, clearly allowing to dissect this signaling component from oxygen radical-mediated toxicity.

The generation of ROS is an established response during inflammation or infection and is implicated, among others, in TNF- or platelet-derived growth factor-evoked signal transmission (49–51). Oxygen- or nitrogen-based radicals are produced in part by specialized cells such as macrophages and neutrophils with the implication of immunological host defense (52–54). A delicate balance between formation and detoxification of radicals exists and is regarded an important determinant of cell survival and cell death. Protection of cells involved in immunological host defense such as macrophages against naturally occurring apoptotic cell death must be considered to have a major impact on immunoregulation.

Activation of NF-κB and AP-1 by ROS and reactive nitrogen species is established and, especially in the case of TNF-α-mediated apoptosis activation of NF-κB, seems to convey protection, i.e., prevention of apoptosis (19). In response to DMNQ, we established activation of a functional p50/p65 heterodimer by super-shift analysis, observed IκB-α degradation, substantiated NF-κB activation in a luciferase transactivation assay, and provided evidence that inducible protection as well as Cox-2 expression were sensitive to NF-κB decoy oligonucleotides. This is in line with the work of Wang et al. (55) and Quillet-Mary et al. (56), who noticed NF-κB activation in response to H$_2$O$_2$-elicited oxidative stress. Although we have been working with a O$_2^-$ -generating compound, we cannot exclude the possibility that disproportionation of O$_2^-$ by superoxide dismutase with the resulting formation of hydroperoxide actually causes NF-κB activation. Decay-oligonucleotide approaches were used to scavenge active transcription factors, thereby blocking their binding to the promoter regions in target genes (57–59). Our results are in line with the report of Schmedtje et al. (60), who eliminated hypoxia-induced Cox-2 expression by NF-κB p65 decay oligonucleotides in human endothelial cells.

In addition to NF-κB, we noticed activation of AP-1 in response to DMNQ. Activation of AP-1 by ROS has been described by Zwacka and coworkers (13), who reduced ischemia/reperfusion-based redox activation of AP-1 by adenoviral-enforced overexpression of mitochondrial superoxide dismutase. Moreover, Manna et al. (61) showed that manganese superoxide dismutase overexpression attenuated TNF-induced AP-1 activation by quenching reactive oxygen intermediates. Several potential oxygen radical-generating sources such as NAD(P)H oxidases, cyclooxygenases, the mitochondrial respiratory chain, or xanthine oxidase are known that may initiate radical signal transmission (1, 4, 62, 63). One possible mechanism for Cox-2 in attenuating NO-induced apoptosis may stem from its peroxidase function that generates peroxy radicals (1, 62). These radicals may directly scavenge NO, thereby eliminating potential damaging species. However, based on observations that Cox-derived prostanoids that provoke an intracellular cAMP increase reproduce protection make the radical interaction theory less favorable.

Activation of NF-κB and AP-1 is inevitably related to macrophage protection. Attenuating NF-κB or AP-1 activation by decoy oligonucleotides or transfection of a dominant-negative c-jun mutant resulted in a parallel block of inducible protection and Cox-2 expression. Our results substantiate the obligatory role of NF-κB in promoting Cox-2 expression, which is fully compatible with the observation of a NF-κB binding site in the promoter region of the murine Cox-2 enzyme (34). Binding of AP-1 to the murine Cox-2 promoter is rationalized and most probably achieved via a CRE site because AP-1-enforced Cox-2 expression through this promoter site has been noticed (28). This is verified when we showed a time- and concentration-dependent AP-1 activation by DMNQ that was blocked by the ERK-specific kinase inhibitor PD98059 and in part by the p38 kinase inhibitor SB203580. Both inhibitors were described to be selective at the concentrations used (64, 65). Our results are in line with the report of Hwang and coworkers, in which LPS-induced Cox-2 expression was inhibited by the two MAPK kinase inhibitors PD98059 and SB203580 (14).

The potential antiapoptotic role of Cox-2 is in analogy to examinations in which Cox-2 blocked butyrate-mediated apoptosis (47). Further proof for a survival-promoting function of Cox-2 came from genetic studies that point to the early involvement of the protein in the progression leading to colon cancer or from correlative investigations showing a high incidence of Cox-2 expression in human tumors (4). For macrophages, it seems conceivable to assume PGE$_2$ formation as a result of Cox-2 expression that, in a self-regulatory feedback loop, will enhance intracellular cAMP formation. Interestingly, intervention in the macrophage cyclic nucleotide system, i.e., supplementation of lipophilic cAMP analogues (59) or the addition of PGE$_2$ will attenuate apoptosis (66). In some analogy, preactivation of macrophages with LPS/IFN-γ/4'-monomethyl-L-arginine (NAE) promoted protection from NO-elicted apoptosis, which was Cox-2 mediated (5). Attenuating macrophage apoptosis reminds of the unique property of endotoxin to achieve low responsiveness, a phenomenon known as endotoxin tolerance (67–69). Control of endotoxin responsiveness may limit proinflammatory macrophage responses. Low level ROS formation may be used as an indicator for oxidative stress and may signal gene expression and thus protective protein synthesis. This rescue system allows macrophages to evade apoptosis. We show that NF-κB and AP-1 activation promotes Cox-2 expression and protects RAW 264.7 macrophages against NO-induced apoptosis. Protection is reversed by AP-1 or NF-κB inhibition, thus restoring a functional p53 response, which is indicative for NO-mediated apoptosis. Murine and human macrophages may use ROS as an autocrine-regulatory pathway to maintain cell viability, a process that contributes and fulfills a fundamental role during immunologic homeostasis.
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References


