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Nitric oxide and superoxide induced p53 and Bax accumulation during mesangial cell apoptosis

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Nitric oxide and superoxide induced p53 and Bax accumulation during mesangial cell apoptosis. During proliferative glomerulonephritis, the early phase of mesangiolysis is linked to increased nitric oxide ($NO^{\cdot}$) production. $NO^{\cdot}$ as well as superoxide (O_2^{-}) are inflammatory mediators that are generated by mesangial cells (MC) after cytokine stimulation. Added individually, both radicals induce MC apoptosis. However, the co-existence of a defined $NO^{\cdot}/O_2^{-}$ ratio is cross-protective. Apoptosis is characterized by specific features such as chromatin condensation, DNA strand breaks, and the occurrence of apoptotic regulating proteins. The tumor suppressor p53 and Bax (Bcl-2 associated protein x) are considered to be classical death promotors, which accumulate after toxic insults. To study p53 and Bax protein accumulation in $NO^{\cdot}$ and/or O_2^{-}-induced apoptosis, we used the NO-donor S-nitrosoglutathione (GSNO) and the redox cycler 2,3-dimethoxy-1,4-naphtoquione (DMNQ). Both agonists initiated DNA fragmentation in a concentration dependent manner associated with transient p53 and Bax up-regulation. Co-generation of $NO^{\cdot}/O_2^{-}$ resulted not only in reduced DNA fragmentation, but also in decreased Bax accumulation. Comparable to the $NO^{\cdot}/O_2^{-}$ co-generation, cytokines failed to induce apoptosis. In contrast, cytokines in combination with pyrrolidine dithiocarbamate, which blocks endogenous superoxide dismutase, allowed p53 and Bax accumulation as well as DNA fragmentation. Our results demonstrate p53 and Bax as early components in $NO^{\cdot}$ and O_2^{-}-induced rat MC apoptosis and point to the $NO^{\cdot}/O_2^{-}$ interaction as a naturally occurring cell defense mechanism.

Apoptosis, or programmed cell death, is a physiological occurring process during tissue development, differentiation, and homeostasis [1]. It is characterized and distinguished from necrosis by morphological and biochemical features such as cell shrinkage, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies [2]. Dysregulation of apoptosis seems closely associated with clinical symptoms of cancer, Alzheimer, or acquired immunodeficiency syndrome (AIDS). Apoptosis also occur in glomeruli of patients with proliferative glomerulonephritis [3]. In the experimental model of Thy 1.1 glomerulonephritis mesangial cell lysis followed by compensating cell proliferation are important features [4–6], although the mechanisms of mesangiolysis during early and late phase of Thy 1.1 nephritis are largely unknown. Mesangial cells (MC) exhibit features of specialized smooth muscle cells and participate to regulate blood filtration, to produce extracellular matrix, and to secrete inflammatory mediators such as nitric oxide ($NO^{\cdot}$) [7, 8] and superoxide (O_2^{-}) [9]. A significant and sustained rate of $NO^{\cdot}$ production is found during the early phase of Thy 1.1 mesangiolysis [10, 11] and is blocked by administration of the NOS inhibitor N^G-monomethyl-L-arginine (NMMA) [10].

Nitric oxide is produced by a family of NO-synthase (NOS) isoenzymes that utilize L-arginine and oxygen in the formation of citrulline and $NO^{\cdot}$ [12]. For reasons of simplicity NOS-isoenzymes are distinguished as Ca^{2+}-dependent and constitutively expressed versus Ca^{2+}-independent and cytokine-induced. The radical $NO^{\cdot}$ participates in divergent biological signaling that can either be cGMP-dependent or cGMP-independent. Mechanistically, the biological action of $NO^{\cdot}$ is determined by its interaction with oxygen, superoxide, or transition metals leading to the formation of various nitrogen derived species [13]. During inflammation the inducible NOS generates large amounts of $NO^{\cdot}$ over an extended period of time that may lead to pathophysiological alterations in association with tissue damage.

In several cell systems such as thymocytes [14, 15], macrophages [16, 17], pancreatic β-cells [18, 19], neurons [20, 21], or MC [22], among others, $NO^{\cdot}$ induces apoptotic cell death. Executive pathways are largely unknown but seem to overlap with the damaging potential of superoxide or $ONOO^{-}$.

Apoptosis is an evolutionary conserved process found in different cells and species. Based on investigations in the nematode *C. elegans* there are cell death promotors such as Ced-3 and suppressor proteins like Ced-9 [1]. The mammalian homologous proteins to Ced-3 are ICE-like proteases, whereas Bcl-2 resembles Ced-9 [23]. The bcl-2 (B-cell lymphoma/leukemia-2) gene was identified at the breakpoint site of the t(14;18) chromosomal translocation that is associated with follicular lymphoma. Bcl-2-like proteins have grown to a family of different proteins that regulate both cell death and survival [24]. Generally, one assumes that the ratio of Bcl-2 to Bax-like proteins determines the cellular susceptibility towards apoptosis. Bcl-2 itself is a suppressor of apoptosis that homo- or heterodimerizes with the homologous protein Bax [25]. In contrast to Bcl-2, Bax (Bcl-2 associated protein x), a 21 kDa protein, is considered a death promotor, which is neutralized by heterodimerization with Bcl-2 or corresponding proteins. In some cells Bax can be expressed in a p53-dependent manner by γ-radiation, chemotherapeutic drugs,

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and other genotoxic stressors [26, 27]. The nuclear phosphoprotein p53 is a tumor suppressor that functions as a transcription factor. Accumulation of wt p53 is sometimes required for the G1 cell cycle checkpoint after DNA damage, and its up-regulation in the case of severe DNA damage is often related to apoptosis. In RAW 264.7 macrophages and RINm5F pancreatic β-cells p53 accumulates after endogenous $NO^{\cdot}$ production that can be blocked by the addition of the NOS inhibitor NMMA [28]. Increased p53 mRNA expression was also documented in rat kidneys with unilateral ureteral obstruction, an experimental model of progressive tubulointerstitial fibrosis [29]. Previous experiments implied $NO^{\cdot}$-mediated p53 up-regulation in MC [22]. However, protein accumulation in response to O_2^- and $NO^{\cdot}/O_2^-$ co-administration remains unknown. Moreover, regulation of Bax in rat MC after radical ($NO^{\cdot}$, O_2^-) generation and/or cytokine treatment is unclear.

To address p53 and Bax expression in response to radical ($NO^{\cdot}$, O_2^-) formation, we stimulated rat MC with the NO-donor S-nitrosoglutathione (GSNO) and the redox cycler 2,3-dimethoxy-1,4-naphtoquione (DMNQ). Increasing concentration of exogenously supplied GSNO and DMNQ induced a progessive p53 and Bax accumulation followed by apoptotic DNA fragmentation. Intriguingly, a timely balanced production of both radicals was inefficient in Bax up-regulation. Moreover, despite endogenous $NO^{\cdot}$ production, cytokines failed to induce apoptosis. However, cytokines in combination with the Cu/Zn-SOD inhibitor, pyrrolidine dithiocarbamate (PDTC), again promoted apoptosis as well as p53 and Bax accumulation. Obviously, p53 and Bax are essential and early inducible components in $NO^{\cdot}$ and O_2^--induced apoptotic mesangial cell death.

METHODS

Materials

Insulin, diphenylamine, Hoechst 33258, poly-L-lysine, sulfanilamide, N-naphtylethylendiamine, lipopolysaccharide, and pyrrolidine dithiocarbamate were purchased from Sigma (Deisenhofen, Germany). Interleukin 1β, terminal desoxynucleotidyl transferase, and interferon-γ were bought from Boehringer Mannheim (Mannheim, Germany). RPMI 1640 and medium supplements were ordered from Biochrom (Berlin, Germany), and streptavidine/rhodamine from Pierce (München, Germany). Secondary antibodies came from Promega/Serva (Heidelberg, Germany) and the α-Bax antibody from Santa Cruz (Heidelberg, Germany). Fetal calf serum was purchased from Gibco (Berlin, Germany). DMNQ was kindly provided by Prof. Dr. Nicotera (University of Konstanz, Konstanz, Germany) and the monoclonal antibody Ab122 was donated by Prof. Dr. Stahl (University of Saarland, Homburg, Germany) [30]. All other chemicals were of the highest grade of purity commercially available.

Culture of mesangial cells

Rat mesangial cells were cultured, cloned and characterized as described previously [31]. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and bovine insulin at 5 μg/ml. One day before and during the experiments the control and stimulated cells were kept in medium with 0.5% fetal calf serum. Previous studies have shown that variable serum concentrations do not effect NO-induced DNA fragmentation. Moreover, unstimulated cells kept in low (0.5%) or high (10%) serum show comparable fragmentation values. For the experiments, passages 8 to 20 of mesangial cells were used.

GSNO synthesis

S-nitrosoglutathione was synthesized as described previously [32]. Briefly, glutathione was dissolved in 0.625 N HCl at 4°C to a final concentration of 625 mM. An equimolar amount of $NaNO_2$ was added and the mixture was stirred for 40 minutes. After the addition of 2.5 volumes of acetone, stirring was continued for another 20 minutes, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, and finally three times with diethylether, and dried under vacuum. Freshly synthesized GSNO was characterized by UV spectroscopy.

Quantification of DNA fragmentation

DNA fragmentation was assayed as reported [33]. Briefly, following incubations, cells were centrifuged, resuspended in 250 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysed by adding 250 μl cold lysis buffer, containing 2 mM EDTA, 0.5% (vol/vol) Triton X-100, and 5 mM Tris-HCl, pH 8.0. Samples were allowed to lyse for 30 minutes at 4°C prior to centrifugation (15 min at 14,000 × g) to separate intact chromatin (pellet) from DNA fragments (supernatant). Pellets were resuspended in 500 μl TE buffer and the DNA content of pellets and supernatants were measured using the diphenylamine reagent.

Nitrite determination

Nitrite, a stable NO oxidation product, was determined using the Griess reaction. Cell free culture supernatants were collected (200 μl), adjusted to 4°C, mixed with 20 μl sulfanilamide (dissolved in 1.2 M HCl) and 20 μl N-naphthylethylenediamine-dihydrochloride. After five minutes at room temperature the absorbance was measured at 560 nm with a reference wavelength at 690 nm. Nitrite concentrations were calculated using a $NaNO_2$ standard.

p53 and Bax quantification

p53 and Bax amount was quantified by Western blot analysis. Briefly, 7.5×10^6 cells were incubated for the times indicated, scraped off and lysed in 150 μl lysis buffer (50 mM Tris/HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0). Lysed cells were sonicated with a Branson sonifier (10 seconds, duty cycle 100%, output control 1). After centrifugation (14,000 × g, 15 min) supernatants were transfered and protein content was analyzed. Finally, samples (100 μg for Bax and 500 μg for p53) were resuspended in the same volume of 2x sample buffer (125 mM Tris/HCl, 2% SDS, 10% glycerin, 1 mM DTT, 0.002% bromephenol blue, pH 6.9) and boiled for five minutes. Proteins were resolved on 10 or 15% SDS-polyacrylamide gels, and blotted onto nitrocellulose sheets. To detect the different molecular weights, we used a molecular weight rainbow marker. Sheets were washed twice with TBS (140 mM NaCl, 50 mM Tris/HCl, pH 7.2) containing 0.1% Tween-20 before blocking unspecific binding with TBS/2% skim milk. The p53 (hybridoma supernatant against p53; clone PAb122; 1:5 in TBS/0.2% milk) or Bax (0.5 μg/ml in TBS/0.2% milk) antibody was added and incubated overnight at 4°C. Nitrocellulose sheets were washed five times and unspecific binding was blocked as described. For detection, blots were

incubated with goat anti-mouse or goat anti-rabbit conjugated with peroxidase (1:10,000 in TBS/0.2% milk) for one hour followed by ECL detection.

Determination of lactate dehydrogenase release

Following incubations, medium of 2.5×10^5 MC was collected and cells were supplemented with 0.2% (vol/vol) Triton-X 100 (in PBS), followed by lysis for four hours at 4°C. A 500 μl reaction mix containing 50 mM triethanolamine was dissolved in 5 mM EDTA, pH 7.6, 127 mM pyruvate, and 14 mM NADH in 1% $NaHCO_3$ was added to the 300 μl cell medium. LDH activity was monitored by the oxidation of NADH following the decrease in absorbance at 334 nm. The percentage of LDH release was defined as the ratio of LDH activity in the supernatant to the sum of the LDH amount released plus the activity measured in the cell lysate.

In situ detection of apoptosis by TUNEL

To detect apoptosis, we used the terminal desoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. Following incubations, cells (3×10^3 cells/well) were washed with PBS and fixed on chamber slides with 1% paraformaldehyde for 30 minutes at 4°C. Cells were kept in 70% ethanol at −20°C overnight, washed with PBS, incubated with terminal desoxynucleotidyl transferase (10 U/100 μl) and 20 μM biotin-dUTP for one hour at 37°C. After washing, staining solution (1 μg/100 μl streptavidine/rhodamine) was added and kept in a dark humid chamber for one hour. Washed and dried chamber slides were embedded and end labeling was analyzed with excitation 560 nm/emission 570 nm wavelength using a fluorescence microscope.

Staining of nuclei with Hoechst 33258

Confluent mesangial cells were stimulated according to experimental protocols, scraped off the culture plates followed by fixation with 3% paraformaldehyde for five minutes onto glass slides. Samples were washed with PBS, stained for five minutes with 8 μg/ml of the Hoechst dye H33258, washed with PBS and mounted in moviol. Nuclei were visualized using a Leitz microscope [17].

Statistical analyses

Each experiment was performed at least three times and statistical analysis were performed using the two-tailed Student's *t*-test. Normal distribution of data are ensured. Otherwise representative data are shown.

RESULTS

NO-induced DNA fragmentation, chromatin condensation, p53 and Bax accumulation

Previous studies have shown that different NO donors such as spermine-NO, GSNO, S-nitroso-N-acetylpenicillamine or a NO·-saturated solution caused apoptosis in rat mesangial cells (MC) [22]. Here we primarily used GSNO to study p53 and Bax accumulation in correlation to apoptotic DNA fragmentation. GSNO induced DNA fragmentation in a concentration dependent manner that was detected and quantitated with the diphenylamine assay after a 24 hours incubation (Fig. 1A). Considering typical morphological alterations, chromatin condensation served as an additional apoptotic parameter. Control incubations (Fig. 1B) lack chromatin condensation, whereas typical morphological alterations were initiated by 500 μM spermine-NO (Fig. 1C). The number of apoptotic cells with chromatin condensation correspond approximately to the percentage of DNA fragmentation. Control studies revealed that quantitative DNA fragmentation as well as morphological alterations are equivalent in low (0.5% FCS: 28% ± 6 DNA fragmentation/24 hr following 1 mM GSNO addition; mean value ± SEM, $N = 4$) and high (10% FCS: 27% ± 2 DNA fragmentation/24 hr following 1 mM GSNO addition; mean value ± SEM, $N = 4$) serum and that serum removal does not trigger apoptotic alterations. Changes in the steady-state of apoptotic signal-conveying proteins such as p53 and Bax were examined by Western blot analysis after NO· treatment. p53 as well as Bax accumulated dose-dependently in response to GSNO (Fig. 2 A, B). A residual background level of p53 was occasionally present in control cells, but significant increases were detected four hours after the addition of 1 or 2 mM GSNO. In contrast, Bax was undetectable in controls, but accumulated in response to GSNO when quantitated two hours after stimulation.

Time-dependent p53 and Bax up-regulation

During apoptosis the sequential activation of specific markers and signaling components becomes apparent. In NO-treated MC, chromatin condensation, a TUNEL-positive reaction, and DNA fragmentation appear after 16 to 24 hours only. In contrast, 1 mM GSNO stimulated p53 accumulation within two hours following its addition (Fig. 2C). p53 levels continued to increase up to eight hours after GSNO challenge, before a decline was seen after 24 hours. Bax accumulation (Fig. 2D) showed a similar time-dependency. The protein appeared two to eight hours after GSNO addition, while the protein band disappeared 16 to 24 hours after the initial challenge.

Superoxide initiated DNA fragmentation, DNA cleavage (TUNEL reaction), and up-regulation of p53 and Bax

Redox cyclers such as DMNQ are valuable tools to study the action of superoxide irrespective to cytokine-mediated activation of NADPH-like oxidases. DMNQ exclusively generated O_2^- within the cell (2 μM DMNQ generated ~260 pmol $O_2^-/10^6$ cells/min) [18] and induced DNA fragmentation in rat MC as measured by diphenylamine assay 24 hours post-stimulation. Controls revealed 5 ± 2% fragmentation, whereas DMNQ at a concentration of 1 μM initiated 26 ± 3% ($N \geq 5$, $P < 0.01$ vs. controls) DNA cleavage; 5 μM of the redox cycler evoked less fragmentation (16 ± 3%, $N \geq 5$, $P < 0.01$ vs. controls) but sustained release of lactate dehydrogenase (LDH; 28 ± 2%, $N \geq 5$, $P < 0.01$ vs. 4 ± 1% LDH release of untreated controls), a typical necrotic marker. Detection of DNA strand breaks (TUNEL reaction) further supported O_2^- induced MC apoptosis. Controls (Fig. 3A) lacked a TUNEL positive reactivity, thereby indicating intact DNA. In contrast, a TUNEL positive reactivity was assured by the exposure to 1 μM DMNQ (Fig. 3B). Timely preceding to DNA fragmentation and LDH release, we found p53 and Bax accumulation in response to increasing DMNQ concentrations (Fig. 3 C, D). p53 was analyzed by Western blots four hours and Bax eight hours following stimulation.

Transient increases of p53 and Bax after superoxide generation

Similar to NO-induced apoptosis in cultured rat MC, chromatin condensation and DNA strand breaks (TUNEL reaction) were detectable 16 hours after DMNQ treatment, whereas DNA

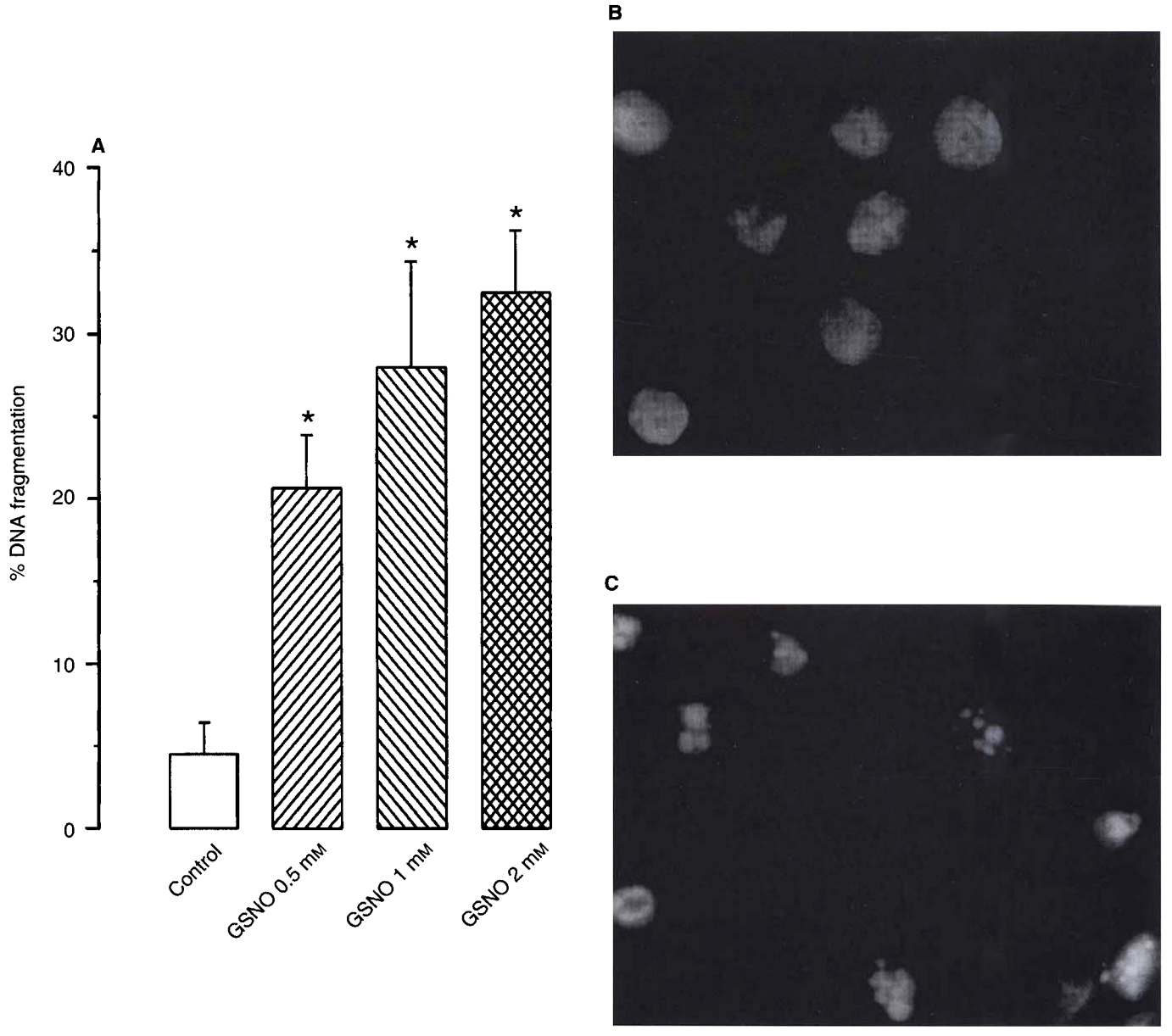

Fig. 1. GSNO-induced DNA fragmentation and chromatin condensation. (*A*) Rat mesangial cells (2.5×10^5 cells/assay) were cultured for 24 hours with increasing GSNO concentrations. DNA fragmentation was quantitated using the diphenylamine assay described in the **Methods** section. All data are mean values ± SEM of at least five separate experiments, $^*P < 0.01$ versus control. (*B* and *C*) Representative photographs show spermine-NO induced chromatin condensation. Mesangial cells were stimulated with vehicle (control, B) or with 500 μM spermine-NO (C) for 16 hours. Cells were fixed and stained using Hoechst dye H33258 as described in the **Methods** section.

fragmentation was observed 24 hours after the initial challenge. The time-response of p53 and Bax up-regulation following DMNQ addition were analyzed by video densitometry of representative Western blots. DMNQ (1 μM) transiently caused p53 accumulation. Maximal protein levels were detected one hour following O_2^- generation, while p53 slowly disappeared within the next 24 hours (Fig. 4A). Up-regulation of Bax was somehow slower compared to p53. In the Western blot shown in Figure 4B, residual Bax was relatively high. However, DMNQ (1 μM) promoted a further increase during the next eight hours. After 24 hours, Bax declined even below the control level. These experiments revealed that late O_2^--induced apoptotic events such as chromatin condensation, and DNA fragmentation are preceded by a transient p53 and Bax accumulation.

Simultaneous generation of NO/O_2^- is non-destructive for rat MC and is accompanied by reduced Bax accumulation

For these studies we stimulated MC with DMNQ and GSNO at the same time. Both agonists have been shown to induce DNA fragmentation when added individually. During these experiments, we kept DMNQ constant at 5 μM, which led to 18 ± 5% ($N \geq 5$, $P < 0.01$ vs. control cleavage that amounted to 5 ± 2%) DNA fragmentation measured with the diphenylamine assay after

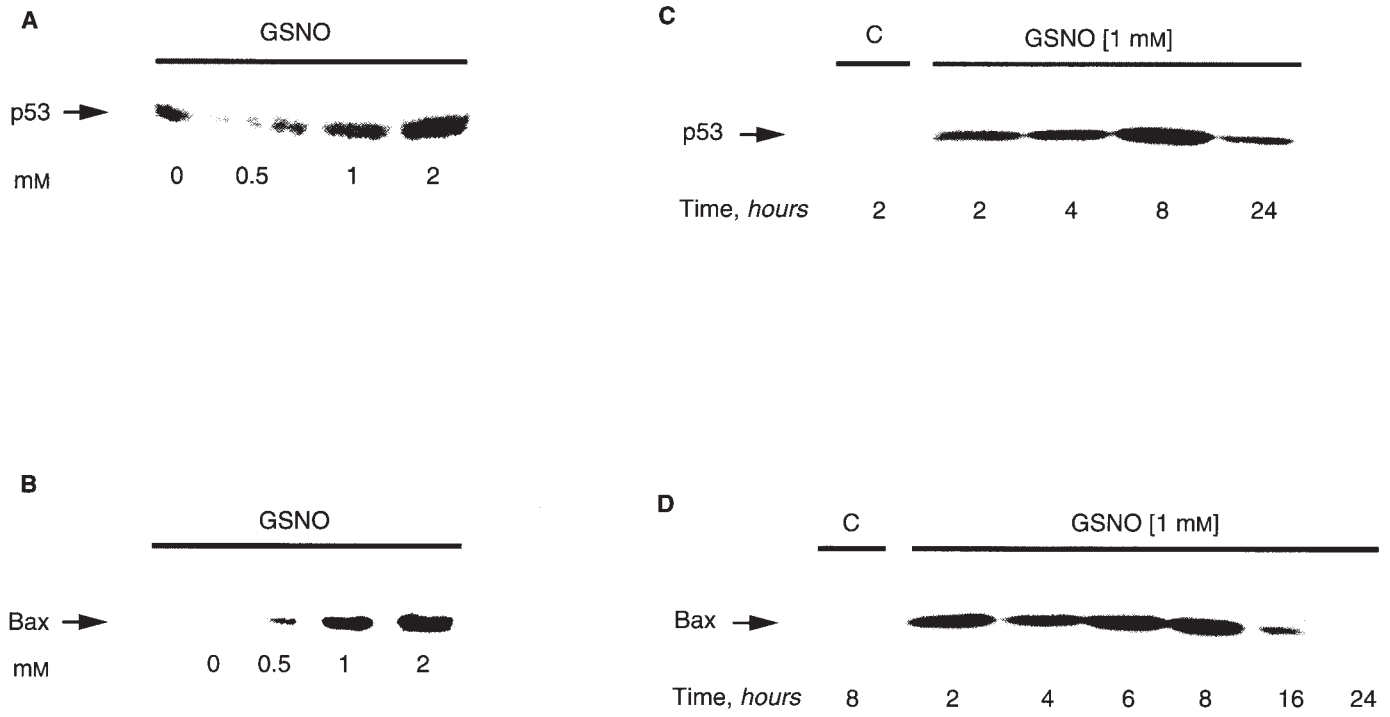

Fig. 2. Concentration- and time-dependent p53 and Bax accumulation in response to GSNO. (*A* and *B*) Mesangial cells (5×10^6 cells/assay) were stimulated with or without GSNO followed by Western blot analysis of p53 (53 kDa) and Bax (21 kDa) as outlined in the **Methods** section. p53 was detected four hours and Bax two hours after GSNO addition. (*C* and *D*) For the time-dependency, mesangial cells (5×10^6 cells/assay) were cultured without the addition of (C, control) or with 1 mM GSNO. Incubations were terminated at times indicated followed by p53 (53 kDa) and Bax (21 kDa) Western blot analysis as described in the **Methods** section. Blots are representative of three similar experiments.

24 hours. Intriguingly, DMNQ in combination with 0.25 mM GSNO evoked only 9 ± 2% ($N \geq 5$, $P < 0.01$ vs. fragmentation elicitated by 5 μM DMNQ) DNA fragmentation and at the same time reduced LDH release compared to cellular damage produced by single application of DMNQ or GSNO. With increasing NO-donor concentrations of 0.5 mM or 1 mM the apoptotic rate rose again to 17 ± 3% and 23 ± 2% (for both determinations $N \geq 5$, $P > 0.01$ vs. cleavage initiated by DMNQ/0.25 mM GSNO). Therefore, the co-generation of $NO^{\cdot}/O_2^-$ in a balanced ratio in part protected cultured rat MC from apoptosis. Extending these examinations, Bax Western blot analysis reflected similar changes as observed with the diphenylamine assay. Figure 5 shows that 5 μM DMNQ promoted Bax up-regulation. Bax accumulation was reduced when DMNQ was co-administed with 0.25 mM and 0.5 mM GSNO. This is surprising, because GSNO at these concentrations triggered a positive Bax response as seen in Figure 2. A further increase of the NO-donor concentration again resulted in an enhanced expression of the Bax steady-state level.

Increased p53 and Bax accumulation, as well as DNA fragmentation after SOD inhibition

The generation of $NO^{\cdot}$ and O_2^- by different cytokines, especially IL-1β and TNF-α, in rat MC is well known [7, 9]. Despite high production of $NO^{\cdot}$ and its oxidation to nitrite, as measured by the Griess assay (Table 1), no apoptotic features became detectable after cytokine (25 U/ml interleukin 1β, 100 U/ml interferon γ, 10 μg/ml lipopolysaccharide, 500 ng/ml tumor necrosis factor α) stimulation. In order to disturb the endogenous formation of superoxide we stimulated MC with cytokines and 10 μM of the NFκB and SOD inhibitor PDTC. PDTC action prevented endogenous $NO^{\cdot}$ production (Table 1) and resulted in accumulation of O_2^-, due to SOD inhibition [34, 35]. Under these conditions significant DNA fragmentation was exclusively detected with the combination of cytokines plus PDTC, but not with either stimuli alone (Table 1). p53 and Bax Western blot analysis confirmed these results (Fig. 6). No apoptotic promoting proteins, such as p53 or Bax, were detectable in control cells. PDTC (10 μM) as well as cytokines (25 U/ml interleukin 1β, 100 U/ml interferon γ, 10 μg/ml lipopolysaccharide, 500 ng/ml tumor necrosis factor α) induced a slight increase in the steady-state level of p53 and Bax. In contrast, cytokines in combination with PDTC evoked a massive accumulation of the corresponding apoptotic proteins. It can be assumed that up-regulation of p53 and Bax after cytokine/PDTC addition lead to DNA fragmentation and apoptosis in rat MC.

DISCUSSION

Programmed cell death participates in the regulation of cell homeostasis in healthy and infected tissue. The characteristics of apoptosis were described in glomerular mesangial cells, endothelial cells, and epithelial cells [22] as well as in glomeruli of patients with proliferative glomerulonephritis [3], and in different experimental renal disease models [4–6]. Apoptotic agonists such as $NO^{\cdot}$, Fas [36, 37], cAMP [38], or deprivation of growth factors [4] are known, although the underlying signal transducing pathways largely remain unknown.

Here, we investigated the steady-state level of two cell death promoting proteins, p53 and Bax, in $NO^{\cdot}$ and O_2^--induced apoptosis in cultured rat MC. To study radical signal transduction

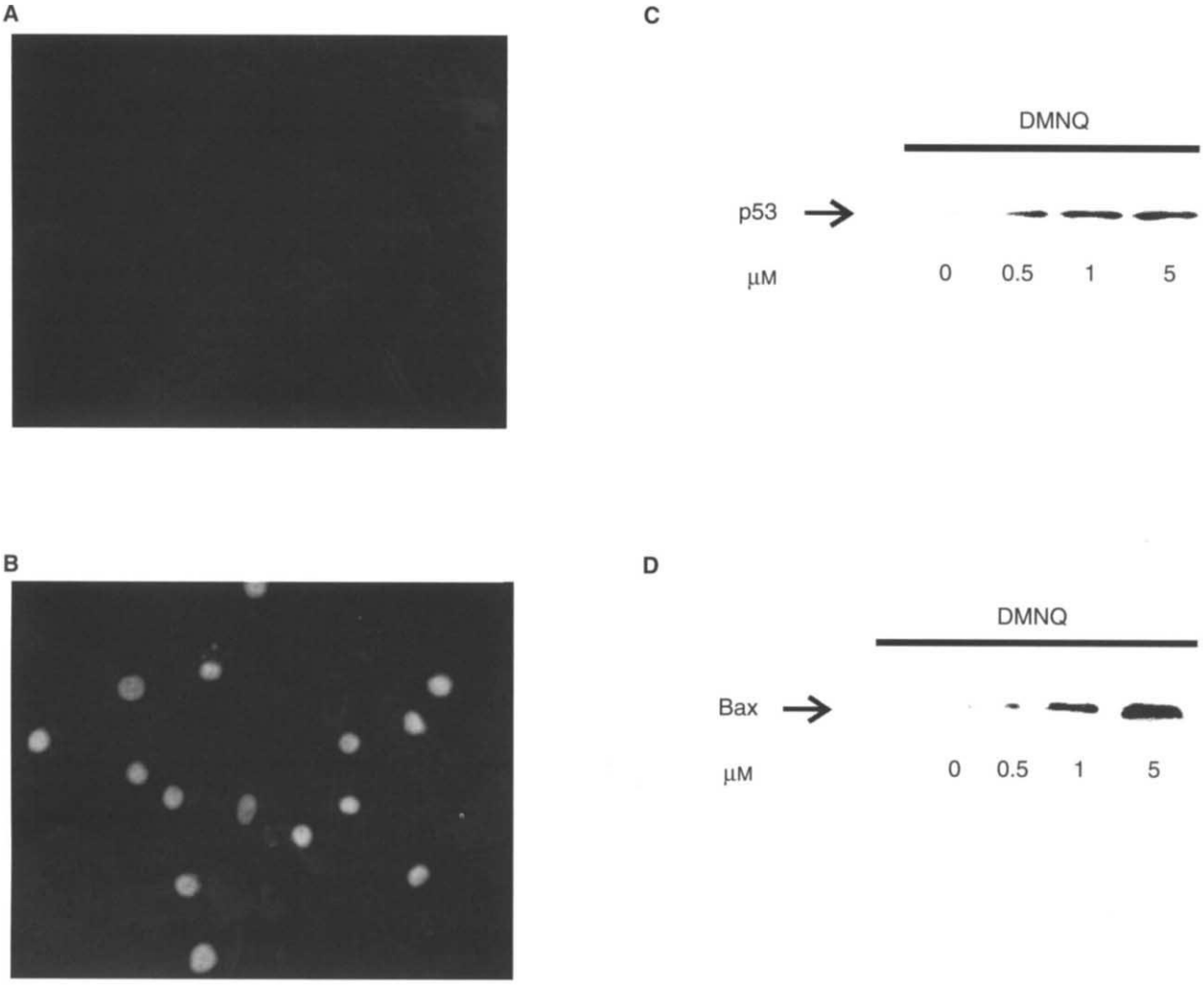

Fig. 3. DNA strand break and concentration-dependent p53 and Bax up-regulation by DMNQ. (*A* and *B*) Mesangial cells (3×10^3 cells/assay) were kept as controls (A) or stimulated with 1 μM DMNQ (B) for 16 hours. DNA strand breaks were detected by the TUNEL technique as outlined in the **Methods** section. (*C* and *D*) Five $\times 10^6$ mesangial cells/assay were stimulated with 0, 0.5, 1, or 5 μM DMNQ. Following incubations, p53 (53 kDa) was detected after four hours by Western blot analysis (C) as outlined in the **Methods** section, while (D) the amount of Bax (21 kDa) was examined eight hours after DMNQ stimulation. The Figure is representative of 3 similar blots.

irrespective of iNOS up-regulation or NADPH-like oxidase activation, we used the NO-donor GSNO, which is known to decompose to nitrite/nitrate as oxidation end products, and the redox cycler DMNQ. Both $NO^{\cdot}$ and O_2^- evoked chromatin condensation and DNA strand breaks after 16 to 24 hours, thereby indicating late apoptotic events. In contrast, up-regulation of classical apoptotic promoting proteins such as p53 and Bax occurred much earlier. Bax and p53 accumulated in a concentration dependent manner after GSNO or DMNQ treatment (Fig. 2 A, B and 3 C, D). $NO^{\cdot}$-mediated p53 up-regulation and induction of apoptosis is exemplified for RAW 264.7 macrophages and RINm5F β-cells [39]. Changes in the p53 mRNA level were reported for experimental models of progressive tubulointerstitial fibrosis [29] where apoptosis occurs [40]. Our data document a transient p53 protein accumulation following $NO^{\cdot}$-intoxication that suggests p53 as an early signal promoting protein. p53 also functions as a transcription factor for growth-arrest and DNA-damage inducible genes including gadd45, $p21^{WAF1/Cip1}$, and Bax [41]. Down-regulation of bax-α mRNA was observed in breast epithelium of cancer patients, while gene transfer of bax-α in breast cancer restored the sensitivity towards serum starvation and Fas-triggered apoptosis [42]. Our data show not only p53 accumulation, but also Bax protein up-regulation following $NO^{\cdot}$ and O_2^- treatment, which clearly preceded DNA fragmentation. Intriguingly, there was no or little residual Bax in MC compared to RAW 264.7 macrophages [43] or breast epithelium [42]. For GSNO we noticed up-regulation of p53 and Bax almost simultaneously (Fig. 2), whereas p53 preceded Bax accumulation in the case of DMNQ (Fig. 4). Further experiments are needed to establish a cause effective relation between apoptosis promoting proteins themselves as well as a definite role of these proteins for the initiation of down-stream signals. Our experiments suggest p53 and Bax up-regulation as early components that precede late apoptotic events after $NO^{\cdot}$ or O_2^- intoxication.

$NO^{\cdot}$ is an ambivalent molecule and its damaging potential is not immediately realized. $NO^{\cdot}$ can induce apoptosis, but its formation

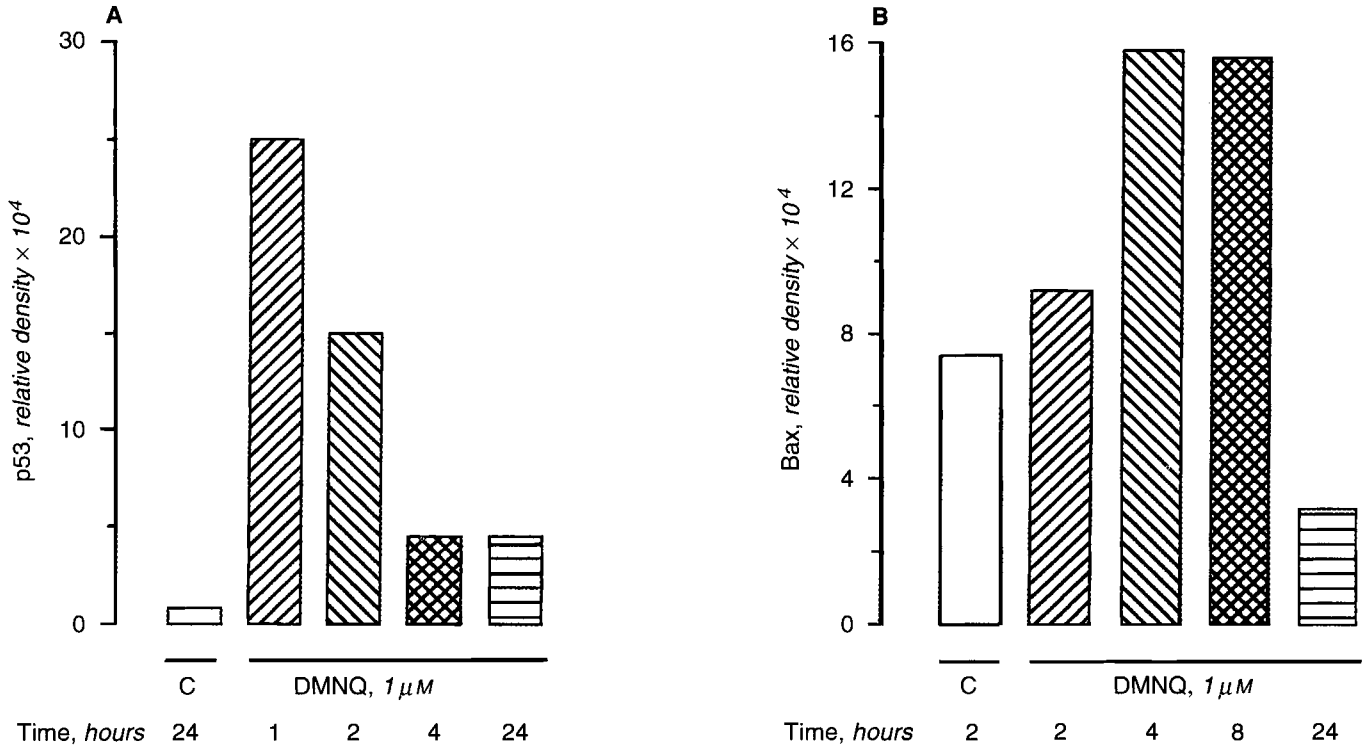

Fig. 4. Time-dependent p53 and Bax increase following DMNQ stimulation. Mesangial cells (5×10^6 cells/assay) were treated without the addition of (C, control) or 1 μM DMNQ. (*A*) p53 expression was determined by Western blot analysis at times indicated, followed by video densitometry of corresponding bands and values are expressed as relative density $\times 10^4$. (*B*) The amount of Bax was determined correspondingly using an anti-Bax antibody as described in the **Methods** section. Relative densities (density $\times 10^4$) are given. Experiments are representative of three similiar examinations.

can also signal cellular protection during ischemia-reperfusion, peroxide-induced toxicity, lipid-peroxidation, or myocardial injury [35, 44–46]. Such diverse biological effects can be explained, at least in part, by the interaction of NO· with its targets oxygen, superoxide, and transition metals. The co-existence of NO· and O_2^- assumingly leads to the diffusion controlled formation of peroxynitrite ($ONOO^-$) [47, 48], which is believed to be highly toxic. However, $ONOO^-$ toxicity differs among cell types as documented in studies with cerebrocortical cultures, PC12 cells, and endothelial cells [48–50] where $ONOO^-$ ranges from a highly toxic to a non-toxic compound. We exposed MC to both NO· and O_2^- by co-stimulation with increasing concentrations of GSNO and O_2^--generating systems (DMNQ, 5 μM). The radical co-exposure protected MC from apoptosis rather than potentiated the destructive effect of the agents added individually. Western blot analysis substantiated protection by radical co-administration at the level of Bax expression (Fig. 5). Bax increased after DMNQ treatment, whereas this was blocked in combination with moderate concentrations of GSNO. This implies that the level of Bax serves as an apoptotic indicator in rat MC and most likely participates in downstream death signaling. Moreover, our results point to efficient termination of destructive NO· or O_2^- signaling by radical-radical interaction. Obviously, excessive NO· or O_2^- generation promotes apoptosis by closely related pathways, involving p53 and Bax. A finely tuned generation of radicals with the likely formation of $ONOO^-$ is cross protective, no longer conveying destructive and thus apoptotic signals to downstream proteins like Bax.

Despite a massive nitrite accumulation, which is a stable NO·-oxidation end-product (Table 1), endogenous NO· production in response to cytokines neither produced apoptosis nor caused necrosis. We assumed that after cytokine stimulation NO· and O_2^- are formed, which antagonize each other, thereby blocking apoptosis. To manipulate the endogenous NO·/O_2^- ratio, we used pyrrolidine dithiocarbamate (PDTC), an inhibitor of NFκB and Cu/Zn superoxide dismutase (SOD) [35, 50]. Consequently, NO· generation is prevented [34] and O_2^- accumulates because its conversion to H_2O_2 is blocked. Previously we reported that diphenyliodonium, a selective NADPH-oxidase inhibitor, blocked the PDTC response by lowering the PDTC evoked O_2^- response [51]. With cytokines in combination with PDTC, DNA fragmentation accompanied by p53 and Bax accumulation (Fig. 6) was detected. These events most likely reflect endogenous O_2^- formation and underscore p53 and Bax as essential apoptotic MC components.

Our study demonstrates NO· and O_2^--induced apoptosis in cultured rat MC are in close association with the up-regulation of the cell death-promoting proteins p53 and Bax. Intriguingly, the co-generation of NO· and O_2^- functions as a natural protection system for MC against endogenously or exogenously produced radicals, suppressing Bax expression and concomitant DNA fragmentation. Further investigations will establish a cause effective role of p53 and Bax in MC apoptosis.

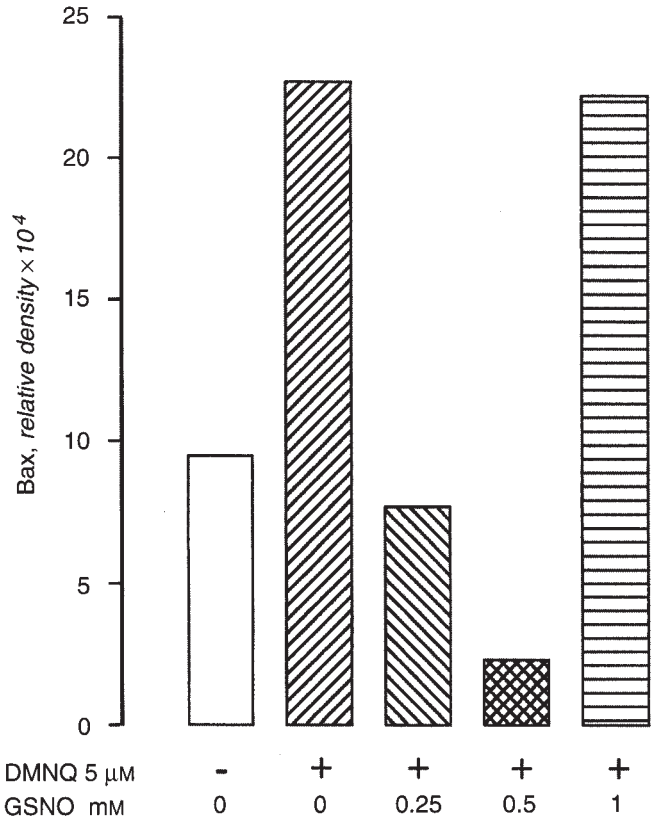

Fig. 5. Alterations of the Bax steady-state level by $NO^{\cdot}/O_2^-$ co-administration. Mesangial cells (5×10^6 cells/assay) were cultured without addition, with 5 μM DMNQ, or a combination of DMNQ and increasing concentrations of GSNO (0, 0.25, 0.5, 1 mM). After four hours, Bax was determined by Western blot analysis as described in the **Methods** section. Bax steady-state levels were quantitated by video densitometry of a representative Western blot as described in Figure 4.

Table 1. Pyrrolidine dithiocarbamate promotes DNA fragmentation in cytokine-stimulated mesangial cells

	% DNA fragmentation	Nitrite accumulation *μM/24 hr*
Control	9 ± 1	2 ± 2
Cytokines	10 ± 1	51 ± 4[a]
PDTC *10 μM*	8 ± 2	1 ± 1
Cytokines + PDTC *10 μM*	35 ± 2[a]	8 ± 4

Mesangial cells (2.5×10^5 cells/assay) were stimulated with vehicle (control), cytokine mix (25 U/ml interleukin 1β, 100 U/ml interferon γ, 10 μg/ml lipopolysaccharide, 500 ng/ml tumor necrosis factor α), 10 μM PDTC, or cytokines in combination with PDTC. DNA fragmentation and nitrite accumulation was determined after 24 hours, as outlined in the **Methods** section. Data are mean values ± SEM of at least five separate experiments.

[a] $P < 0.01$ versus control

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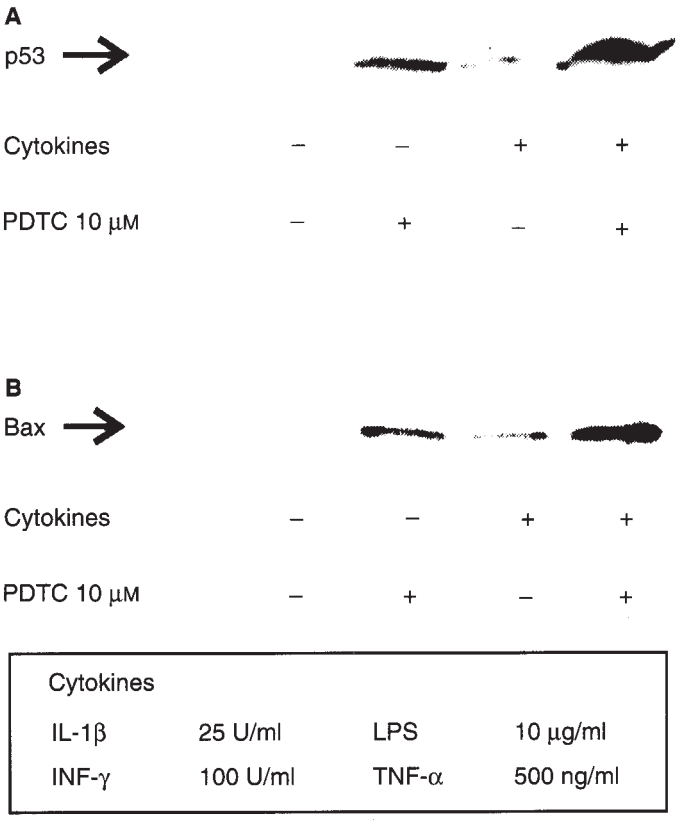

Fig. 6. Pyrrolidine dithiocarbamate promotes p53 and Bax accumulation in cytokine-stimulated mesangial cells. Mesangial cells (5×10^6 cells/assay) were incubated without addition, a cytokines mix (25 U/ml interleukin 1β, 100 U/ml interferon γ, 10 μg/ml lipopolysaccharide, 500 ng/ml tumor necrosis factor α), 10 μM PDTC, or the combination of cytokines and PDTC. (*A*) Following a four hour exposure, p53 (53 kDa) was detected by Western blot analysis. (*B*) Bax (21 kDa) accumulation was analyzed after eight hours. Blots are representative of three similar experiments.

APPENDIX

Abbreviations are: DMNQ, 2,3-dimethoxy-1,4-naphtoquinone; GSNO, S-nitrosoglutathione; LDH, lactate dehydrogenase; MC, mesangial cells; NOS, nitric oxide synthase; PDTC, pyrrolidine dithiocarbamate; SOD, Cu/Zn superoxide dismutase.

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