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Nitrosative and oxidative stress induced heme oxygenase-1 accumulation in rat mesangial cells

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Abstract

The formation of nitric oxide ($NO^{\cdot}$) and superoxide (O_2^-) promotes rat mesangial cell death. Apoptotic death is characterized by DNA fragmentation, caspase-3 activation and concomitant poly(ADPribose) polymerase cleavage, as well as accumulation of the tumor suppressor protein p53. In close association with apoptotic parameters we noticed upregulation of heme oxygenase by the NO donor *S*-nitrosoglutathione (GSNO) and the redox cycler 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) in a time- and concentration-dependent manner. In response to the $NO^{\cdot}$ donor, heme oxygenase-1 expression was more easily obtained than initiation of apoptosis. Radical ($NO^{\cdot}/O_2^-$) cogeneration abrogated DNA fragmentation, suppressed caspase activation and lowered p53 accumulation, thereby promoting cell survival of mesangial cells. In contrast, heme oxygenase-1 expression remained elevated under conditions of GSNO/DMNQ coadministration. Conclusively, heme oxygenase-1 is a stress marker for both nitrosative and oxidative stress. Accumulation of heme oxygenase-1 is found under conditions of both, apoptotic cell death and cell survival, thereby questioning a specific cytoprotective role of heme oxygenase-1 under conditions of $NO^{\cdot}$ and/or O_2^- formation in rat mesangial cells. © 1998 Elsevier Science B.V.

Keywords: Nitric oxide ($NO^{\cdot}$); Superoxide (O_2^-); Heme oxygenase (HO); Apoptosis; Mesangial cell

1. Introduction

The functional integrity of an organism demands multiple cellular interactions which are affected in part by the regulation of cell proliferation and programmed cell death. This is exemplified during tissue development, embryogenesis and differentiation (Ellis et al., 1991). Uncontrolled proliferation or excessive cell death is considered the basis for severe medical diseases such as cancer, Alzheimer, or glomerulonephritis. Apoptosis, a synonym for programmed cell death, is a well defined process with typical biochemical and morphological features such as cell shrinkage, chromatin condensation, active endonucleases, DNA fragmentation and the formation of apoptotic bodies (Schwartzman and Cidlowski, 1993). In contrast to necrosis, also termed accidental cell death, apoptosis is an energy-demanding process which requires an intact membrane potential.

Apoptosis is often found during inflammation, e.g. during glomerulonephritis, in close association with increased nitric oxide ($NO^{\cdot}$) production (Cattel et al., 1993; Baker et al., 1994; Narita et al., 1995). $NO^{\cdot}$ is generated by a family of $NO^{\cdot}$-synthase isoenzymes which, for simplistic reasons, are discriminated as either constitutively or inducibly expressed. All $NO^{\cdot}$-synthase isoenzymes utilize L-arginine and oxygen in the formation of citrulline and $NO^{\cdot}$ (Marletta, 1994). Once generated $NO^{\cdot}$ signals as a physiological mediator by activating soluble guanylyl cyclase, or under pathophysiological conditions by inhibition of essential proteins or by promoting DNA damage. Diverse biological functions of $NO^{\cdot}$ are determined by interactions with oxygen, superoxide, or transition metals (Stamler, 1994).

In several cell systems such as thymocytes (Fehsel et al., 1995; Sandau and Brüne, 1996), macrophages (Albina et al., 1993; Meßmer et al., 1995), pancreatic β-cells (Dypbukt et al., 1994; Kaneto et al., 1995), neurons (Dawson et al., 1993; Lipton et al., 1993), or mesangial cells

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(Mühl et al., 1996), among others, $NO^{\cdot}$ induces apoptotic cell death. As previously shown for rat mesangial cells, apoptosis is defined by increased DNA fragmentation, chromatin condensation and upregulation of apoptotic promoting proteins such as p53 and Bax (Sandau et al., 1997b). The nuclear phosphoprotein p53 is a tumor suppressor which accumulates after DNA damage and is often associated with initiation of apoptosis (Levine, 1997). Generally, the extension of cell death is based on the regulation of different apoptosis promoting proteins and survival factors, which are in part cell-specific. Heat shock proteins among others are described to be cell protective (Schlesinger, 1990). One family member of small heat shock proteins is heme oxygenase (HO) (Tenhunen et al., 1968; Shibahara et al., 1987; Mitani et al., 1989). Two isoforms have been identified, an inducible form heme oxygenase-1 (32 kDa) and a constitutive form heme oxygenase-2 (34 kDa) (Schacter, 1988). Oxidative stress is implicated a major heme oxygenase-1 inducing factor, besides cytokines, heavy metals, endotoxins and hormones (Keyse and Tyrrell, 1989; Cantoni et al., 1991; Alam and Dem, 1992; Mitani et al., 1992; Nascimento et al., 1993; Camhi et al., 1995; Tetsuka et al., 1995). Several observations led to the assumption that heme oxygenase-1 provides cellular resistance against oxidative injury although precise mechanisms remained unclear. Heme oxygenase catalyzes the rate-limiting step in heme degradation, thereby generating biliverdin and carbon monoxide (CO). Biliverdin is further reduced by biliverdin reductase to bilirubin (Tenhunen et al., 1968, 1969; Kutty and Maines, 1981), which is considered to be an antioxidant (Stocker et al., 1987). CO in analogy to $NO^{\cdot}$ shares the ability to activate guanylyl cyclase, thereby generating cGMP (Brüne and Ullrich, 1987; Marks et al., 1991). Both, antioxidants and cyclic nucleotides are possible factors that confer cellular resistance.

To investigate the role of heme oxygenase-1 during apoptosis in rat mesangial cells, we initiated apoptotic cell death with $NO^{\cdot}$ donors such as *S*-nitrosoglutathione (GSNO) or superoxide (O_2^-) generating agents such as 2,3-dimethoxy-1,4-naphtoquinone (DMNQ). $NO^{\cdot}$- and O_2^--exposure led to a concentration- and time-dependent heme oxygenase-1 accumulation. Further we correlated heme oxygenase-1 upregulation with caspase activation and the occurrence of apoptosis promoting proteins such as p53. Whereas coincuba-tion of rat mesangial cells with $NO^{\cdot}$ and O_2^- in a balanced ratio produced cross-protection against the toxicity elicited by each compound individually in close association with reduced p53 accumulation and suppressed caspase activity, the expression of heme oxygenase-1 remained elevated. We suggest that heme oxygenase-1 primarily is a marker for oxidative and nitrosative stress, but its cytoprotective ability is still elusive. For protection the balance of apoptosis promoting proteins versus survival factors, other then heme oxygenase-1, determine the decision between cellular life and death.

2. Materials and methods

2.1. Chemicals

Insulin and diphenylamine were purchased from Sigma, Deisenhofen, Germany. Interleukin 1β was bought from Boehringer Mannheim, Mannheim, Germany. RPMI 1640 and medium supplements were ordered from Biochrom, Berlin, Germany. Secondary antibodies came from Promega/Serva, Heidelberg, Germany and the heme oxygenase-1 antibody from Stress Gene, Victoria, Canada. The mouse monclonal anti-poly(ADPribose) polymerase antibody (clone C-II-10) was used with the kind permission of Professor Dr. Poirier, Department of Molecular Endocrinology, Centre Hospitalier de l'Université Laval Research Center and Laval Univerity, Quebec, Canada and provided by Dr. Bürkle, German Cancer Research Center, Heidelberg, Germany. Fetal calf serum was purchased from Gibco, Berlin, Germany. DMNQ was kindly provided by Professor Dr. Nicotera, University of Konstanz, Konstanz, Germany and the monoclonal antibody Ab122 directed against p53 was from Professor Dr. Stahl, University of Saarland, Homburg, Germany. All other chemicals were of the highest grade of purity commercially available.

2.2. Culture of mesangial cells

Rat mesangial cells were cultured, cloned and characterized as described previously (Pfeilschifter and Vosbeck, 1991). The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and bovine insulin (5 μg/ml). One day before and during the experiments, controls 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 10–25 of mesangial cells were used.

2.3. GSNO synthesis

S-nitrosoglutathione was synthesized as described previously (Hart, 1985). Briefly, glutathione was dissolved in 0.625 M 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 min. After the addition of 2.5 volumes of acetone, stirring was continued for another 20 min, followed by filtration of the precipitate. GSNO was washed once with 80% acetone, two times with 100% acetone, three times with diethylether, and dried under vacuum. Freshly synthesized GSNO was characterized by UV spectroscopy.

2.4. Quantification of DNA fragmentation

DNA fragmentation was assayed as reported (McConkey et al., 1989). Briefly, following incubations, cells (2.5×10^5 cells/assay) 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 (2 mM EDTA, 0.5% Triton X-100 (v/v), 5 mM Tris–HCl, pH 8.0). After 30 min at 4°C, disintegrated cells were centrifuged ($14\,000 \times g$, 15 min) to separate intact chromatin (pellet) from DNA fragments (supernatant). Pellets were resuspended in 500 μl TE buffer and the DNA content of pellets versus supernatants were measured using the diphenylamine reagent.

2.5. Heme oxygenase-1, poly(ADPribose) polymerase and p53 quantification

Heme oxygenase-1, poly(ADPribose) polymerase and p53 were quantified by Western blot analysis. Briefly, 2×10^6 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 phenylmehtylsulfonylfluoride, pH 8.0). Lysed cells were sonicated with a Branson sonifier (10 s, duty cycle 100%, output control 1). After centrifugation ($14\,000 \times g$, 15 min) the protein content in the supernatant was analyzed. Finally, 100 μg protein was resuspended in the same volume of 2 × sample buffer (125 mM Tris/HCl, 2% sodium dodecyl sulfate, 10% glycerin, 1 mM dithiothreitol, 0.002% bromephenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5, 10 or 15% sodium dodecyl sulfate-polyacrylamide gels and blotted onto nitrocellulose sheets. Molecular weights were calibrated in proportion to molecular weight rainbow markers. Accurate protein loading is assured by protein determination of each individual sample, while correct protein transfer to nitrocellulose was routinely quantitated by ponceau SS staining. Transblots 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 heme oxygenase-1 (1:1000 in TBS/0.2% milk), poly(ADPribose) polymerase (hybridoma supernatant against poly(ADPribose) polymerase, clone C-II-10, 1:5 in TBS/0.2% milk), or p53 (hybridoma supernatant against p53; clone PAb122; 1:5 in TBS/0.2% milk) antibody was added and incubated overnight at 4°C. Nitrocellulose sheets were washed 5 times and unspecific binding was blocked as described. For protein detection, blots were incubated with goat anti-rabbit or goat anti-mouse scondary antibodies conjugated with peroxidase (1:10000 in TBS/0.2% milk) for 1 h, followed by emission chemiluminescence detection.

2.6. 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 is ensured. Otherwise representative data are shown.

3. Results

3.1. Nitric oxide and superoxide induced DNA fragmentation and heme oxygenase-1 accumulation in a time- and concentration-dependent manner

It was previously shown that radicals such as nitric oxide ($NO^{\cdot}$) or superoxide (O_2^-) induced apoptosis in rat mesangial cells (Sandau et al., 1997a). Here we focused on the ability of $NO^{\cdot}$- and O_2^--releasing compounds to upregulate heme oxygenase-1 versus their potency to promote apoptosis. As revealed by Western blot analysis (Fig. 1A) 500 μM GSNO induced heme oxygenase-1 protein accumulation in a time-dependent manner. Unstimulated cells (C) showed an insignificant level of heme oxygenase-1 which is upregulated within 60 min after the NO challenge. Expression of heme oxygenase-1 increased with time, reached a maximum after 6 h, and remained elevated for at least 24 h before the amount of protein declined (timepoint not shown).

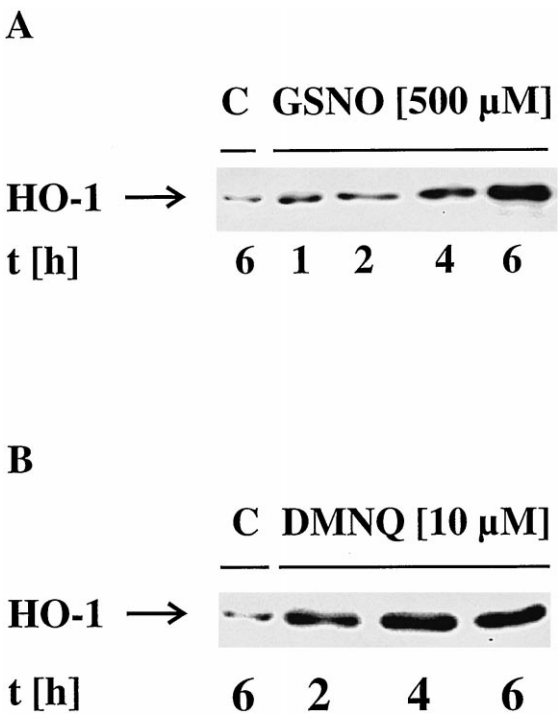

Fig. 1. $NO^{\cdot}$ and O_2^--induced heme oxygenase-1 (HO-1) accumulation. Mesangial cells (5×10^6 cells/assay) were stimulated with or without 500 μM GSNO (A) or 10 μM DMNQ (B). Incubations were terminated at times indicated followed by heme oxygenase-1 Western blot analysis as described in Section 2. Blots are representative of 3 similar experiments.

DMNQ is a redox cycler which penetrates the plasma membrane and exclusively generates O_2^- inside the cell. DMNQ at a concentration of 10 μM initiated heme oxygenase-1 accumulation within 2 h. Protein expression further increased after 4 h and remained upregulated 6 h after DMNQ treatment (Fig. 1B). Besides a time-dependent heme oxygenase-1 response, expression was concentration-dependently affected by GSNO/DMNQ as well. For these experiments heme oxygenase-1 was detected by Western blot analysis after 6 h, which was established as an optimal time point (Fig. 1).

Low GSNO concentrations (0.05 and 0.1 mM) promoted significant heme oxygenase-1 expression which further increased in a concentration-dependent manner until a maximum steady-state level was attained with 1 mM GSNO. DMNQ at 1 and 5 μM initiated heme oxygenase-1 accumulation as well but to a less extent than 10 μM of the O_2^--donor. Comparing the potency of $NO^{\cdot}$ at inducing DNA fragmentation versus heme oxygenase-1 expression, it was striking that small concentrations of GSNO (0.05 and 0.1 mM) lead to significant accumulation of heme oxygenase-1, whereas their apoptotic promoting ability was insignificant (Table 1). This relationship was reversed at higher $NO^{\cdot}$ donor concentrations. Expression of heme oxygenase-1 levelled out at 0.5 mM GSNO, whereas the amount of fragmented DNA still increased with higher $NO^{\cdot}$ concentrations.

For these calculations DNA fragmentation was measured with the diphenylamine assay 24 h after agonist addition, while representative heme oxygenase-1 Western blots were analyzed by video densitometry. To compare DNA fragmentation with heme oxygenase-1 expression, parameters were calculated as percent increase relative to unstimulated controls. Specifically, 10.8% DNA fragmentation of unstimulated cells served as the control value (100%) and increased fragmentation was calculated proportionally. Relative heme oxygenase-1 accumulation refers to the volume of heme oxygenase-1 bands compared to the background level of unstimulated cells. These observations corroborate heme oxygenase-1 as a stress marker which is extremely sensitive for small $NO^{\cdot}$ or O_2^- concentrations.

3.2. Heme oxygenase-1 expression in correlation to caspase activation and p53 accumulation

Mesangial cells incubated with either $NO^{\cdot}$ or O_2^- produced apoptotic cell death. Cogeneration of both radicals in a balanced way was cross-protective and only a surplus of either $NO^{\cdot}$ or O_2^- produced mesangial cell death (Sandau et al., 1997a). Radical cogeneration was simulated by the addition of exogenous $NO^{\cdot}$- and O_2^--releasing compounds. Therefore we applied DMNQ and GSNO as $NO^{\cdot}/O_2^-$-donors in combination and followed the activation of caspases as a specific apoptotic response. Caspases cleave various substrates after an aspartate residue (Alnemri et al., 1996), which is best exemplified by degradation of poly(ADPribose) polymerase by caspase 3, commonly known as CPP32 (Orth et al., 1996). As seen in Fig. 3 poly(ADPribose) polymerase is cleaved in rat mesangial cells after $NO^{\cdot}$ or O_2^- challenge.

Stimulation of mesangial cells with 500 μM GSNO or 1 μM DMNQ led to the cleavage of the 116 kDa holoprotein with the occurrence of the 85 and 31 kDa cleavage products after an 8 h incubation period. The poly(ADPribose) polymerase antibody recognized the 116 kDa protein and the 85 kDa fragment only. Variations in the amount of holoenzyme picked up by the antibody may arise by the tight chromatin-poly(ADPribose) polymerase association in the nucleus. This consideration does not apply to cleaved protein fragments. Costimulation with 500 μM GSNO and 1 μM DMNQ for 8 h suppressed poly(ADPribose) polymerase cleavage completely. This is in line with downregulation of other apoptotic parameters such as DNA fragmentation. Specifically, unstimulated cells revealed 5 ± 2% DNA fragmentation which increased to 21 ± 3% after the addition of 500 μM GSNO, while 1 μM DMNQ evoked 29 ± 4% DNA cleavage. Costimulation with GSNO/DMNQ reduced DNA fragmentation to 15 ± 7% ($p < 0.04$ versus DMNQ, $n \geq 4$). To further correlate apoptotic parameters such as p53 accumulation with heme oxygenase-1 expression we treated mesangial cells with GSNO and/or DMNQ for 6 h before proteins were detected by Western blot analysis.

p53 accumulated in response to DMNQ as well as

Table 1
Correlation of $NO^{\cdot}$-induced DNA fragmentation and heme oxygenase-1 accumulation

	GSNO (mM)				
	0	0.05	0.1	0.5	1
% DNA fragmentation (24 h)	11 ± 2	11 ± 1	12 ± 1	$20 \pm 2^*$	$28 \pm 2^*$
DNA fragmentation (24 h) % increase versus control	0	0	9	82	154
Heme oxygenase-1 % increase versus control	0	71	111	119	119

Mesangial cells (2.5×10^5 cells/DNA fragmentation and 5×10^6 cells/Western blot) were stimulated with vehicle (control) or different GSNO concentrations. DNA fragmentation was quantitated after 24 h using the diphenylamine assay described in Section 2. Data are mean values $\pm$ S.E.M. of at least five separate experiments, $^*p < 0.001$ versus control. Unstimulated cells served as controls (100%) and increased fragmentation was calculated proportionally. Heme oxygenase-1 accumulation was detected by Western blot analysis 6 h after GSNO addition. Details are described in Section 2. Heme oxygenase-1 steady-state levels were quantitated by video densitometry of a representative Western blot. Relative heme oxygenase-1 accumulation refers to the density of heme oxygenase-1 bands compared to the background level of unstimulated cells.

GSNO (lane 2 and 4), while coadministration of GSNO and DMNQ significantly abrogated p53 upregulation (lane 3). However, the pattern of heme oxygenase-1 expression differed. Heme oxygenase-1 is upregulated in response to either DMNQ or GSNO and its expression is unaltered by radical cogeneration (lane 3). Clearly heme oxygenase-1 is a sensitive stress marker in response to O_2^-- or $NO^{\cdot}$-formation and its accumulation occurred in settings of both, cell death and survival.

4. Discussion

Heme oxygenase is the rate-limiting step in heme degradation but its upregulation by agonists such as cytokines, endotoxins, metals, or oxidative stress points at additional functions (Tenhunen et al., 1968; Keyse and Tyrrell, 1989; Cantoni et al., 1991; Alam and Dem, 1992; Mitani et al., 1992; Nascimento et al., 1993; Camhi et al., 1995; Tetsuka et al., 1995). Several studies linked heme oxygenase-1 accumulation to cytoprotection by revealing increased damage due to heme oxygenase-1 inhibition. These observations led to the interpretation that heme oxygenase-1 is a cell-protective protein. We intended to follow expression of heme oxygenase-1 in correlation with mesangial cell apoptosis especially as diverse data on heme oxygenase-1 expression in relation to renal injuries exist. Involvement of heme oxygenase-1 refers to the glycerol model of acute renal failure (Zager et al., 1995; Vogt et al., 1996), renal ischemia/reperfusion (Maines et al., 1993; Paller et al., 1993; Raju and Maines, 1996), oxidant-induced nephropathy by cisplatin and gentamicin (Agarwal et al., 1995) and $CoCl_2$ as well as $HgCl_2$ nephrotoxicity (Lin et al., 1990; Nath et al., 1996). During glomerulonephritis sustained release of $NO^{\cdot}$ and O_2^- is linked to the early phase of mesangiolysis (Cattel et al., 1993; Narita et al., 1995). Experimentally, this situation can be studied in cultured mesangial cells by addition of $NO^{\cdot}$ donors and O_2^--generating systems. As shown in Figs. 1 and 2, $NO^{\cdot}$ and O_2^- initiated heme oxygenase-1 accumulation in a time- and concentration-dependent manner. Whereas $NO^{\cdot}$ was earlier described as a heme oxygenase-1 inducer for other cell types (Maines, 1996; Takahashi et al., 1996), redox cyclers such as DMNQ were not directly discussed for oxidative stress-induced heme oxygenase-1 expression. Although DMNQ exclusively generates O_2^- we cannot exclude that heme oxygenase-1 upregulation is forced by superoxide dismutase-dependent H_2O_2 formation (Dypbukt et al., 1994). More effective responses of DMNQ at higher concentrations can be explained by the likely disproportionation of O_2^- by superoxide dismutase and further detoxification of H_2O_2 by catalase in contrast to nitrosative stress. Heme oxygenase-1 emerged as a sensitive stress marker as it accumulated with low GSNO concentrations (Table 1) but not in control experiments with reduced glutathione (GSH). In comparison, initiation

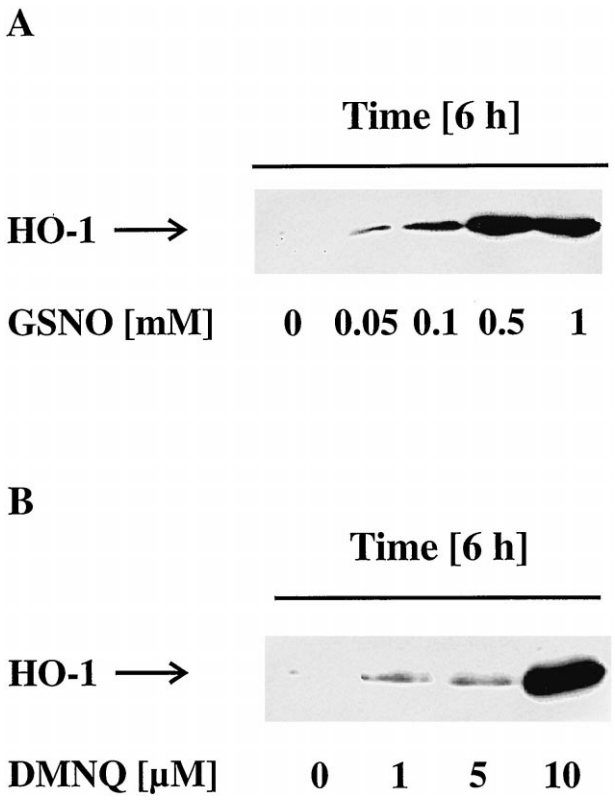

Fig. 2. Concentration-dependent heme oxygenase-1 (HO-1) upregulation in response to GSNO and DMNQ. Mesangial cells (5×10^6 cells/assay) were incubated with different concentrations of GSNO (A) and DMNQ (B). Heme oxygenase-1 expression was determined by Western blot analysis after 6 h as outlined in Section 2. Figures are representative of 3 similar blots.

of apoptotic alterations required much higher concentrations of the $NO^{\cdot}$ donor. Suggestively, nitrosative stress first initiated heme oxygenase-1 expression before triggering apoptosis. In contrast, DMNQ concentrations of 1–10 μM concurrently induced heme oxygenase-1 upregulation and DNA fragmentation. Previously we have shown that $NO^{\cdot}$- and O_2^--induced mesangial cells apoptosis is accompanied by p53 and Bax upregulation, TUNEL positive reactions, chromatin condensation and DNA fragmentation (Sandau et al., 1997a,b). These observations are now corroborated by poly(ADPribose) polymerase cleavage which is considered a prototype caspase substrate (Fig. 3).

The biological effect of $NO^{\cdot}$ is determined by the interaction with biomolecules such as oxygen, superoxide, or transition metals (Stamler, 1994). The diffusion controlled reaction between $NO^{\cdot}$ and O_2^- produces peroxynitrite ($ONOO^-$), a highly reactive oxidant (Pryor and Squadrito, 1995). Its cytotoxic function varies considerably as exemplified for cerebrocortical neurons which are highly susceptible (Bonfoco et al., 1995), whereas endothelium cells seem to be resistant (Lin et al., 1995). In our cell system, the balanced cogeneration of $NO^{\cdot}$ and O_2^- is cell protective as DNA fragmentation was decreased, p53 accumulation was abrogated, and poly(ADPribose) polymerase

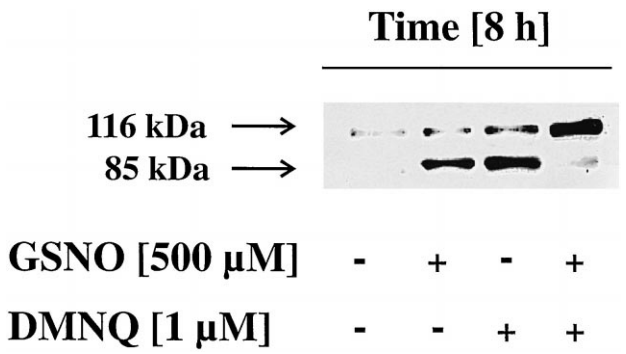

Fig. 3. Poly(ADPribose) polymerase cleavage by $NO^{\cdot}/O_2^-$ coadministration. Mesangial cells (5×10^6 cells/assay) were cultured with 500 μM GSNO, 1 μM DMNQ, the combination of both, or remained as controls. Cleavage of the 116 kDa poly(ADPribose) polymerase holoenzyme to the 85 kDa fragment was detected by Western blot analysis after 8 h as described in Section 2. Experiment is representative of 3 similar examinations.

cleavage was suppressed (Figs. 3 and 4). Wink et al. discussed $NO^{\cdot}/O_2^-$ radical cogeneration by quenching $NO^{\cdot}$-mediated nitrosative reactions, i.e. nitrosation of glutathione, by increasing O_2^- concentrations generated by the xanthine/xanthine oxidase system (Wink et al., 1997). This resembles a shift from nitrosative to oxidative stress. In mesangial cells, maximum $NO^{\cdot}/O_2^-$-mediated protection was achieved by a balanced and timely coordinated administration of radical generators, only (Sandau et al., 1997a). Logically, $NO^{\cdot}$ and O_2^- should interact to form $ONOO^-$ inside the cell as DMNQ penetrates the plasma membrane and exclusively generates O_2^- in association with NADPH-cytochrome P-450 enzymes. Consequently, nitrosative stress is decreased whereas oxidative stress increases. This leads to the intriguing question whether this kind of oxidative stress is offset by cell defense mechanisms such as reduced glutathione, superoxide dismutase, or catalase and whether it specifically induces cell protective protein expression. Costimulation with $NO^{\cdot}$ and O_2^- suppressed p53 accumulation and caspase activation but left heme oxygenase-1 expression elevated (Fig. 4). Nitrosative stress or, alternatively $NO^{\cdot}$ was quenched by O_2^-, whereas oxidative stress must have been increased by $NO^{\cdot}/O_2^-$ cogeneration compared to O_2^-, therefore leaving heme oxygenase-1 expression unaltered (Fig. 4 lane 2 and 3). Some authors correlated heme oxygenase-1 upregulation with a decrease in reduced glutathione (GSH) but this mechanism cannot apply for our situation (Lautier et al., 1992; Ewing and Maines, 1993; Rizzardini et al., 1994; Oguro et al., 1996). In mesangial cells, $NO^{\cdot}$ transiently increased rather than decreased the amount of GSH whereas O_2^- and H_2O_2 lowered GSH (unpublished data). However, heme oxygenase-1 accumulated in response to both, $NO^{\cdot}$ and O_2^-. Clearly, nitrosative and oxidative stress are two diverse processes whereas heme oxygenase-1 is a marker for both. The assumption that heme oxygenase-1 has a cytoprotective effect thereby attenuating apoptosis remain elusive as it accumulates during cell death and survival. Additional experiments with the heme oxygenase inhibitor tin protoporphyrin IX failed to modulate mesangial cell death (data not shown) which is in contrast to models of oxidative stress (H_2O_2) in vascular endothelial cells or tubular injury elicited by nephrotoxic serum (Motterlini et al., 1996; Vogt et al., 1996). In conclusion, heme oxygenase-1 upregulation as a general protective principle must be questioned under our experimental conditions, although its participation during protection in correlation with other apoptotic or survival factors cannot be excluded. In rat mesangial cells, heme oxygenase-1 is an early nitrosative and oxidative stress marker during both cell death and survival.

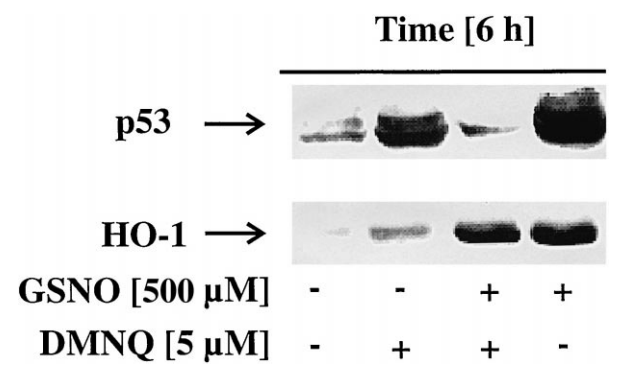

Fig. 4. p53 and heme oxygenase-1 (HO-1) steady-state level alterations by $NO^{\cdot}/O_2^-$ costimulation. Mesangial cells (5×10^6 cells/assay) were stimulated with vehicle (control), 5 μM DMNQ, 500 μM GSNO, or the combination of DMNQ/GSNO. p53 and heme oxygenase-1 were detected after 6 h by Western blot analysis as outlined in Section 2. Blots are representative of 3 similar experiments.

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