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Mesangial cells but not hepatocytes are protected against NO/O_2^- cogeneration: mechanistic considerations

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Abstract

Reactive oxygen and nitrogen species such as superoxide (O_2^-) and nitric oxide (NO) are produced under diverse conditions and provoke distinct signaling reactions. The formation of NO has been shown to induce apoptosis and/or necrosis in mesangial cells and to protect other cells such as hepatocytes. Often, NO and O_2^- are simultaneously generated, which results in their diffusion-controlled interaction and, thus, redirects the signaling properties of either NO or O_2^-. This has been proven for mesangial cells, where O_2^- formation attenuates NO-initiated apoptosis. As the mechanisms involved remained unclear, we studied the potential impact of the glutathione redox system and compared the results obtained with mesangial cells with those obtained with Hep G2 hepatocytes. In contrast to mesangial cells, Hep G2 cells appeared resistant to NO donors but displayed massive cell destruction following NO/O_2^- cogeneration. As a result, we noticed a greater increase in GSSG levels in Hep G2 cells than in mesangial cells. GSH depletion reversed the cell protection in mesangial cells and enhanced the cell damage in Hep G2 cells. NO/O_2^--mediated mesangial protection is associated with an increased glutathione reductase activity and an increase in GSH. In conclusion, NO/O_2^- sensitivity is cell type specific and is determined by the glutathione redox system.

Keywords: Mesangial cell; Hep G2 cell; Cell death; Nitric oxide (NO); Superoxide; Glutathione

1. Introduction

Nitric oxide (NO) and superoxide (O_2^-) are proinflammatory mediators produced by NO synthase, NAD(P)H-like oxidase or alternative pathways such as mitochondrial respiratory isoenzymes. NO-generating enzymes can be roughly classified as constitutively expressed vs. cytokine inducible. Inducible nitric oxide synthase (iNOS) delivers large amounts of NO over an extended period of time, often in association with cell destruction, i.e. apoptosis. Apoptotic cell death by NO occurs in different cells (Lipton et al., 1993; Dypbukt et al., 1994; Takahashi et al., 1996; Vogt et al., 1996) and is characterized by distinctive morphological and biochemical features (Schwartzman and Cidlowski, 1993). Mechanistically, the action of NO is determined by its reaction with oxygen, superoxide or transition metals, which leads to the formation of multiple reactive nitrogen species including peroxynitrite ($ONOO^-$) (Stamler, 1994). $ONOO^-$ and other reactive NO-derived species are proposed to account for NO-mediated toxicity (Liebermann et al., 1995; Blanco et al., 1995).

Mesangial cells are specialized smooth muscle cells in the glomerulus of the kidney that contain, like macrophages and astrocytes, iNOS as well as NAD(P)H-like oxidases (Pfeilschifter et al., 1993; Forrester et al., 1990; Lewis et al., 1995; Harris, 1992). Previous studies with NO donors and O_2^--generating systems have provided evidence that a balanced and simultaneous generation of both radicals is nondestructive for mesangial cells, whereas the unopposed formation of either NO or O_2^- induces apoptotic and in higher concentrations necrotic cell death (Moore et al., 1994). The initiation of apoptosis was accompanied by increased p53 and Bax expression, caspase activation and DNA fragmentation. However, these alterations were largely attenuated under conditions of NO/O_2^- coadministration. Moreover, signaling mechanisms as a consequence of the NO/O_2^- interaction circumvented signals initiating apoptosis and promoted cell protection.

In contrast, hepatocytes have been described to be NO-insensitive or to be protected from cell death by NO formation. The antiapoptotic action of NO often is explained

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by the *S*-nitrosylation of caspase-3 (Li and Billiar, 1999). If this holds true for hepatocytes, the question remains why NO initiates caspase-dependent apoptosis in cells such as mesangial cells or macrophages. It can be speculated that differences in the redox state of individual cells account for these fundamental differences and have a major impact on oxidative and/or nitrosative reactions, which in turn influence cell death.

The intracellular redox state is mainly determined by the tripeptide glutathione, the most abundant cellular nonprotein thiol. Through its nucleophilic character, glutathione serves as a reductant in the metabolism of peroxides and free radicals. Glutathione predominantly exists in its reduced form (GSH) and to a much lesser extent as mixed disulfides, thioesters or oxidized glutathione (GSSG). The level of reduced GSH is maintained by a functional glutathione reductase (GSSG reductase) or its synthesis via the rate-limiting enzyme γ-glutamylcysteine synthetase and GSH synthetase (Orrenius and Moldeus, 1984). Importantly, the redox state determined by the GSH/GSSG ratio controls cellular signaling and may affect the activity of transcription factors such as nuclear factor κB or activator protein 1 (AP-1) (Droge et al., 1994), which appear to be regulators of apoptosis (Von Knethen et al., 1999).

To unravel the molecular basis of NO/O_2^- interactions in the regulation of cell death, we used the NO donor *S*-nitrosoglutathione (GSNO) and the redox cycler 2,3-dimethoxy-1,4-naphthoquinone (DMNQ). We noticed that, in contrast to mesangial cells, hepatocytes were severely injured by apoptosis and necrosis after NO/O_2^- stimulation. Analysis of GSSG, GSNO and protein *S*-nitrosothiol formation predicted GSH to be essential for conveying protection against NO/O_2^- coadministration and GSSG recycling by activation of GSSG reductase was suggested to be significantly different between mesangial cells and hepatocytes.

2. Materials and methods

2.1. Materials

Insulin, diphenylamine, triethanolamine, glutathione, 1,3-bis[2-chloroethyl]-1-nitrosourea, 5,5′-dithio-bis(2-nitrobenzoic acid) and L-buthionine sulfoximine were purchased from Sigma, Deisenhofen, Germany. NADH, NADPH, glutathione reductase and pyruvate were bought from Roche, Mannheim, Germany. RPMI 1640 and medium supplements were ordered form Biochrom, Berlin, Germany. Fetal calf serum was purchased from Life Technologies, Berlin, Germany. DMNQ came from Calbiochem, Bad Soden, Germany, and 2-vinyl-pyridine from Aldrich, Steinheim, Germany. *S*-nitrosoglutathione was synthesized as previously described (Hart, 1987). Griess reagent system was purchased from Promega, Mannheim, Germany. All other chemicals were of the highest grade of purity and commercially available.

2.2. Culture of mesangial cells and HEP G2 cells

Rat mesangial cells were cultured, cloned and characterized as previously described (Pfeilschifter and Vosbeck, 1991). 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, cells were kept in medium without fetal calf serum. Passages 10–25 of mesangial cells were used. Hep G2 cells were kept in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Two times a week, cells were harvested by using trypsin/EDTA, divided and replated.

2.3. DNA fragmentation

DNA fragmentation was quantitated with the diphenylamine assay as previously reported (Sandau et al., 1997a). Briefly, following incubation, mesangial cells were resuspended in 250 μl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer), and incubated with an additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 4 °C. After centrifugation (10,000 × *g*, 15 min), intact chromatin (pellet) and DNA fragments (supernatant) were recovered. Pellets were resuspended in 500 μl TE buffer and again precipitated overnight with 500 μl of 10% trichloroacetic acid at 4 °C. Following centrifugation (4000 × *g*, 10 min) the supernatant was removed. After the addition of 150 μl of 5% trichloroacetic acid, samples were boiled for 15 min. DNA contents were quantitated using the diphenylamine reagent. The percentage of cleaved DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

2.4. Lactate dehydrogenase (LDH) release

The percentage of LDH released from cells, expressed as the proportion of LDH released into the medium compared to the total amount of LDH present in intact cells, is a marker of cellular necrosis and possibly derived from incomplete apoptosis due to the lack of phagocytosis. Total LDH was determined following cell lysis with 0.1% Triton X-100. LDH activity was monitored by following the oxidation of NADH as the decrease in absorbance at 334 nm. Reactions were carried out in a triethanolamine buffer (50 mM triethanolamine), pH 7.6, containing 5 mM EDTA, 127 mM pyruvate and 14 mM NADH.

2.5. Glutathione determination

Following incubations, cells were scraped off, centrifuged and washed two times with phosphate-buffered saline (PBS). Cells were lysed by the addition of 150 μl 1% sulfosalicyl acid and incubation for at least 10 min at 4 °C. After centrifugation (10,000 × *g*, 5 min), 130 μl of the supernatant was further processed for GSSG quantification.

GSH content was directly measured. For GSSG analysis, reduced glutathione was derivatized by addition of 5 μl 2-vinylpyridine for 1 h at room temperature. The amount of GSSG in 50 or 40 μl of GSH lysate was measured according to the method of Tietze (1969), which is based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) (150 μM). Intraassay signal amplification was achieved by the reduction of GSSG to GSH by glutathione reductase (8 μg/ml) and NADPH (0.2 mM) dissolved in buffer A (0.1 M KP_i, 1 mM EDTA, pH 7.5). The kinetics of 2-thionitrobenzoic acid (TNB) formation were measured at 405 nm for 2 min and GSH and GSSG concentrations were calculated using GSH or GSSG standards.

2.6. Glutathione reductase activity

After stimulation, cells were washed two times with ice-cold PBS and harvested by centrifugation (10,000 × *g*, 20 s). Cell pellets were resuspended in 400 μl 20 mM potassium phosphate buffer (pH 7), sonified and lysed for 10 min. Afterwards, samples were centrifuged (12,000 × g, 5 min) and supernatants were used for glutathione reductase activity measurements. Therefore, 300 μl supernatant was transferred to a cuvette, and then 164 μl dH_2O and 336 μl reaction buffer with the final concentrations of 100 mM potassium phosphate buffer pH 7, 1 mM EDTA, 0.2 mM NADPH and 1 mM GSSG were added. Glutathione reductase activity was monitored by following the oxidation of NADPH as the decrease in absorbance at 340 nm.

2.7. Detection of S-nitrosothiols

After stimulation, cells were washed two times with ice-cold PBS. Lysis buffer (pH 7.5) containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA and 0.5% NP-40 was added. Cells were scraped off and centrifuged (10,000 × *g*, 10 min). *S*-nitrosothiol content was assayed according to Cook et al. (1996). In brief, proteins in the lysate were precipitated by 0.5 M 5-sulfosalicylic acid. Pellets were dissolved in HEN buffer (250 mM HEPES, pH 7.7, 0.1 mM EDTA and 10 μM neocuproine) and incubated with 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylendiamine in the presence or absence of 0.18 mM $HgCl_2$. The quantity of nitrite derived from *S*-nitrosothiols was measured photometrically. The concentration of protein *S*-nitrosothiols was determined as the quantity of nitrite, concomitantly formed from NO, per milligram of protein.

2.8. GSNO assay

After stimulation, cells were washed two times with ice-cold PBS. Lysis buffer (pH 7.5) containing 50 mM Tris, 150 mM of NaCl, 5 mM of EDTA and 0.5% of NP-40 was added. Cells were scraped off and centrifuged (10,000 × *g*, 10 min). 5-sulfosalicylic acid was added to the supernatant to precipitate proteins, followed by centrifugation (10,000 × *g*, 10 min). The resulting supernatant was used for GSNO detection. This was achieved by adding 1% sulfanilamide and 0.1% of *N*-(1-naphthyl)ethylendiamine in the presence or absence of 0.18 mM $HgCl_2$. Nitrite was quantified photometrically. Differences between the amount of nitrite detected in the presence or absence of $HgCl_2$ correspond to the concentration of GSNO. Results are expressed as picomoles per milligram of protein.

2.9. Statistical analysis

Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student's *t*-test. Statistical probability (*P*) are expressed as * $P < 0.01$. The normal distribution of data was checked.

3. Results

Mesangial cells and Hep G2 cells reacted differently to reactive nitrogen and reactive oxygen species. In the following experiments, we used GSNO as a NO donor and DMNQ as a source of O_2^-. Cells were stimulated with agonists as indicated for 24 h and DNA fragmentation and LDH release were determined to distinguish between apoptosis and necrosis. In mesangial cells, GSNO dose-dependently induced DNA fragmentation and LDH release. This is exemplified for concentrations of 250 and 500 μM GSNO (Table 1). In contrast, GSNO neither promoted apoptosis nor elicited necrosis in Hep G2 cells. The redox cycler DMNQ in concentrations up to 10 μM was nontoxic in both mesangial cells and Hep G2 cells.

In the following studies, we examined the interaction between NO and O_2^- in effecting cell death. For mesangial cells, we chose 250 μM GSNO in combination with 0, 1, 5, 10 or 20 μM DMNQ and stimulated cells for 24 h (Fig. 1A). The simultaneous administration of GSNO and 5 or 10 μM DMNQ significantly attenuated NO-induced DNA fragmen-

Table 1
NO and O_2^- affects apoptosis and necrosis in mesangial cells and Hep G2 cells

		Control	GSNO (mM)		DMNQ (μM)	
			0.25	0.5	5	10
MC	Percent DNA fragmentation	9 ± 2	22 ± 3[a]	27 ± 3[a]	11 ± 2	13 ± 2
	Percent LDH release	6 ± 2	20 ± 3[a]	29 ± 3[a]	8 ± 2	5 ± 1
Hep G2	Percent DNA fragmentation	4 ± 2	8 ± 3	9 ± 3	10 ± 3	13 ± 5
	Percent LDH release	7 ± 2	10 ± 5	13 ± 0	10 ± 1	10 ± 1

Mesangial cells (MC) and Hep G2 cells were stimulated with vehicle, 0.25 vs. 0.5 mM GSNO, or 5 vs. 10 μM DMNQ for 24 h. DNA fragmentation and LDH release were analyzed as described in Section 2. Data are mean values ± S.D. of at least five separate experiments.

[a] $P < 0.01$ vs. control.

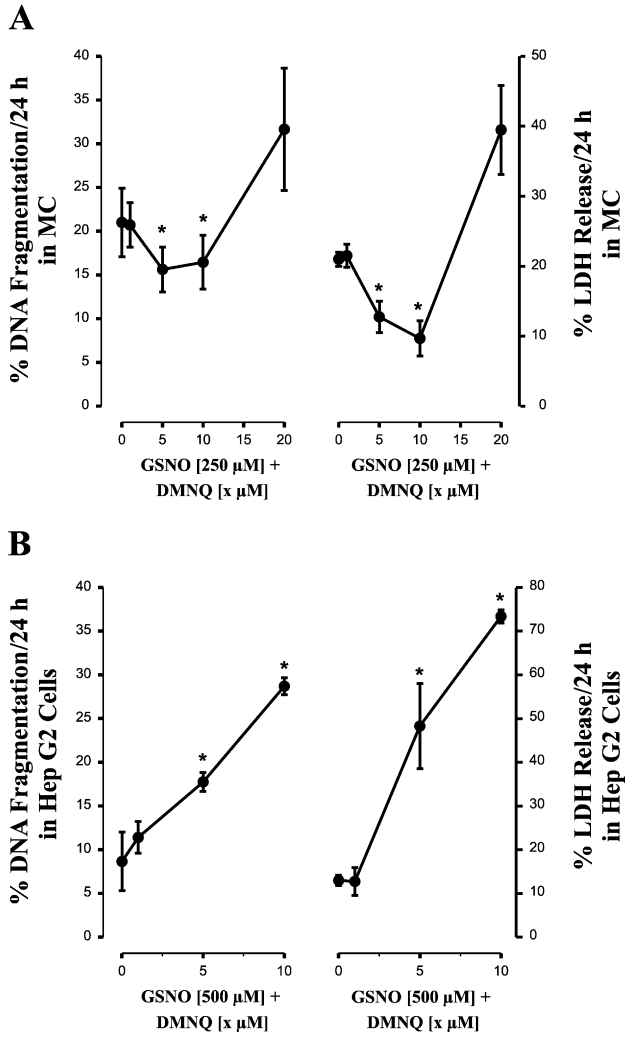

Fig. 1. Impact of NO/O_2^- cogeneration on mesangial cells and Hep G2 cells. (A) Mesangial cells were stimulated with 250 μM GSNO in combination with 0, 1, 5, 10 or 20 μM DMNQ for 24 h. DNA fragmentation and LDH release were determined as described in Section 2. (B) Hep G2 cells were incubated with 500 μM GSNO in the presence of 0, 1, 5 or 10 μM DMNQ for 24 h followed by the analysis of the DNA fragmentation and LDH release. Data are mean values ± S.D. of at least seven separate experiments. * $P < 0.01$ vs. GSNO without DMNQ.

tation and LDH release. However, with increasing concentrations of DMNQ, we noticed more signs of cell death. Previous studies confirmed that combinations of other NO donors and O_2^- generating systems, i.e. xanthine/xanthine oxidase, worked in a similar way (Sandau et al., 1997a). As a result, we used combinations of 250 μM GSNO/5 μM DMNQ or 500 μM GSNO/5 μM DMNQ, which significantly reduced apoptosis (data not shown) in all subsequent experiments. Hep G2 cells behaved differently. A combination of 500 μM GSNO and increasing concentrations of DMNQ evoked up to 30% DNA fragmentation and a strong LDH release (Fig. 1B).

To analyze the molecular mechanisms of NO/O_2^--induced cell destruction in Hep G2 cells in detail, we used the combination of 250 μM GSNO/5 μM DMNQ in subsequent experiments, as seen in Figs. 2 and 5. Under these conditions, there was less apoptosis and necrosis, thus, enabling positive or negative modulatory effects to be seen.

GSH is the major cellular defense system against oxidants such as NO, O_2^- or $ONOO^-$. Therefore, we questioned the role of GSH during NO/O_2^--induced protection/destruction by depleting intracellular GSH with L-buthionine sulfoximine. The pharmacological agent lowers intracellular GSH by blocking γ-glutamyl synthase, the key enzyme for GSH synthesis. A 24-h preincubation with 500 μM L-buthionine sulfoximine reduced glutathione levels by more than 90% (data not shown) but left the rate of spontaneous apoptosis in mesangial cells and Hep G2 cells unaltered (Fig. 2A and B). DNA fragmentation as a result of NO/O_2^- coadministration was reduced in mesangial cells compared to the response elicited by GSNO alone. Pretreatment with 500 μM L-buthionine sulfoximine for 24 h shifted the NO/O_2^- mediated protection to massive DNA fragmen-

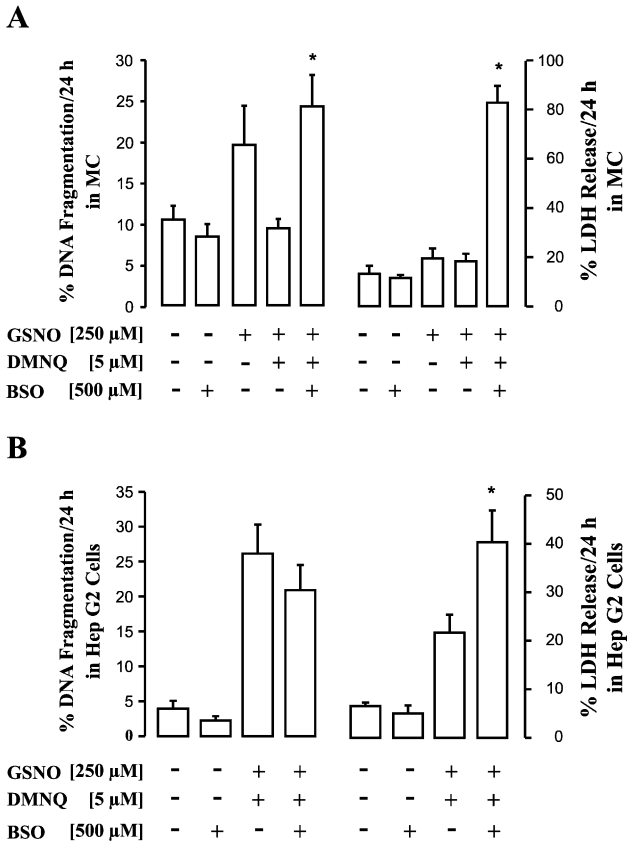

Fig. 2. Cell damage in mesangial cells and Hep G2 cells following GSH depletion. Mesangial cells (A) and Hep G2 cells (B) were cultured with vehicle, 250 μM GSNO, GSNO in combination with 5 μM DMNQ, either in the absence or in the presence of 500 μM L-buthionine sulfoximine for 24 h. L-buthionine sulfoximine was present for 24 h prior to the addition of GSNO/DMNQ. DNA fragmentation and LDH release were measured as described in Section 2. Data are mean values ± S.D. of at least five independent experiments. * $P < 0.01$ vs. GSNO/DMNQ without L-buthionine sulfoximine.

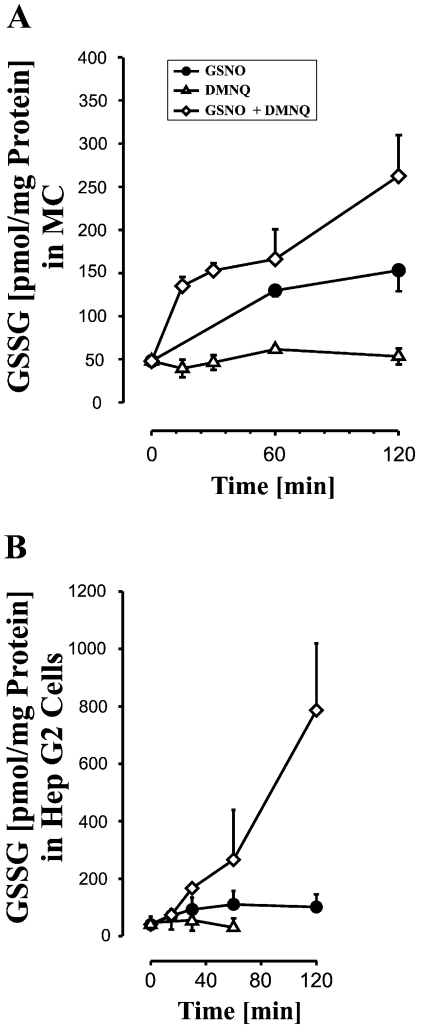

Fig. 3. GSSG formation following NO and/or O_2^- generation in mesangial cells and Hep G2 cells. Mesangial cells (A) and Hep G2 cells (B) were treated with 500 μM GSNO (●), DMNQ (△) (A: 5 μM in case of MC; B: 10 μM in case of Hep G2 cells), or the combination of both (◇). Incubations were terminated at the times indicated and GSSG was analyzed as outlined in Section 2. Data are mean values ± S.D. of at least three separate experiments. GSSG increases in response to GSNO or by GSNO/DMNQ were significantly ($P < 0.01$) different from those of controls.

tation of roughly 25% (Fig. 2A). Further analysis revealed that GSH depletion not only offset the protection against apoptosis but also promoted substantial necrosis, as determined by LDH release. In turn, necrosis under these experimental conditions was successfully reversed from 80% to control values of about 10% by the addition of 1 mM N-acetylcysteine (data not shown). In the case of Hep G2 cells, GSH depletion produced by a 24-h preincubation with L-buthionine sulfoximine insignificantly reduced DNA fragmentation evoked by 250 μM GSNO/5 μM DMNQ (Fig. 2B). This was paralleled by a shift to higher rates of LDH release. Apparently, lowering glutathione levels increased cell damage in both cell types, with a more pronounced effect in mesangial cells.

Having established the importance of intracellular reduced glutathione, especially for NO/O_2^- elicited protection in mesangial cells, we investigated the appearance of oxidized glutathione (GSSG), a general marker of oxidative stress. Under the culture conditions, mesangial cells as well as Hep G2 cells contained about 50 pmol GSSG/mg protein. DMNQ treatment resulted in very minor fluctuations in GSSG levels in mesangial cells or Hep G2 cells when measured after 15, 30, 60 or 120 min. GSNO provoked a minor GSSG increase but this effect was not reproduced with the NO donor spermine-NO (data not shown). Apparently, minor GSSG formation is the result of GSNO breakdown and is not a cell specific response to a low level of NO formation. However, a significant increase in GSSG level in response to GSNO/DMNQ costimulation was detected in mesangial cells as well as Hep G2 cells. Interestingly, Hep G2 cells produced roughly threefold more GSSG (approximately 800

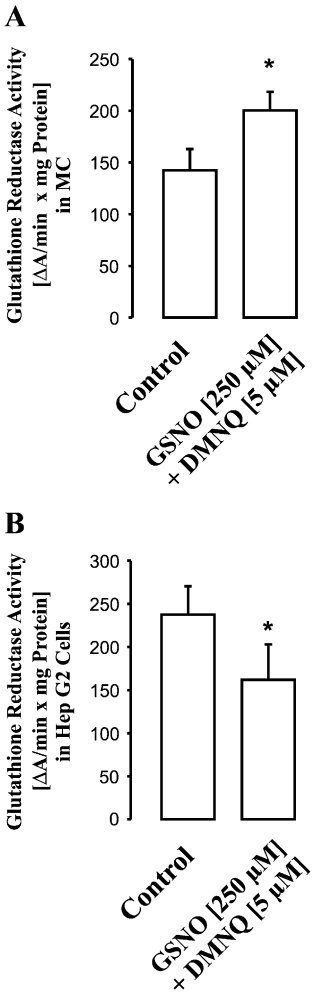

Fig. 4. Glutathione reductase activity in response to NO/O_2^- costimulation. Mesangial cells (A) and Hep G2 cells (B) were stimulated with vehicle or 250 μM GSNO in combination with 5 μM DMNQ. Glutathione reductase activity was determined 30 min after stimulation as described under Section 2. Data are mean values ± S.D. of at least seven individual experiments. * $P < 0.01$ vs. control.

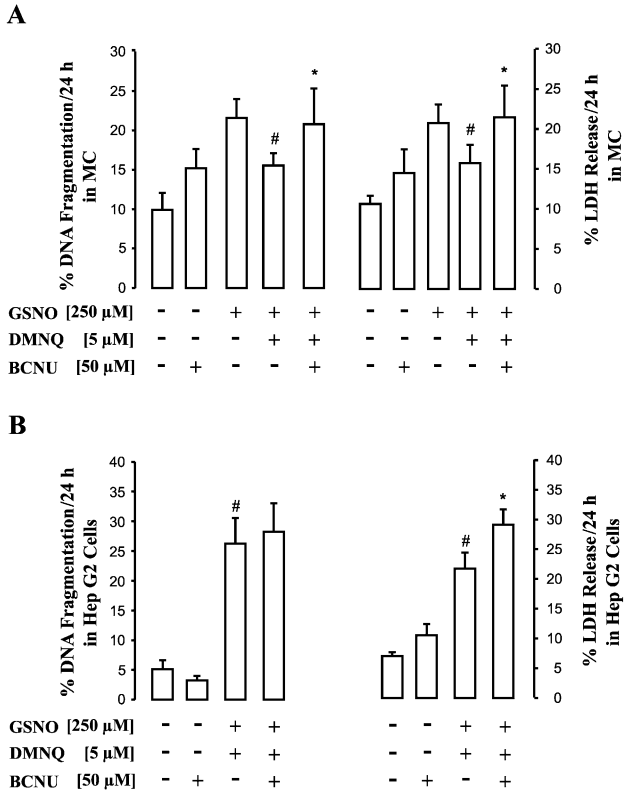

Fig. 5. Cell death under conditions of an attenuated glutathione reductase activity. Mesangial cells (A) and Hep G2 cells (B) were cultured with vehicle, 250 μM GSNO, GSNO in combination with 5 μM DMNQ, either in the absence or the presence of 50 μM BCNU for 24 h. BCNU was added for 30 min prior to the addition of GSNO/DMNQ. DNA fragmentation and LDH release were measured as described in Section 2. Data are mean values ± S.D. of at least five independent experiments. #$P<0.01$ vs. GSNO, *$P<0.01$ vs. GSNO/DMNQ without BCNU.

pmol/mg protein) after GSNO/DMNQ addition than did mesangial cells (approximately 250 pmol GSSG/mg protein) when assayed after a 120-min incubation (Fig. 3A and B).

These results raised the question whether mesangial cells recycled GSSG more efficiently than Hep G2 cells did. Prominent GSSG reduction may refill the GSH pool more quickly, thus, promoting mesangial cell protection. The key enzyme for GSSG recycling is GSSG reductase. We measured the activity of GSSG reductase in mesangial cells and Hep G2 cells under basal conditions and after 30 min in response to 250 μM GSNO/5 μM DMNQ. Mesangial cells and Hep G2 cells displayed different basal activities with a roughly twofold higher activity in Hep G2 cells than in mesangial cells. Intriguingly, GSNO/DMNQ stimulation significantly enhanced GSSG reductase activity in mesangial cells whereas GSSG reductase activity was significant lowered in Hep G2 cells (Fig. 4A and B).

To gain further insight into the functional role of GSSG reductase, we used 50 μM 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU), a selective GSSG reductase inhibitor. First, we determined GSSG reductase activity after BCNU supplementation. BCNU treatment for 30 min reduced GSSG reductase activity by 40–50% compared to unstimulated control. In both cell types GSSG reductase activity was less than 20% of control activity after a 15-h incubation with 50 μM BCNU (data not shown). In the next set of experiments, we pretreated cells for 30 min with 50 μM BCNU prior the addition of 250 μM GSNO/5 μM DMNQ. DNA fragmentation and LDH release were measured 24 h later. Similar to previous experiments, NO-induced DNA fragmentation and LDH release were lowered by costimulation with DMNQ in mesangial cells. The reduction seen for apoptotic and necrotic parameters was fully offset when GSSG reductase was blocked with 50 μM BCNU (Fig. 5A). BCNU alone marginally but not significantly increased the rate of apoptosis and necrosis. When we examined Hep G2 cells, BCNU pretreatment left DNA fragmentation evoked by GSNO/DMNQ unaltered but slightly enhanced LDH release (Fig. 5B). To conclude, attenuating GSSG reductase reversed the cell protection in mesangial cells and increased necrosis in Hep G2 cells.

An enhanced activity of GSSG reductase following GSNO/DMNQ stimulation may explain the less pronounced GSSG increase in mesangial cells than in Hep G2 cells. However, depletion of GSH or attenuation of GSSG reductase activity with BCNU elicited similar effects in Hep G2 cells and mesangial cells. This implies that an additional mechanism, besides functional GSSG reductase, contributes to the protection seen in mesangial cells under coformation of NO/O_2^-. This was apparent when we determined the level of reduced GSH. Unexpectedly, mesangial cells displayed a pronounced but transient increase in GSH level 15, 30 and 60 min after GSNO/DMNQ stimulation. This effect was not detected in Hep G2 cells (Fig. 6). The fivefold GSH increase in mesangial cells can hardly be explained by elevated GSSG reductase activity as the total GSSG reductase activity was much lower than the GSH concentrations.

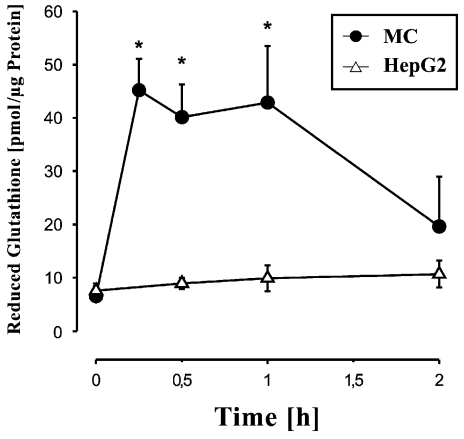

Fig. 6. Alterations in GSH after GSNO/DMNQ addition in mesangial cells and Hep G2 cells. Mesangial cells and Hep G2 cells were incubated with vehicle or 500 μM GSNO/5 μM DMNQ for the times indicated. Reduced glutathione was determined as outlined in Section 2 and expressed as picomole GSH per microgram of total protein. Data are mean values ± S.D. of at least five separate experiments. *$P<0.01$ vs. control.

We considered it of importance to study the amount of *S*-nitrosylated proteins, which reflect interactions between nitric oxide and cellular proteins. Based on our results, we suggest that GSH interacts with nitric oxide or peroxynitrite. When NO reacts with GSH, GSNO is formed. GSNO is then cleaved by GSH, giving rise to GSSG and HNO. When peroxynitrite reacts with GSH, GSSG and nitrite are formed. On the bases of these considerations we stimulated mesangial cells with 250 μM GSNO in the absence and presence of 5, 10 and 20 μM DMNQ. Hep G2 cells were treated with 500 μM GSNO in the absence and presence of 2.5, 5 and 10 μM DMNQ. Following a 24-h incubation, protein *S*-nitrosothiols and GSNO were measured as outlined in Section 2. Stimulation of mesangial cells with GSNO caused a strong increase in the amount of *S*-nitrosylated proteins (Fig. 7A),

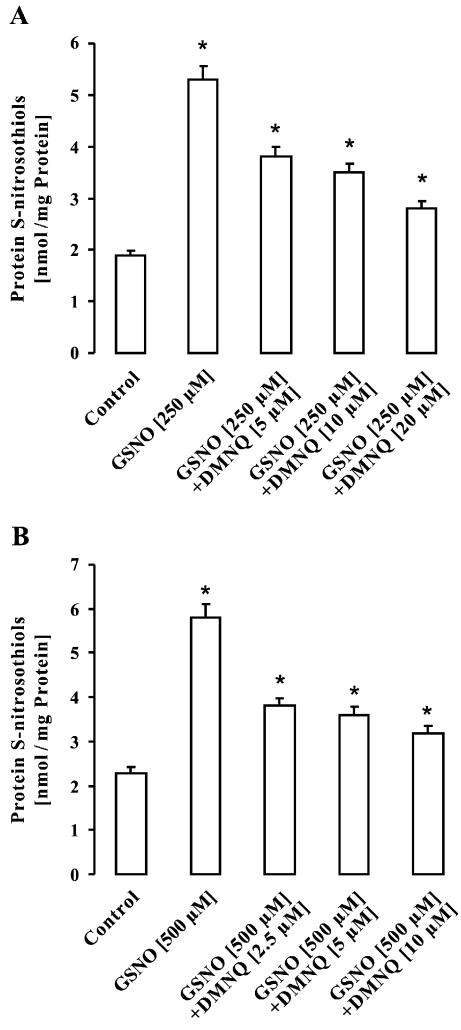

Fig. 7. Protein *S*-nitrosothiols in mesangial cells and Hep G2 cells treated with GSNO/DMNQ. Mesangial cells (A) were stimulated with 250 μM GSNO in the absence and presence of 5, 10 and 20 μM DMNQ. Hep G2 cells (B) were treated with 500 μM GSNO in the absence and presence of 2.5, 5 and 10 μM DMNQ. *S*-nitrosothiols were quantified as outlined in Section 2. Results are expressed as nanomoles of NO_2^- released from *S*-nitrosothiols per milligram of total protein. Data are mean values ± S.D. of at least five separate experiments. * $P < 0.01$ vs. control.

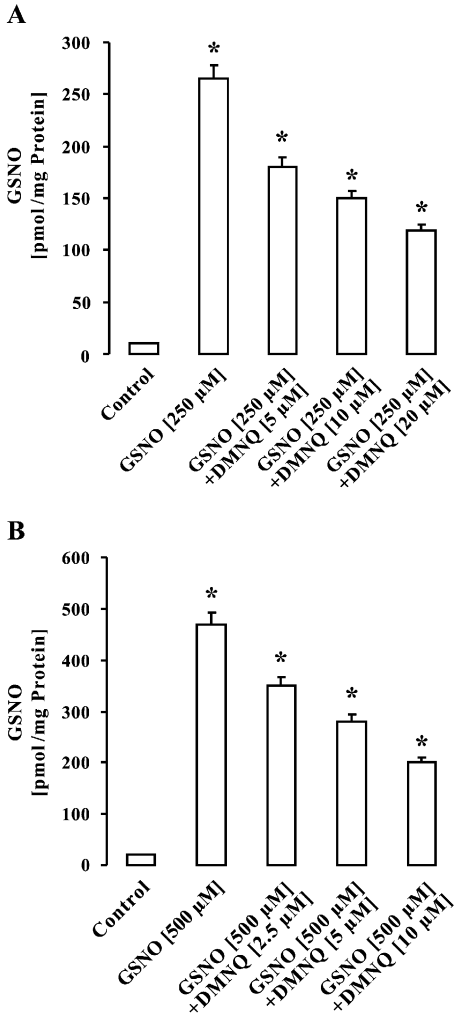

Fig. 8. Intracellular GSNO in mesangial cells and Hep G2 cells treated by GSNO/DMNQ. Mesangial cells (A) were stimulated with 250 μM GSNO in the absence and presence of 5, 10 and 20 μM DMNQ. Hep G2 cells (B) were treated with 500 μM GSNO in the absence and presence of 2.5, 5 and 10 μM DMNQ. The amount of intracellular GSNO was assayed as mentioned in Section 2 and expressed as picomole GSNO per milligram protein. Data are mean values ± S.D. of at least five separate experiments. * $P < 0.01$ vs. control.

whereas stimulation of Hep G2 cells with GSNO caused a smaller increase in the amount of *S*-nitrosylated proteins (Fig. 7B), based on the double amount of GSNO used in Hep G2 cells compared to mesangial cells (Fig. 7A and B).

Furthermore, in mesangial cells and Hep G2 cells treated with GSNO the amount of intracellular GSNO significantly increased (Fig. 8A and B). Treatment of mesangial cells and Hep G2 cells with GSNO and DMNQ decreased the quantity of protein *S*-nitrosothiols (Fig. 7A and B) as well as the formation of intracellular GSNO (Fig. 8A and B).

In conclusion, coadministration of NO and O_2^- protected mesangial cells against NO-induced cell damage. The protective mechanism required intracellular glutathione and was associated with an increased GSSG reductase activity, which may lead to efficient GSSG recycling. In addition, the

level of reduced GSH was elevated. In contrast, Hep G2 cells responded with enhanced cell damage after NO/O_2^- stimulation which was associated with increased levels of GSSG and an attenuated GSSG reductase activity. The level of GSH was not elevated.

4. Discussion

Apoptosis is an essential program for scavenging genetically modified or toxicologically injured cells in an effort to maintain genomic stability and to keep a homeostatic cell number. Its regulation is complex and dysregulation is associated with multiple diseases.

In this and previous studies, we established that NO-mediated apoptotic cell death occurs in rat mesangial cells (Nitsch et al., 1997). However, NO is an ambivalent molecule and provides cellular protection under conditions such as ischemia–reperfusion, peroxide-induced toxicity, lipid peroxidation, or myocardial injury (Nicholson and Thronberry, 1997; Messmer et al., 1994; Zhang et al., 1996; Estevez et al., 1995; Lin et al., 1995). Unfortunately, unifying concepts for a role of NO in either causing cell destruction or cell protection remain elusive. In our experimental set-up, we used two cell types, rat mesangial cells and Hep G2 cells, that are characterized as NO-sensitive vs. NO-resistant. This diversity allowed us to focus on the signaling mechanisms that are elicited in mesangial cells and Hep G2 cells as a result of radical, i.e. NO, and superoxide coformation. Radical formation was initiated by the NO donor GSNO and the O_2^- producing redox cycler DMNQ. In agreement with earlier experiments, the generation of O_2^- along with NO protected mesangial cells from entering apoptosis or necrosis, which normally occurred as a result of NO formation (Fig. 1A). As noticed previously, protection required the simultaneous generation of both NO and O_2^- (Moore et al., 1994). Under these conditions, $ONOO^-$ formation as a result of a diffusion-controlled NO/O_2^- interaction must be assumed (Stamler, 1994). Generally, $ONOO^-$ leaves as hallmarks of its action the oxidation and modification of as yet poorly characterized targets, which may account for its toxicity. In cerebrocortical cultures $ONOO^-$ induces apoptosis and necrosis (Bonfoco et al., 1995). This situation is seen in Hep G2 cells, where NO/O_2^- costimulation initiated massive cell death, whereas NO alone was not toxic (Fig. 1B). In biological systems, the action of reactive oxygen species and reactive nitrogen species such as O_2^- and NO is determined by the interaction with distinctive partners. Among possible reaction partners the interaction of NO with O_2^- appears of utmost importance because this reaction is kinetically more favorable than all other possibilities. As a consequence, the biological milieu influences the reactivity of NO and O_2^- and, as a result, the damaging potential is not immediately realized. Radical formation may account for toxicity but may be protective as well.

Cell survival under conditions of NO-derived or oxidative stress requires specific self-defense mechanisms. An important determinant of free radical-derived oxidative stress is glutathione which serves as an antioxidant to maintain the cellular redox balance. Importantly, the essential role of GSH in facilitating NO/O_2^- mediated protection was noticed in L-buthionine sulfoximine-treated cells. Similar results showing that L-buthionine sulfoximine, i.e. GSH depletion, enhances peroxynitrite-mediated cell toxicity (Fici et al., 1997; Cuzzocrea et al., 1998) have been reported. GSH depletion eliminated protection from apoptosis in association with the occurrence of massive necrosis in mesangial cells and further promoted necrotic cell death in Hep G2 cells (Fig. 2A and B).

The assumption that radicals, and especially their coformation, initiate severe oxidative stress was underscored by GSSG measurements. NO/O_2^- costimulation promoted immediate, substantial, and long-lasting GSSG accumulation in Hep G2 cells and mesangial cells (Fig. 3A and B). GSNO induced a moderate GSSG increase but far less than that induced by NO/O_2^-. In contrast, DMNQ did not cause GSH oxidation, presumably due to the continuous release of small amounts of O_2^- that are efficiently detoxified by superoxide dismutase. The shift toward massive oxidative stress as a result of NO/O_2^- cogeneration, as seen in our experiments, is in line with in vitro experiments performed by Wink et al. (1997). They observed GSSG formation in the presence of a NO donor, a O_2^- generating system, and GSH. The authors suggested that nitrosative reactions as a result of NO formation are quenched by increasing the concentration of O_2^-, thus, causing oxidative stress reactions to predominate. In mesangial cells, NO/O_2^- mediated reactions acted as a chain breaker during apoptotic signaling. We conclude that $ONOO^-$ generated by NO/O_2^- coformation may react faster with GSH than NO or O_2^-. As a result, it seems conceivable that during NO intoxication the relative rate of O_2^- formation will redirect signaling pathways towards protection as long as GSH is available. GSH may in fact detoxify $ONOO^-$, since GSH scavenges $ONOO^-$ prior to the formation of the potent oxidant HOONO (Nath et al., 1996).

The availability of GSH and/or the GSH/GSSG ratio seems to be important for the decision whether NO/O_2^- cogeneration confers cellular protection or destruction. The GSH/GSSG status is mainly regulated by enzymes responsible for glutathione synthesis and/or GSSG reduction. Our results provide evidence that the regulation of the corresponding enzymes occurs in a cell-type specific manner. NO/O_2^- coadministration enhanced the activity of the GSSG reductase in mesangial cells but decreased it in Hep G2 cells. Although a mechanistic explanation remains elusive at this time, our observation explains the more pronounced GSSG increase in Hep G2 cells compared to mesangial cells following the addition of NO/O_2^-. As assumed, attenuation of the recycling of GSSG by the GSSG reductase inhibitor BCNU promoted cell damage,

either by attenuating protection from apoptosis in mesangial cells or by promoting necrosis in Hep G2 cells (Fig. 5A and B). We suggest that NO/O_2^- cogeneration protected against NO-mediated cell damage in mesangial cells and triggered destruction in Hep G2 cells. Apparently, GSH is important for cellular protection because GSH depletion resulted in massive cell damage in both experimental systems. Differences between mesangial cells and Hep G2 cells became apparent when GSSG concentrations were measured, which were higher in Hep G2 cells than in mesangial cells. This was paralleled by antagonistic regulation of GSSG reductase, which increased in mesangial cells but decreased in Hep G2 cells. However, the activity of GSSG reductase cannot be responsible for the GSH increase seen in mesangial cells because the total amount of GSSG reductase was far below the basal level of GSH. We conclude that NO/O_2^- evoked mesangial cell protection uses other mechanisms beside GSSG reductase. An increase of GSH in mesangial cells has been described in the literature under various conditions (White et al., 1995; Imanishi et al., 1997; Sengupta and Bhattacharyya, 1996; Meredith et al., 1998). Some investigations provided evidence for enhanced GSH synthesis whereas others provided no explanation for the increase. White et al. (1995) detected a GSH increase in lung fibroblasts and pulmonary artery smooth muscle cells following treatment with NO donors. The GSH increase, seen in mesangial cells (Fig. 6), was only partly reduced by L-buthionine sulfoximine (data not shown) and, therefore, unlikely to be due to GSH synthesis only. γ-Glutamyl transferase removes the γ-glutamyl moiety of GSH, thereby regulating the degradation of GSH. As the biological half-life of glutathione is less than 1 h in the kidney, mainly because of a high activity of γ-glutamyltransferase, inhibition of glutathione degradation appears an alternative explanation that will be tested in future studies.

The generation of nitric oxide enhances *S*-nitrosothiol formation. In mesangial cells treated with 250 μM GSNO we observed a drastic increase in protein *S*-nitrosothiols. In Hep G2 cells exposed to 500 μM GSNO, protein *S*-nitrosothiols increased as well but their levels were much lower than those in mesangial cells (Fig. 7A and B). One may speculate whether protein nitrosylation contributes to cell death, considering the correlation between protein nitrosylation and susceptibility to NO in mesangial cells. In addition, the quantity of intracellular GSNO increased to a similar extent in both mesangial cells and Hep G2 when stimulated with GSNO (Fig. 8A and B). In turn, GSNO may interact with remaining GSH, promoting the formation of GSSG and HNO. The formation of GSSG may not be limited because the concentration of GSNO was low com-

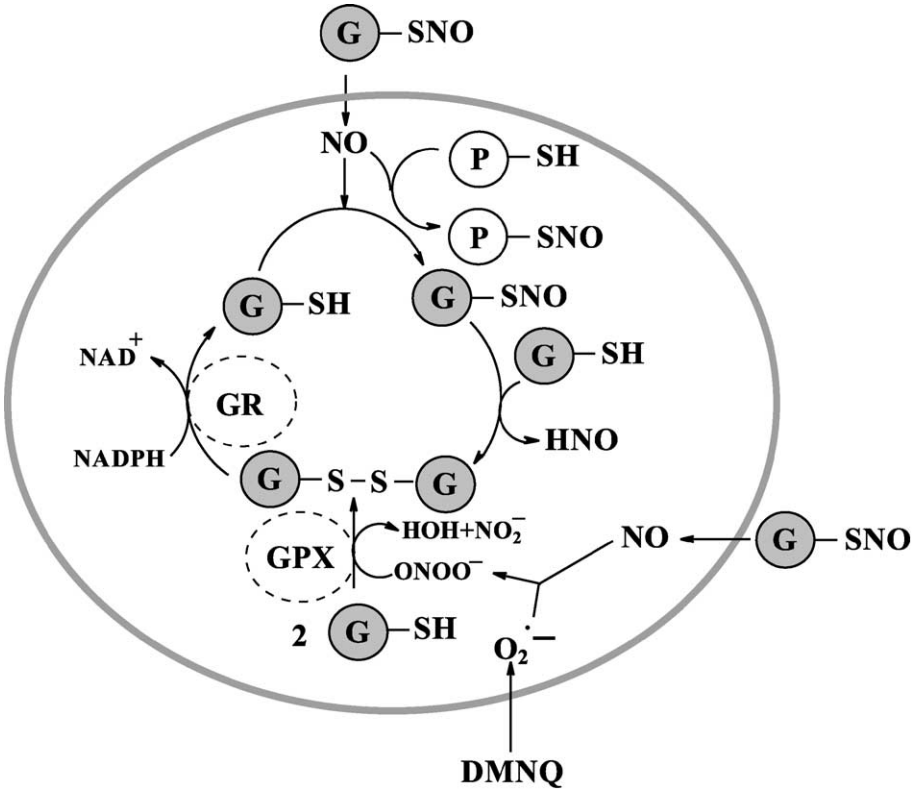

Fig. 9. Proposed pathways of GSH in facilitating protection against $NO/ONOO^-$. NO supplied by extracellular GSNO (G-SNO) interacts with proteins to generate protein *S*-nitrosothiols (P-SNO) or with GSH to form intracellular GSNO, which reacts with a second molecule of GSH to produce GSSG and HNO. GSSG is reduced by glutathione reductase (GR). Peroxynitrite produced by GSNO/DMNQ cotreatment is cleaved by glutathione peroxidase (GPX) to H_2O and nitrite. Oxidative stress is handled more efficiently in mesangial cells because of the higher amounts of GSH as well as activation of glutathione reductase. See text for details.

pared to that of GSH in both cell lines. Stimulation of mesangial cells and Hep G2 cells with a NO donor together with DMNQ decreased the amount of intracellular *S*-nitrosothiol compared to single treatment with a NO donor (Figs. 7 and 8). We assume that in mesangial cells the cleavage of peroxynitrite formed during GSNO/DMNQ cotreatment is more pronounced, based on the higher amount of GSH present, thus, the handling of NO/O_2^- coformation is more efficient in mesangial cells than in Hep G2 cells. Additionally, if O_2^- is present, *S*-nitrosothiol formation is reduced at the expense of GSSG production, which is handled more efficiently in a reducing environment, perhaps as found in mesangial cells. Basic mechanistic considerations are summarized in the scheme presented in Fig. 9.

The results presented in this work are consistent with those of studies with mesangial cells showing that endogenous cogeneration of NO and superoxide protected these cells from apoptotic death, whereas elimination of superoxide production following cytokine treatment allowed cell death in close association with endogenous NO formation (Sandau et al., 1997a,b).

We conclude that NO/O_2^- sensitivity mainly depends on the glutathione system and is regulated in a cell-specific manner. This adds to the controversy and difficulty in predicting the destructive vs. the protective signaling actions of NO.

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