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Increased oxidative stress in the RAW 264.7 macrophage cell line is partially mediated via the S-nitrosothiol-induced inhibition of glutathione reductase

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Abstract We investigated whether endogenously or exogenously produced nitric oxide (NO) can inhibit cellular glutathione reductase (GR) via the formation of S-nitrosothiols to decrease cellular glutathione (GSH) and increase oxidative stress in RAW 264.7 cells. The specificity of this inhibition was demonstrated by addition of a NO-synthase inhibitor, and met- or oxyhemoglobin. Using isolated GR we found that only certain NO donors inhibit this enzyme via S-nitrosothiol. Furthermore, we found that cellular GSH decrease is paralleled by an increase of superoxide anion production. Our results show that the GR enzyme is a potential target of S-nitrosothiols to decrease cellular GSH levels and to induce oxidative stress in macrophages.

Key words: S-nitrosothiol; Glutathione; Glutathione reductase; Nitric oxide; Oxidative stress

1. Introduction

Nitric oxide (NO), a free radical generated by at least three isoforms of NO synthase (NOS) has a remarkable range of physiological and pathophysiological effects. In some cases, such as the liver high levels of nitric oxide synthesis are cytoprotective while in other instances NO has cytostatic or cytotoxic effects exemplified by the microbicidal or tumoricidal activity of NOS2 expressing cells [1]. The mechanism(s) by which nitric oxide (NO) exerts its cytotoxic activities includes suppression of DNA synthesis, profound reduction in protein synthesis, inhibition of the mitochondrial transport chain, the inhibition of GAPDH and cytochrome P450 activity as well as apoptosis [1]. Many of these responses are associated with the modification of proteins by NO itself or by physiological NO carriers such as S-nitrosothiols (RSNO) or metal conjugates [2]. One possible mechanism by which RSNO may exert its (patho)physiological effect is the inhibition of glutathione reductase (GR) and glutathione S-transferase [3–7]. It has been well established that the intracellular flavoenzyme glutathione reductase recycles oxidized glutathione (GSSG) to maintain high levels of reduced glutathione (GSH) which is important in the detoxification processes of various cells. Increased oxidative stress or reduced cell proliferation has been observed after endogenous and exogenous applied NO and associated with diminished levels of total GSH [8–11]. In the same line of evidence, it was demonstrated that S-nitrosoglutathione and diglutathionyl-dinitroso-iron (DINC-$[GSH]_2$), two major carriers of NO, can inhibit the enzyme GR through the oxidation of the Cys-63 site of the enzyme [6]. However, all GR inhibition studies presented so far used the isolated enzyme. Therefore, the aim of the present study was to investigate whether endogenously or exogenously produced NO can inhibit cellular glutathione reductase via the formation of RSNO to decrease cellular glutathione levels and increase oxidative stress in the murine macrophage cell line RAW 264.7.

2. Materials and methods

2.1. Cell culture conditions

The mouse macrophage cell line RAW 264.7 was obtained from ATCC, Rockville, MD, USA. Cells were cultured in 60 mm Petri dishes in William's medium E, containing calf serum, penicillin/streptomycin, HEPES, insulin (GIBCO BRL, Paisley, Scotland), hydrocortisone and sodium-pyruvate (Sigma, Deisenhofen, Germany), in a humidified atmosphere of 5% CO_2 and 95% air at 37°C [12]. Endogenous NO production was generated with 200 U/ml rat recombinant interferon-γ (IFN-γ; Genzyme, Cambridge, MA, USA), and 5 μg/ml lipopolysaccharide (LPS, from *Escherichia coli* 0111:B4, Sigma), in the presence of L-arginine and in the absence of CS and hydrocortisone. Inhibition of NO-synthase activity was performed using 1 mM N^Gmonomethyl-L-arginine (NMA, Sigma). In order to scavenge NO, cell cultures were simultaneously incubated with either methemoglobin or oxyhemoglobin (Sigma). Extracellular NO production was generated using S-nitroso-*N*-acetylpenicillinamine (SNAP), S-nitroso-L-glutathione (SNOG, Alexis, Germany), S-nitroso-L-glutathione-monoethylester (SNOG-MEE, Alexis), S-nitroso-cysteine (SNOC, [13]), or diethylenetriamine/nitric oxide (DETA/NO) at various concentrations. In addition, we incubated macrophages with a reactive oxygen intermediate (ROI) generating system (1 mM hypoxanthine and 0.5 U/ml xanthine-oxidase, Boehringer, Mannheim, Germany). In order to accentuate the protective contribution of GSH in cellular damage following oxidative stress we depleted GSH from cells with 0.2 mM L-buthionine sulfoxime (BSO, Sigma) and measured the formation of NO derivatives, and reactive oxygen intermediates.

2.2. Determination of NO_2^- plus NO_3^-, S-nitrosothiols, and lactate dehydrogenase (LDH) leakage in supernatants

Cell culture supernatants were assayed for the stable end products of NO oxidation (NO_2^- plus NO_3^-) and RSNO levels using modified procedures based on the Griess reaction as recently described [14]. In order to evaluate the cellular damage following various treatments, supernatants were measured for LDH leakage using a Cobas Bio autoanalyzer (Hoffmann La Roche, Basel, Switzerland). In addition, cell viability was determined by a routine trypan blue exclusion assay.

2.3. Determination of intra- and extracellular glutathione reductase (GR) enzyme activity

GR enzyme activity was determined in cell pellets and culture supernatants using the method recently published by Becker et al. [3]. In order to verify whether the inhibition of GR by NO donors

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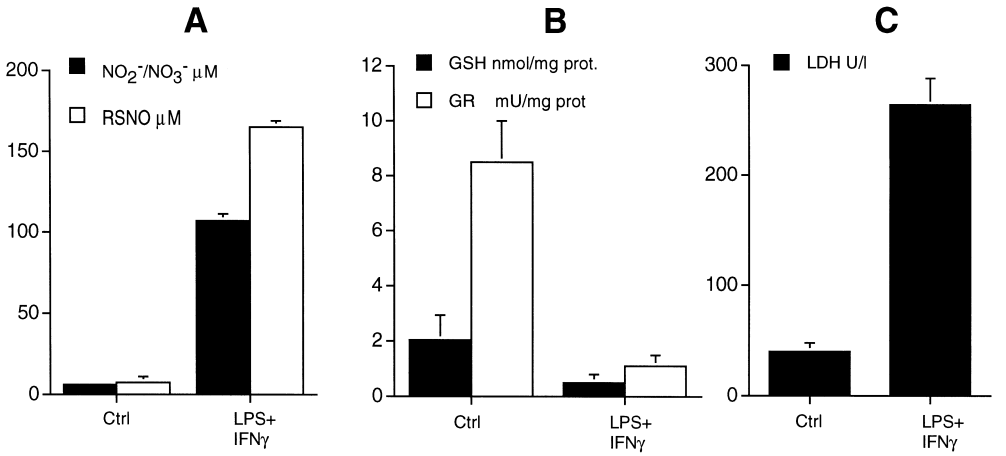

Fig. 1. Endogenous nitric oxide (NO) production (A) is directly linked to loss of the cellular glutathione levels (GSH, B), glutathione reductase activity (GR, B) and increased cell injury (C). RAW 264.7 cells were exposed either to LPS+IFN-γ or medium alone (Ctrl) for 24 h. Then, supernatants were taken for determination of nitrite/nitrate (NO_2^-/NO_3^-), S-nitrosothiols (RSNO), and lactate dehydrogenase (LDH). Cells were pelleted, washed twice and resuspended either in 3% metaphosphoric acid for GSH or in potassiumphosphate-EDTA buffer for GR assay. Results are expressed as the mean ± S.E.M. of the determination on three separate experiments.

is an oxidative process we incubated GR plus NO donors with 10.0 mM DTT as recently described [6].

2.4. Determination of total intra- and extracellular GSH levels

For evaluating total intra- and extracellular GSH levels (representing GSH+GSSG), cell pellets were resuspended in 1 ml metaphosphoric acid buffer (3%), centrifuged and aliquots of the supernatants were adjusted to pH 7.5–8.0 with K_2CO_3 (cell pellets were used for protein determination). Total cellular GSH was assayed by a modified method reported by Griffith [15] using an enzymatic recycling procedure in which reduced GSH was sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic-acid) (DTNB) to GSSG which is then reduced by NADPH back to GSH in the presence of glutathione reductase to react again with DTNB. The rate of DTNB formation is monitored at 412 nm and the glutathione present was evaluated from a standard curve. GSSG was determined by first masking reduced GSH with 2-vinylpyridine, then GSSG was measured as described above. Oxidized and reduced GSH, that may be released in the supernatant were measured in the same way. All data were normalized per mg protein.

2.5. Determination of superoxide in cell culture supernatants

The release of superoxide ($O_2^{\cdot-}$) in the medium was measured spectrophotometrically by monitoring the superoxide dismutase inhibitable reduction of 160 µM ferricytochrome c at 550 nm at 37°C. One mol $O_2^{\cdot-}$ reduces 1 mol ferricytochrome c [16].

2.6. Statistical analysis

Values are expressed as mean ± S.E.M. Significance of differences was determined by using the Anova test (Statview statistics program, Abacus Concept, Inc.). Statistical significance was established at a P value < 0.05.

3. Results

3.1. Diminished cellular GSH levels and GR enzyme activity after endogenous NO production or exogenously produced reactive oxygen intermediates

Recent evidence suggests that induction of the inducible NOS isoform has a potent effect on the cellular GSH content [16]. We found that the increase of NOx (nitrite+nitrate, and RSNO) production (Fig. 1A) in RAW cell supernatant after LPS+IFNγ incubation parallels a decrease of both the total cellular GSH (Fig. 1B) levels and the GR enzyme activity (Fig. 1B). In addition, we observed an increased LDH membrane leakage after stimulation with LPS+IFNγ (Fig. 1C). The treatment of RAW cells with LPS+IFNγ leads to cell death of 20 ± 2.4% when compared to untreated cells. In order to prove whether NO is directly linked to the cellular loss of GSH levels and a reduction in GR enzyme activity, cell cultures were simultaneously incubated with NMA, met- and oxyhemoglobin. As depicted in Fig. 2 we found that either NMA, met- or oxyhemoglobin could partially reconstitute the LPS+IFNγ-induced loss of GSH and GR enzyme activity. In addition, we measured whether GSH levels or GR activity could be determined in culture supernatants. As shown in Table 1 we found no differences in GSH levels between the supernatants of control cultures and IFNγ+LPS or IFNγ+LPS+NMA treated cell cultures. In addition, we were unable to detect any GR activity in the supernatant. Like the NO-mediated cellular loss of GSH and GR activity, we observed that ROI generating compounds can equally deplete the cellular GSH content (2.04 ± 0.9 vs. 1.1 ± 0.5), and GR enzyme activity (8.5 ± 1.5 vs. 4.1 ± 1.2).

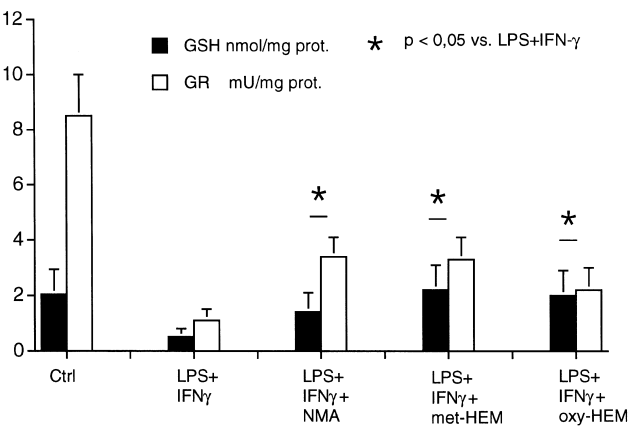

Fig. 2. Raw 264.7 cells were exposed for 24 h either to normal medium (Ctrl) or LPS/IFNγ in the presence or absence of NMA, oxyhemoglobin, and methemoglobin. Cellular GSH levels were determined as a direct result of GR inhibition caused by LPS/IFNγ. In the presence of NMA, oxyhemoglobin or methemoglobin the inhibition of GR activity, and GSH levels were partially restored. Results are expressed as mean of triplicates ± S.E.M. of four separate experiments.

Table 1
Intra- and extracellular GSH/GSSG levels, and GR activity in RAW 264.7 cell cultures

Treatment	Intracellular GSH (nmol/mg prot.)	Intracellular GSSG (nmol/mg prot.)	Extracellular GSH (nmol/mg prot.)	Extracellular GSSG (nmol/mg prot.)	Intracellular GR activity (mU/mg prot.)	Extracellular GR activity (mU/mg prot.)	Cell viability (%)
Control	3.4 ± 0.7	<[a]	1.52 ± 0.3	0.08 ± 0.05	11.8 ± 1.5	<[a]	95.6 ± 0.5
IFNγ+LPS	1.2 ± 0.2	<	1.39 ± 0.25	0.11 ± 0.1	3.7 ± 0.9	<	75.3 ± 3.6
IFNγ+LPS+NMA	2.5 ± 0.3	<	1.34 ± 0.1	0.06 ± 0.05	7.7 ± 1.8	<	82.0 ± 2.5
SNOG	0.9 ± 0.4	<	n.d.	3.1 ± 0.6	4.8 ± 1.3	<	23.3 ± 1.5
SNOC	0.4 ± 0.1	<	3.45 ± 0.5	0.3 ± 0.1	6.5 ± 1.2	<	18.6 ± 3.5
SNAP	1.2 ± 0.2	<	2.35 ± 0.2	0.6 ± 0.1	5.7 ± 1.0	<	23.0 ± 2.3

[a]Below detection limit; n.d., not done.
Cells were exposed for 24 h either to SNOG (1 mM), SNOC (1 mM), SNAP (1 mM), IFNγ+LPS, IFNγ+LPS+NMA or medium alone (Control). Then, GSH (reduced and oxidized GSH) and GR activity were determined as described. All data were normalized per mg protein. Results are expressed as mean of triplicates ± S.E.M. of three separate experiments.

3.2. Depletion of GSH leads to reduced NO levels but increased superoxide formation

Several recent publications have shown that the exposure of cells to NO leads to GSH depletion and reduced cell growth [9–11,17]. It is thought that one consequence of the GSH depletion is an increased oxidative stress. Therefore, we have investigated the formation of nitrite/nitrate, and superoxide in culture supernatants after LPS+IFNγ exposure in the presence or absence of BSO (inhibitor of the γ-glutamylcysteine synthase). As shown in Fig. 3 the cellular depletion of GSH (0.2 ± 0.1 nmol/mg prot. vs non-depletion 2.0 ± 0.4) leads to a significant ($P < 0.01$) loss of nitrite/nitrate production (Fig. 3A) while simultaneously a significant ($P < 0.01$) increase of superoxide anion (Fig. 3B) formation was observed.

3.3. Decrease of cellular GSH and GR enzyme activity by various NO donors in RAW 264 cells

Various NO donors have been reported to interfere directly or indirectly with cellular GSH [10,18]. As shown in Fig. 4 various NO donors decrease both the cellular GSH contents and the GR enzyme activity between 60 and 95% (Fig. 4A) while their parent compounds have no significant inhibitory activity (data not shown). In addition, it was observed that all NO donors lead to a sharp increase of membrane leakage measured as a LDH release in the supernatant (Fig. 4B) and cell death of about 80% (Table 1). Cell lysis can lead to the loss of cellular GSH and GR through leakage from the cells when cell viability is reduced. Therefore, we have determined whether the loss of cellular GSH and GR activity could be found in the supernatants of NO donor treated cells. As shown in Table 1 some parts of the diminished cellular GSH levels can be explained by the leakage of GSH and GSSG in the supernatant. However, the sum of intra- and extracellular GSH in NO donors treated cell cultures was not equal to the amount found in untreated control cultures. Only when cells were exposed to GSNO high amounts of GSH and GSSG were found in the supernatant. In addition, we found no extracellular GR activity in culture supernatants of NO donor treated cell cultures. The sum of intra- and extracellular GR activity was not equal to the amount found in control cultures. We concluded from these results that the loss of cellular GSH and GR through simple leakage from the cells represents only a small portion and that GR is inhibited by S-nitrosothiols.

3.4. SNOC and SNOG but not DETA/NO and SNAP inhibit the isolated GR enzyme activity

Several authors have suggested that the formation of S-nitrosothiols is necessary to inhibit the GR enzyme activity [4–7]. Since we have shown in Fig. 4 that all investigated NO donors inhibit the cellular GR enzyme activity in RAW 264.7 cells we investigated whether they would also exert an inhib-

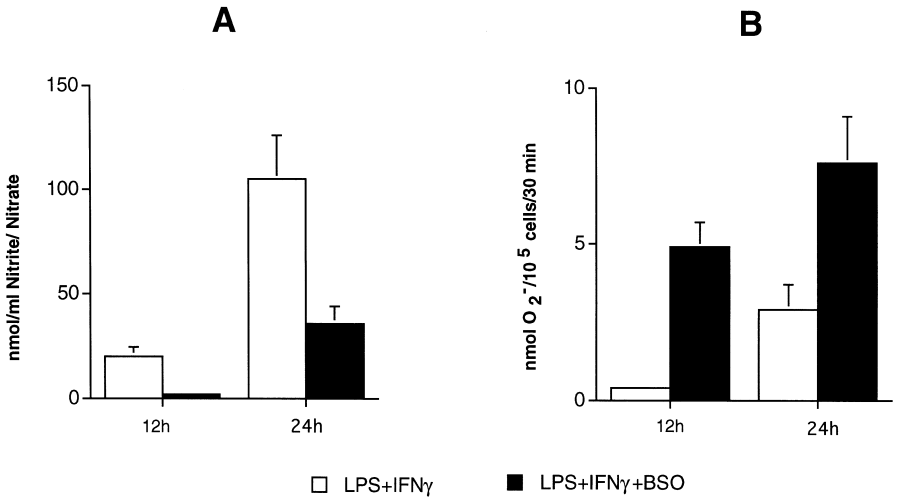

Fig. 3. Increased superoxide anion (O_2^-) formation in RAW 264.7 cells after GSH depletion. Cells were depleted of GSH with 0.2 mM L-butionine sulfoximine (BSO), then cell cultures were exposed to LPS/IFN-γ for 12 h and 24 h. Then, we measured O_2^- production (B) and NO_2^-/NO_3^- levels (A) at the same time points as described. Results are expressed as mean of triplicates ± S.E.M. of three separate experiments.

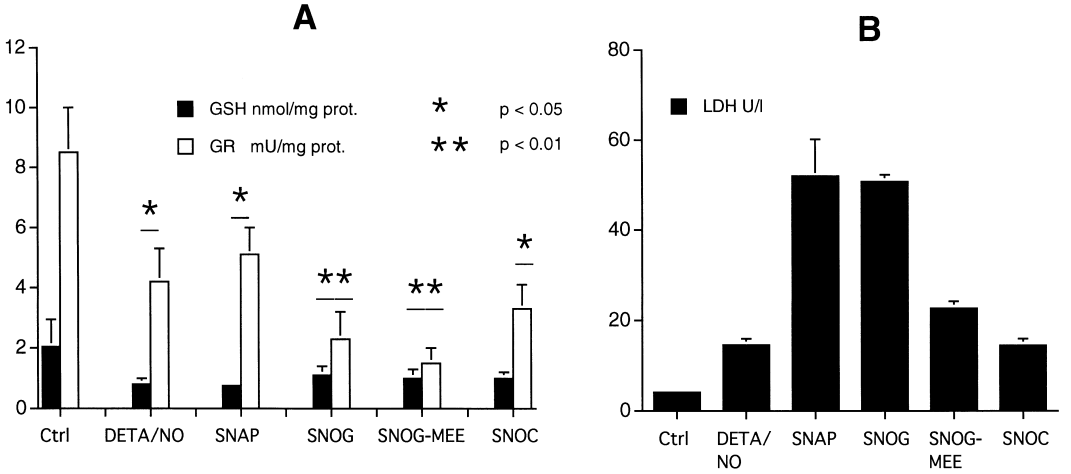

Fig. 4. RAW 264.7 cells were incubated with various NO donors at 1 mM for 24 h. All NO donors down regulated the cellular GSH contents, and GR enzyme activity (A). In addition, we observed increased cell injury in the supernatants (B) as compared with untreated control cultures (Ctrl). Results are the mean of triplicates ± S.E.M. of three separate experiments.

itory effect on the isolated enzyme. We found that DETA/NO and SNAP do not inhibit significantly ($P > 0.05$) the GR enzyme activity while the S-nitrosothiols SNOC and SNOG showed an inhibition of approximately 40%. Recently, Becker et al. have reported that the presence of albumin would be necessary in order to stabilize recombinant human GR to achieve inhibition by S-nitrosoglutathione [3]. We found that the addition of albumin to SNOG (Fig. 5A) and SNOG (Fig. 5B) reduces the inhibitory effects of both NO donors towards the enzyme activity of GR significantly ($P < 0.05$) by approximately 50% independently of the NO donor concentration. Furthermore, we investigated whether the inhibition of GR enzyme activity is an oxidative process as recently suggested [6]. We found that a simultaneous 2 h incubation of GR with SNOC and 10 mM DTT partially reversed the SNOC-induced inhibition of GR enzyme activity (17.8 ± 1.0% vs. 3.1 ± 0.8% in the presence of DTT).

4. Discussion

In recent years numerous publications have demonstrated a link between nitric oxide production, cellular damage, and the cellular glutathione content [10,11,18,19]. The consequence of a decrease in cellular GSH levels is an increase of reactive oxygen intermediates leading to oxidative stress and potent cellular damage [20,21]. The mechanisms of ROI and/or NOI-mediated cellular damage include suppression of DNA and protein synthesis, inhibition of the mitochondrial electron

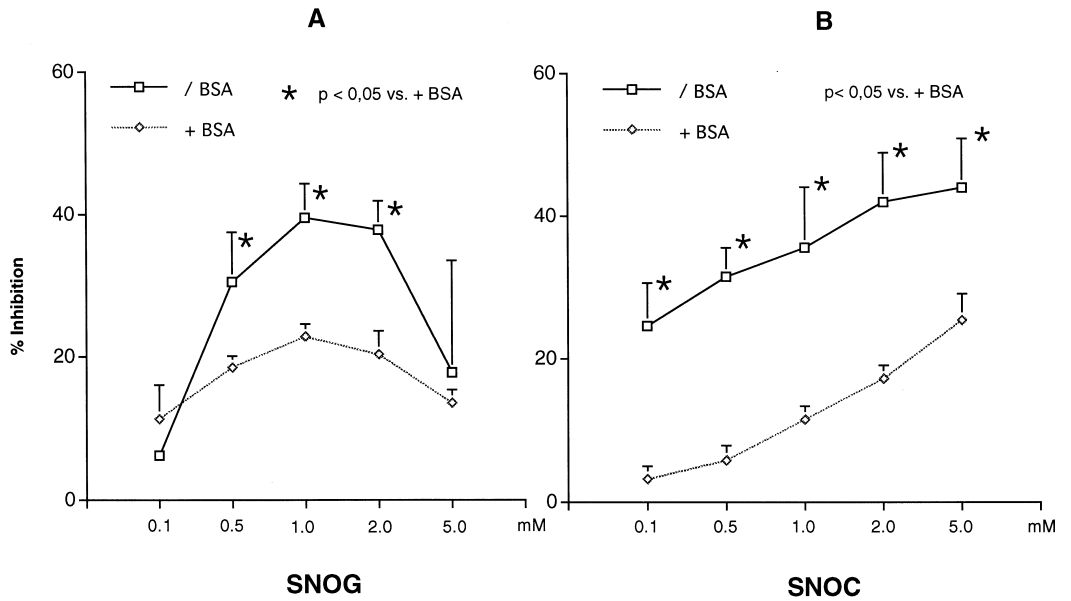

Fig. 5. Inhibition of recombinant glutathione reductase in the presence or absence of 5 mg/ml bovine serum albumin (BSA) in a glutathione reductase standard assay as described above. Recombinant glutathione reductase (2.5 U/ml) was incubated with S-nitroso-glutathione (SNOG), and S-nitroso-cysteine (SNOC) in a concentration range of 0.1 to 5 mM in the presence of 1 mM NADPH in a total incubation volume of 1 ml. Aliquots were taken after 1 h and assayed for GR enzyme activity. Both SNOG and SNOC showed a dose-dependent inhibition of the GR enzyme activity. However, addition of BSA to the incubation mixture resulted in a reduced inhibition of recombinant GR enzyme activity. Results are expressed as the mean of triplicates ± S.E.M. of three separate experiments.

transport chain and apoptosis [1,21]. The modification of proteins by radicals is associated with the formation of S-nitrosothiols or metal conjugates [2]. Recently, several investigators shed light upon possible pathophysiological mechanisms of how S-nitrosylation of proteins may interfere with glutathione metabolism [5,6]. It was observed that the formation of S-nitrosoglutathione and DINC-GSH can inhibit the enzyme glutathione reductase and glutathione S-transferase [3–7]. In the present study, we show that endogenously or exogenously produced nitric oxide can inhibit cellular glutathione reductase activity via the formation of S-nitrosothiols. Furthermore, we demonstrated that the inhibition of NOS by the L-arginine analogue NMA or by trapping nitric oxide by met- and oxyhemoglobin can partially reverse the inhibition of glutathione reductase activity and consequently the loss of cellular GSH levels. This suggests that the presence of hemoglobin in the blood may function as a control mechanism of NO overproduction in order to control cellular damage.

Furthermore, in the present study we show that a decrease in cellular GSH levels parallels the decrease of NO production. This observation is not surprising since Stuehr et al. demonstrated that GSH is a potent co-factor for NOS enzyme activity [22]. Thus, it seems conceivable to assume that NO regulates its own enzyme activity through various negative feedback mechanisms [23,24], including the depletion of cellular GSH to diminish NOS activity. The latter reaction might be of interest in the treatment of chronic inflammatory diseases such as arthritis [25] where continuous NO overproduction leads to potent cellular damage. It was also demonstrated that the inhibition of NOS by oral application of NMA improved murine autoimmune arthritis by reducing cellular damage [26]. Whether this treatment also improves the cellular GSH synthesis thus reducing oxidative stress in chronic inflammation needs to be further elucidated. Although the beneficial or detrimental role of NO in various inflammatory diseases is still a matter of debate, it seems that cellular GSH closely controls both the production of toxic ROI and the NO production. In support of this conclusion, we found that lower levels of cellular GSH lead to increased oxidative stress and subsequent increased cellular damage.

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