

# Heme oxygenase-1 induction by endogenous nitric oxide: influence of intracellular glutathione

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**Abstract** To investigate the influence of glutathione (GSH) on cellular effects of nitric oxide (NO) formation, human colon adenocarcinoma cells were transfected with a vector allowing controlled expression of inducible nitric oxide synthase (iNOS). Protein levels of oxidative stress-sensitive heme oxygenase-1 (HO-1) were analyzed in the presence or absence of GSH depletion using L-buthionine-[S,R]-sulfoximine and iNOS induction. While no effect was observed in the presence of iNOS activity alone, a synergistic effect on HO-1 expression was observed in the presence of iNOS expression and GSH depletion. This effect was prevented by addition of N-methyl-L-arginine. Therefore, targeting of endogenous NO may be modulated by intracellular GSH.

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**Key words:** Heme oxygenase-1; Nitric oxide; Glutathione; Oxidative stress

## 1. Introduction

Nitric oxide (NO) is a signaling molecule involved in a wide variety of biological processes [1]. The cellular redox environment plays an important role in NO effects. Indeed, direct effects depend on reactions between NO radical and specific biological molecules, such as metal centers or other radicals, while indirect effects will result from reactions of reactive nitrogen oxide species (RNOS), derived from NO auto-oxidation, with various biological targets including thiols [2].

The intracellular environment is generally in a reducing state because of glutathione (GSH), which is present in virtually all cells in millimolar concentration [3]. GSH depletion has often been used to simulate oxidative conditions [4,5].

In order to investigate modulations of NO effects by the redox environment, the expression of heme oxygenase-1 (HO-1), the inducible form of heme oxygenase, was determined. HO-1 is a microsomal membrane and a stress response protein that is induced by a variety of stimuli including heme, salt

of heavy metals, GSH depleters or NO donors. Most of the known HO-1 inducers stimulate the production of reactive oxygen species or lead to a low GSH level [6].

Instead of delivering NO using an exogenous donor, which may not reflect endogenous delivery, NO exposure was achieved by transfection of inducible nitric oxide synthase (iNOS) which produces a sustained amount of NO. To have a controlled production of NO, the bacterial isopropyl- $\beta$ -D-thiogalactoside (IPTG)-regulated lac system was used [7].

## 2. Materials and methods

### 2.1. DLD-1 cells transfected with a vector allowing controlled expression of iNOS

Human adenocarcinoma cells, DLD-1 (ATCC No. CCL-221), grown as described previously [8], were transfected with the plasmid placOP-iNOS. The latter allows IPTG-inducible expression of iNOS and was constructed by insertion of murine iNOS cDNA (kind gift of Dr. Lowenstein, The Johns Hopkins University School of Medicine, Baltimore, MD, USA) into placIOP [9]. Several clones were isolated by addition of hygromycin 200  $\mu$ g/ml (Roche Applied Biosciences, Rotkreuz, Switzerland). Cells were plated at 36 000/cm<sup>2</sup> and iNOS expression was induced by addition of 60  $\mu$ M IPTG (Eurogentec, Seraing, Belgium) during 72 h. The clone with the highest inducible expression was selected for investigation of HO-1 induction.

### 2.2. GSH depletion

To deplete the GSH level, cells were incubated for 48 h with 6  $\mu$ M L-buthionine-[S,R]-sulfoximine (BSO, Sigma, Buchs, Switzerland) [10]. Cellular GSH content was verified using the 5,5'-dithiobis-2-nitrobenzoic acid reduction assay [11].

### 2.3. Western blot analysis

Preparation of cellular extracts, protein determination and Western blot analysis were performed as previously described [8,12]. The equivalent of 20  $\mu$ g of total protein was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyclonal anti-iNOS (Transduction Laboratories, Lexington, KY, USA), anti-actin (Sigma), and anti-HO-1 (Stressgen Biotechnologies, Victoria, BC, Canada) were diluted at 1:1000, 1:2000 and 1:4000, respectively. Detection was achieved by enhanced chemiluminescence (Amersham Pharmacia, Dubendorf, Switzerland) and densitometry was performed on non-saturated films.

### 2.4. iNOS activity

Activity of iNOS was assessed indirectly by measuring nitrite accumulation in conditioned medium [12]. To inhibit NO production, N-methyl-L-arginine (L-NMA, Alexis Biochemicals, Lausen, Switzerland) was used at 1 mM concentration [13].

### 2.5. Real-time polymerase chain reaction (PCR)

Total RNA was isolated as previously described [12] from  $2 \times 10^7$  cells 48 h and 24 h after IPTG or BSO addition, respectively. Reverse transcription (RT) was performed on 1  $\mu$ g using AMV reverse transcriptase (Promega). The resulting cDNA was amplified by the SYBR-Green PCR assay, and products were detected on a Prism 5700 de-

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**Abbreviations:** NO, nitric oxide; RNOS, reactive nitrogen oxide species; GSH, glutathione; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; IPTG, isopropyl- $\beta$ -D-thiogalactoside; BSO, L-buthionine-[S,R]-sulfoximine; L-NMA, N-methyl-L-arginine; DETA/NO, diethylenetriamine/nitric oxide

tection system (SDS, ABI/Perkin Elmer). Histone RNA was used to standardize the total amount of cDNA. Primers for HO-1 (CTGCG-TTCCTGCTCAACATC and GGTCCTTGGTGTGCATGGGTC) and for histone (GGTAAAGCACCCAGGAAGCA and CCTCCAGTA-GAGGGCGCAC) yielded PCR products of 69 and 64 bp, respectively. Specificity of PCR was checked by analyzing the melting curve. Relative mRNA levels were determined by comparing (a) PCR cycle threshold between cDNA of HO-1 and histone ( $\Delta C$ ), and (b)  $\Delta C$  values between treated and untreated conditions ( $\Delta\Delta C$ ).

#### 2.6. Cell exposure to NO donor

In order to compare endogenous versus exogenous NO exposure, parental DLD-1 cells were treated for 9 h with 20 or 200  $\mu\text{M}$  of the NO donor diethylenetriamine/NO (DETA/NO, kind gift of Dr. Larry Keefer, NCI, Frederick, MD, USA) in the presence or absence of GSH depletion. Exposure time to DETA/NO was selected from a time course experiment (3–24 h).

#### 2.7. Statistical analysis

Statistical comparisons were carried out using a two-way analysis of variance or a Mann–Whitney multiple comparison test.

### 3. Results

#### 3.1. GSH depletion does not affect endogenous synthesis of NO

iNOS-transfected DLD-1 cells were plated in the presence or absence of IPTG and treated with BSO to deplete GSH content. iNOS protein could only be detected in the presence

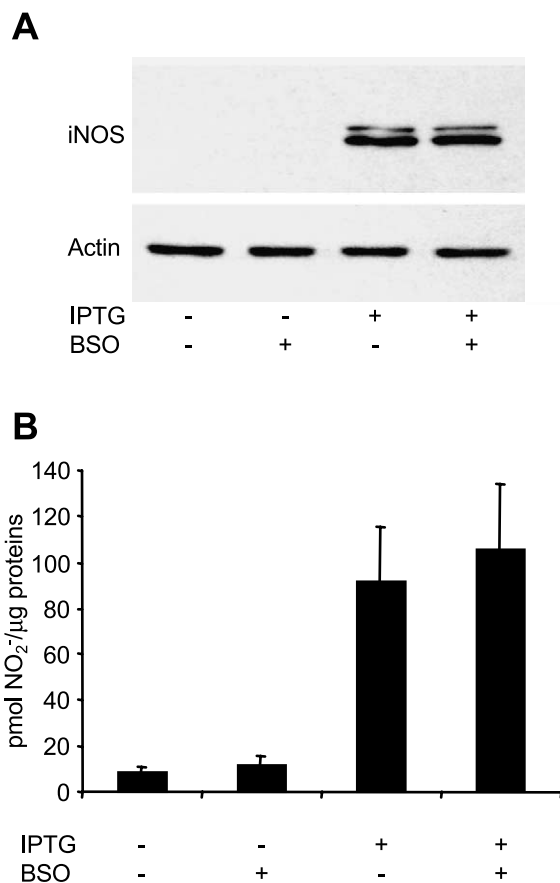


Fig. 1. Characterization of DLD-1 cells transfected with a vector allowing controlled expression of iNOS. A: Western blot analysis of cellular extracts from iNOS-transfected DLD-1 cells cultured in the presence or absence of IPTG (60  $\mu\text{M}$ , 72 h) and/or BSO (6  $\mu\text{M}$ , 48 h). B: Nitrite accumulation in the conditioned medium. Mean  $\pm$  S.E.M.,  $n = 7$ .

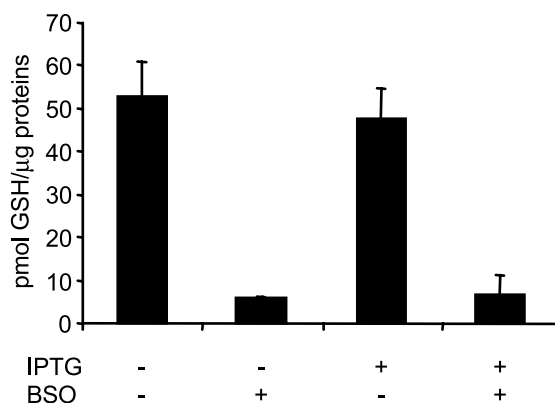


Fig. 2. Measurement of GSH depletion in iNOS-transfected DLD-1 cells exposed to 6  $\mu\text{M}$  BSO added 24 h after IPTG (60  $\mu\text{M}$ , 72 h). Mean  $\pm$  S.D.,  $n = 3$ .

of IPTG as shown by Western blot analysis (Fig. 1A). The capacity of this newly synthesized iNOS to produce NO was assessed by nitrite ( $\text{NO}_2^-$ ) accumulation in conditioned medium, which was used as a reflection of NO production. After 72 h in the presence of IPTG, a significant 11-fold increase in nitrite accumulation was observed ( $F$  ratio = 21.5;  $\text{df} = 1, 24$ ;  $P < 0.005$ ; Fig. 1B). No differences in iNOS expression (Fig. 1A) or nitrite measurement (Fig. 1B) were observed with BSO.

The total amount of GSH was  $52.7 \pm 8.3$  pmol GSH/ $\mu\text{g}$  of proteins ( $n = 3$ ) in iNOS-transfected DLD-1 cells (Fig. 2). Assuming that proteins correspond to 16% of cellular content, the intracellular GSH level was estimated approximately to be 5 mM, consistent with values available in the literature [3]. This concentration was not significantly different in parental DLD-1 cells (data not shown). Incubation for 48 h with 6  $\mu\text{M}$  BSO resulted in a significant 89% decrease of GSH content ( $F$  ratio = 178.9;  $\text{df} = 1, 8$ ;  $P < 0.005$ ; Fig. 2). IPTG alone had no effect on GSH level nor did it modify the BSO effect on this parameter (Fig. 2).

#### 3.2. Modulation of HO-1 expression by NO production and GSH depletion

HO-1 expression in iNOS-transfected DLD-1 cells was determined by Western blot analysis (Fig. 3A). Cellular extracts from DLD-1 cells exposed to cadmium chloride (50  $\mu\text{M}$ , 6 h) were used as positive control [14]. Normalized HO-1 expression showed a significant induction of  $2.0 \pm 0.3$ -fold ( $n = 9$ ) with BSO ( $F$  ratio = 60.5;  $\text{df} = 1, 32$ ;  $P < 0.005$ ; Fig. 3A,B). NO produced in the presence of IPTG had no inducing effect per se (Fig. 3A,B). However, IPTG had a synergistic effect on BSO-dependent induction of HO-1 expression ( $3.1 \pm 0.4$ -fold

Table 1  
Real-time PCR of HO-1 expression

	$\Delta C$	Fold induction
Control	$6.90 \pm 0.38$	
BSO	$6.79 \pm 0.11$	$1.1 \pm 0.1$
IPTG	$7.30 \pm 0.27$	$0.8 \pm 0.1$
IPTG+BSO	$6.32 \pm 0.08$	$1.5 \pm 0.1$

Relative mRNA levels were determined by comparing the PCR cycle threshold between cDNA of HO-1 and histone ( $\Delta C$ ), and the fold induction was calculated by  $2^{\Delta\Delta C}$ , where  $\Delta\Delta C$  is the difference between untreated and treated conditions. Mean  $\pm$  S.D.,  $n = 3$ .

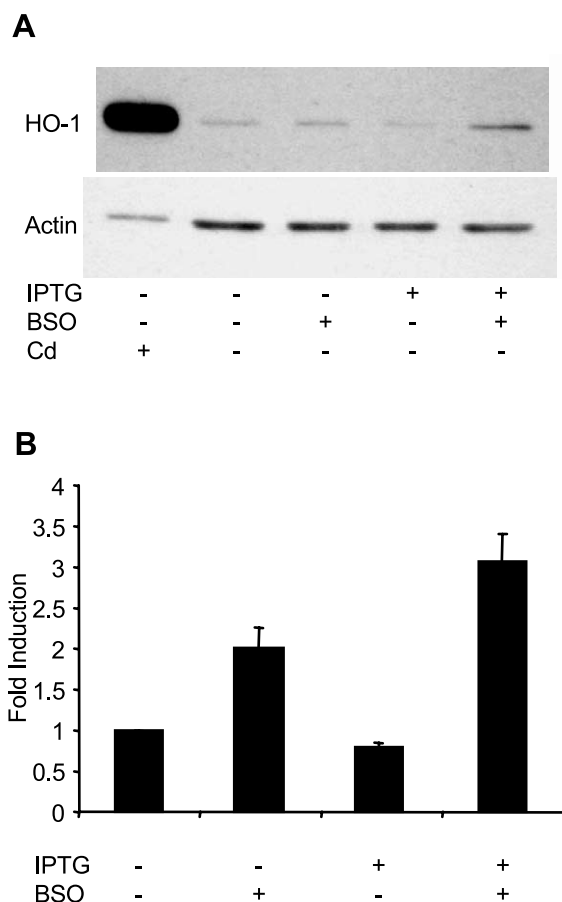


Fig. 3. Synergistic effect of GSH depletion and iNOS expression on HO-1 induction. A: Investigation of HO-1 expression by Western blot analysis of cellular extracts from iNOS-transfected DLD-1 cells cultured in the presence or absence of IPTG (60  $\mu$ M, 72 h) and/or BSO (6  $\mu$ M, 48 h). Cellular extracts from cadmium chloride (50  $\mu$ M, 6 h)-exposed DLD-1 cells were used as positive control. B: Quantification normalized by actin. Mean  $\pm$  S.E.M.,  $n=9$ .

induction in IPTG+BSO condition,  $n=9$ ;  $F$  ratio=9.0;  $df=1,32$ ;  $P<0.01$ ; Fig. 3A,B).

This synergism between IPTG and BSO treatments was buttressed by real-time RT-PCR of HO-1 expression ( $F$  ratio=9.8;  $df=1,8$ ;  $P<0.05$ ; Table 1).

To verify that the IPTG effect was due to NO production, L-NMA, an inhibitor of iNOS, was employed. Addition of 1 mM L-NMA to iNOS-transfected DLD-1 cells in the presence of IPTG led to 86% inhibition of nitrite accumulation in the conditioned medium ( $P<0.01$ , Mann–Whitney test; Fig. 4A). The same range of decrease in nitrite measurement due to L-NMA was observed in the presence of IPTG and BSO treatments (Fig. 4A). L-NMA had no effect on HO-1 expression in the presence of IPTG alone as shown by Western blot analysis (Fig. 4B,C). On the other hand, L-NMA significantly decreased HO-1 expression from  $3.9 \pm 0.7$ -fold induction ( $n=18$ ) to  $2.1 \pm 0.4$ -fold ( $n=8$ ) in the presence of IPTG and BSO treatments ( $P<0.05$ , Mann–Whitney test; Fig. 4B,C).

The specificity of NO implication was further confirmed by the absence of an IPTG effect on BSO-induced HO-1 expression in DLD-1 parental cells (data not shown).

### 3.3. Exposure to NO donor

In order to compare the effect of endogenous production of

NO in iNOS-transfected DLD-1 cells with exposure to exogenous NO, parental DLD-1 cells were treated with the NO donor DETA/NO. An accumulation of nitrite in the conditioned medium was observed only in DLD-1 cells exposed to DETA/NO (Fig. 5A). Normalized HO-1 expression determined by Western blot analysis (Fig. 5B) showed a significant induction of  $2.8 \pm 0.2$ -fold ( $n=11$ ) with BSO ( $F$  ratio=19.5;  $df=1,28$ ;  $P<0.01$ ; Fig. 5C). Only the highest DETA/NO concentration used (200  $\mu$ M) had an inducible effect on HO-1 ( $F$  ratio=7.8;  $df=1,28$ ;  $P<0.01$ ; Fig. 5C), and an

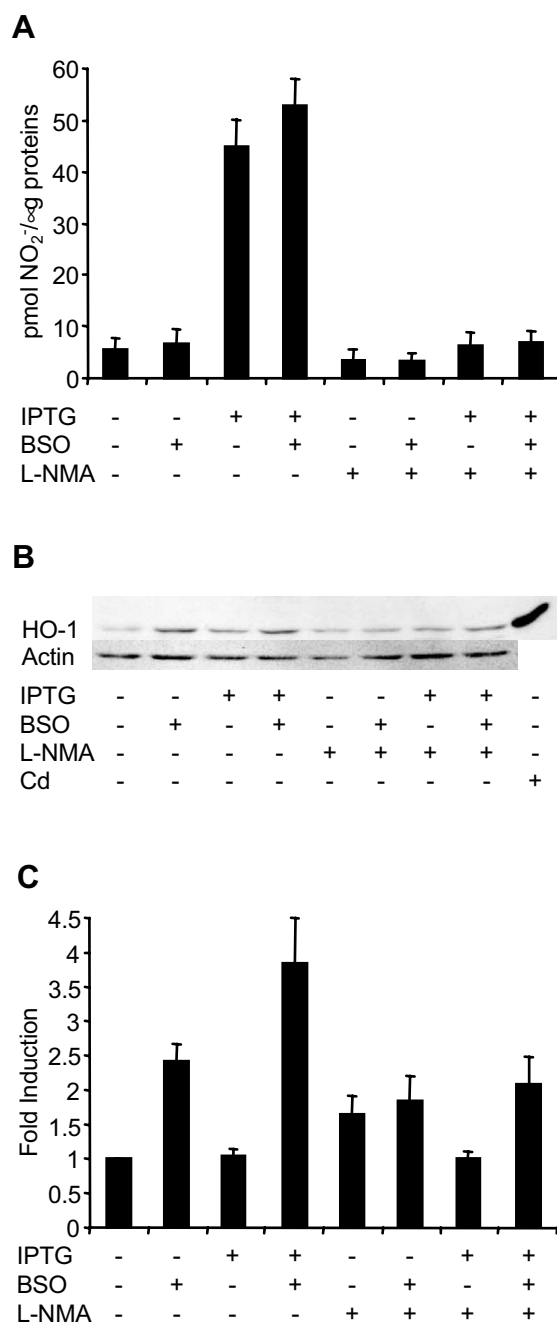


Fig. 4. Effect of iNOS inhibitor. A: Nitrite accumulation in conditioned medium of iNOS-transfected cells cultured in the presence or absence of IPTG (60  $\mu$ M, 72 h), BSO (6  $\mu$ M, 48 h) and L-NMA (1 mM, 72 h). Mean  $\pm$  S.E.M.,  $n=8$ . B: Western blot analysis of HO-1 expression in cellular extracts. C: Quantification of Western blot analysis normalized by actin. Mean  $\pm$  S.E.M.,  $n=8-18$ .

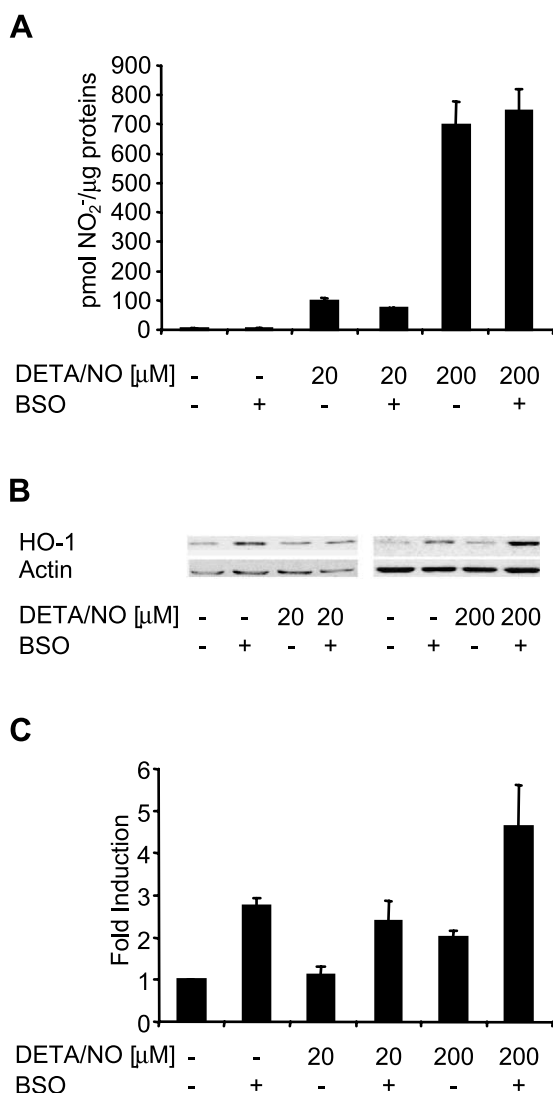


Fig. 5. Effect of exposure to NO donor. A: Nitrite accumulation in conditioned medium of DLD-1 cells treated with DETA/NO 20 or 200  $\mu$ M, 9 h and/or BSO (6  $\mu$ M, 48 h). Mean  $\pm$  S.E.M.,  $n=3-8$ . B: Western blot analysis of HO-1 expression in cellular extracts. C: Quantification of Western blot analysis normalized by actin. Mean  $\pm$  S.E.M.,  $n=3-11$ .

additive effect with BSO. DETA/NO 20  $\mu$ M had no effect per se and did not modify BSO-driven induction (Fig. 5C).

#### 4. Discussion

Investigation of HO-1 expression has often been used as the endpoint to document cellular response to changes in redox homeostasis [15,16]. The synergistic effect of GSH depletion and oxidative stress on HO-1 induction and the reversibility of these effects by *N*-acetyl-cysteine have also been documented [17], whereas the influence of the redox environment on NO produced endogenously is less well characterized. In addition, most investigations on HO-1 induction by NO have been performed with NO donors [14,18,19], which may not reflect actual regulation of HO-1 expression by NO in vivo. Indeed, experimental evidence suggests modulation of NO-dependent HO-1 induction by the in vivo environment. In fact, when HO-1 expression was observed in endothelial cells exposed

to reduced oxygen tension for 18 h, it was associated with increased levels of nitrosothiols and iNOS expression. However, only *N*-acetyl-cysteine but not iNOS inhibitors were able to completely prevent the hypoxia effect [20]. Similarly, in rat hepatocytes stimulated with cytokines and endotoxins, increased HO-1 activity was not completely prevented by NOS inhibitors [21].

In order to determine the individual contribution of endogenous NO independently of other cellular modifications, a simple system was therefore set up. DLD-1 cells were transfected with a vector allowing controlled expression of iNOS. A significant induction of HO-1 was observed with BSO, which depleted cellular GSH even in the absence of iNOS activity. This result is consistent with previous observations, where BSO was either added to fibroblasts in culture or administered intraperitoneally to rats [10,22]. In the presence of IPTG alone, iNOS was induced, but no effect on HO-1 expression was detected. This result does not seem to be related to cell type since the same observations were obtained with the ecdysone-inducible expression system transfected in human fetal kidney cells developed by Xu et al. [23] (data not shown). On the other hand, a synergistic effect on HO-1 expression was shown in the presence of IPTG and GSH depletion. Moreover, addition of L-NMA decreased nitrite accumulation in conditioned medium and was able to prevent this IPTG-dependent effect, demonstrating the involvement of NO production. Therefore, modulation of cellular GSH content was able to modify targeting of endogenous NO. This is consistent with the observation that NO contributes to HO-1 induction under hypoxia, where a decrease in GSH level was also observed [20].

Possible mechanisms of NO-dependent potentiation of GSH depletion can be inferred from recent knowledge on regulation of HO-1 expression. At the transcriptional level, HO-1 is regulated mainly by alleviation of repression mediated by the hemoprotein Bach1 [24]. Upon increase of the heme level, Bach1 is released and allows activators such as Nrf2 to bind enhancer regions [25]. A transcription-independent increase of HO-1 expression, due to stabilization of mRNA, has also been described [18]. As mentioned above, HO-1 induction has been demonstrated using NO donors and both transcription-dependent and -independent effects have been attributed to RNOS, in particular nitrosothiols [14,18,20]. In our system, endogenously produced NO is not efficiently metabolized to species able to induce HO-1, although the levels of NO production achieved are comparable to those obtained by cytokine-stimulated human cells or other recombinant systems [23,26,27]. These results are consistent with the observation that the NO donor DETA/NO 20  $\mu$ M, which releases NO levels similar to endogenous production, was not able to induce HO-1. On the other hand, exposure to 200  $\mu$ M DETA/NO led to HO-1 induction as described in other studies [18,19]. However, exogenous donor was not able to elicit potentiation of the BSO effect at these concentrations, highlighting the importance of intracellular NO delivery.

To explain the synergistic induction observed with iNOS expression and GSH depletion, different scenarios can be considered. One possibility is that low GSH content increases formation of nitrosothiols. However, no detectable increase in intracellular 4,5-diaminofluorescein-2 fluorescence, which is sensitive to nitrosating agents [28], was observed (Espey,

personal communication). In addition, different redox modifications of cysteine residues have been recently suggested to mediate a graduated response, but changes due to RNOS were less effective compared to other stresses [29], rendering this hypothesis difficult to reconcile with a synergistic effect. Alternatively, NO would potentiate the GSH depletion effect by acting via a nitrosation-independent mechanism. Indeed, HO-1 is negatively regulated by Bach1 in most tissues including intestine [24]. Therefore it is possible that NO disrupts this negative regulation by interaction with the heme prosthetic group of Bach1, thereby leading to a potentiation of GSH depletion. Interestingly, another recent study shows that while NO has no effect per se, it potentiates Angeli's salt-dependent HO-1 induction [19]. Finally, it is possible that stabilization of HO-1 mRNA, previously observed using NO donors [18], is achieved by endogenous NO only in the presence of a low GSH level.

In conclusion, this study showed that endogenous NO per se is not sufficient to induce HO-1, but potentiates the effects of GSH depletion. Although the mechanisms involved have not been identified yet, the set-up of a controlled model of endogenous NO production will make it possible to address these questions.

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