HIF-1α protein as a target for S-nitrosation

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Abstract Hypoxia-inducible factor-1α (HIF-1α) is a master regulator to sense decreased oxygen partial pressure. HIF-1α stability regulation initiates a complex biological response that allows cells to act appropriately to meet patho-physiological situations of decreased oxygen availability. Recently, nitric oxide emerged as a messenger with the ability to stabilize HIF-1α and to transactivate HIF-1 under normoxia. Considering that reactive nitrogen species are recognized for post-translation protein modifications, among others S-nitrosation, we asked whether HIF-1α is a target for S-nitrosation. In vitro NO+ donating NO donors such as GSNO and SNAP provoked massive S-nitrosation of purified HIF-1α. All 15 free thiol groups found in human HIF-1α are subjected to S-nitrosation. Thiol modification is not shared by spermine-NONOate, a NO radical donating compound. However, spermine-NONOate in the presence of O_2^-, generated by xanthine/xanthine oxidase, regained S-nitrosation, most likely via formation of a N_2O_3-like species. In vitro, S-nitrosation of HIF-1α was attenuated by the addition of GSH or ascorbate. In RCC4 and HEK293 cells GSNO or SNAP reproduced S-nitrosation of HIF-1α, however with a significantly reduced potency that amounted to modification of three to four thiols, only. Importantly, endogenous formation of NO in RCC4 cells via inducible NO synthase elicited S-nitrosation of HIF-1α that was sensitive to inhibition of inducible NO synthase activity with *N*-monomethyl-L-arginine. NO-stabilized HIF-1α was susceptible to the addition of *N*-acetyl-cysteine that destabilized HIF-1α in close correlation to the disappearance of S-nitrosated HIF-1α. In conclusion, HIF-1α is a target for S-nitrosation by exogenously and endogenously produced NO.
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Key words: Hypoxia; Nitric oxide; S-Nitrosothiol; Post-translational modification; Superoxide

1. Introduction

Hypoxia-inducible factor 1 (HIF-1) is an essential transcription factor involved in oxygen-dependent gene regulation. The HIF-1 transcriptional system senses decreased oxygen availability and transmits this signal into patho-physiological responses such as angiogenesis, erythropoiesis, vasomotor control, an altered energy metabolism, as well as cell survival decisions. This makes HIF-1 a master regulator of oxygen homeostasis [1–3].

HIF-1 is a heterodimeric transcription factor composed of α and β subunits. While HIF-1β is constitutively expressed in many cell types, HIF-1α is present at low or undetectable amounts under normal oxygen supply because the protein is rapidly degraded by the ubiquitin–proteasome system. Studies in von Hippel–Lindau protein (pVHL) defective renal cell carcinomas indicated a critical role of pVHL in HIF-1α degradation [4,5]. Recent evidence suggests that prolyl hydroxylases sense oxygen and function as putative oxygen sensors. These enzymes target highly conserved proline residues at position 564 and/or 402 of HIF-1α to hydroxylate them. Proline hydroxylation appears necessary and sufficient for binding of pVHL to HIF-1α with concomitant degradation of HIF-1α by the ubiquitin–proteasome system. Under hypoxia proline hydroxylation of HIF-1α is impaired, ubiquitination by pVHL is attenuated with the consequence of HIF-1α stabilization. Following its heterodimerization with HIF-1β the protein complex translocates to the nucleus, binds to promoter specific sites (HRE, hypoxia response elements) and drives classical hypoxia responsive gene activation [6,7].

Besides hypoxia, HIF-1 can be activated by transition metals and by reagents that chelate iron such as desferroxamine often chosen to mimic hypoxia. For these compounds including hypoxia it is rationalized that direct inhibition of proline hydroxylation accounts for HIF-1α stabilization. More recently, it appeared that HIF-1 can be activated by growth factors, cytokines, or hormones under normoxic conditions [8–10]. In addition, the modulatory role of nitric oxide (NO) and/or superoxide (ROS, reactive oxygen species) emerged. Regulation of HIF-1 activity by NO is likely to be of (patho)-physiological relevance but details at this point are not clear. Initial observations suggested that NO inhibits hypoxia-induced HIF-1α stabilization and HIF-1 transcriptional activation [11–13]. Later studies indicated that chemically diverse NO donors or endogenous NO formation under normoxic conditions provoked HIF-1α stabilization, HIF-1 DNA-binding, and activation of downstream target gene expression [14–17]. For ROS, the situation is complex as well. There is experimental evidence in support of the hypothesis that mitochondrial generation of superoxide and dismutation to H_2O_2 are required for induction of HIF-1 activity and target gene activation, thus implying ROS generation under hypoxia. An alternative model proposes that hypoxia decreases production of ROS and that mitochondrial derived oxygen species are not involved in HIF-1α stability regulation [18,19].

Protein thiol modification by NO-derived species is now appreciated as an important post-translational regulatory mechanism affecting protein function. Moreover, in many cases thiol modification is compatible with S-nitrosylation/S-nitrosation. Considering that some thiol groups found in

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HIF-1α are particularly reactive, best exemplified for cysteine-800 [20], it came to the question whether HIF-1α is a target for S-nitrosation.

Here we report that in purified human HIF-1α all thiol groups are sensitive to S-nitrosation. In cells, NO donor-evoked or inducible NO synthase-dependent stabilization of HIF-1α is closely paralleled and reduction of S-nitrosation as well as protein stabilization are achieved by *N*-acetyl-cysteine (NAC).

2. Materials and methods

2.1. Materials
RCC4 cells, constitutively expressing HIF-1α, were provided by Professor P.J. Ratcliffe, Oxford, UK. Medium and supplements were purchased from Biochrom (Berlin, Germany). Fetal calf serum was bought from Life Technologies (Berlin, Germany). The nitrite detection assay based on the Griess reaction was purchased from Promega (Mannheim, Germany). Antibodies to biotin were bought from Rockland (Gilbertsville, USA). Methyl-methane-thiosulfonate (MMTS) and *N*-(6-[biotinamido])hexyl-3′-(2′-pyridyldithio)propionamide (biotin-HPDP) were purchased from Pierce (Rockville, USA). HIF-1α antibodies were ordered from Beckton Dickinson (Heidelberg, Germany). GSNO was synthesized as previously described [21]. All other chemicals were of the highest grade of purity and commercially available.

2.2. Cell culture
RCC4 cells expressing HIF-1α were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml) [22]. HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml) [23].

2.3. Expression and purification of HIF-1α
Human HIF-1α was expressed in Sf9 insect cells by infecting them with baculoviruses expressing His-tagged HIF-1α, which was generously provided by Professor J.W. Conaway, Oklahoma City, OK, USA. Infection of the cells and protein purification have been described previously [24]. Briefly, cells were cultured at 27°C in TC100 medium with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were infected with the recombinant baculoviruses at a multiplicity of infection of 10 and collected 60 h post-infection. Cells were lysed in buffer containing 40 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, 1 mM dithiothreitol, 0.5% NP-40, 10% glycerol and protease inhibitors (Roche, Mannheim, Germany). Cell debris was removed by centrifugation at 40 000 rpm at 4°C for 1 h and the supernatant was incubated with Ni^{2+}-agarose over night at 4°C. Beads were stringently washed and protein was eluted with lysis buffer containing 300 mM imidazole. Protein content was determined by Coomassie staining.

2.4. S-Nitrosothiol detection
S-Nitrosothiols were analyzed by a colorimetric assay [25–27].

2.5. Detection of superoxide and nitric oxide
Superoxide produced by xanthine/xanthine oxidase was quantified by the method based on the reduction of ferricytochrome *c* (Fe^{3+}) to ferrocytochrome *c* (Fe^{2+}) [28]. NO production was detected using the oxyhemoglobin assay [29].

2.6. Detection of protein thiols
The amount of protein thiols was titrated by using 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) [30].

2.7. Nitrite measurements
The concentration of nitrite was determined by the Griess assay [31].

2.8. S-Nitrosothiols detection by Western analysis
Western blot analysis of protein S-nitrosothiols was performed as previously described [32]. In brief, following agonist addition, cells were washed two times with ice-cold phosphate-buffered saline (PBS). Lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.5% NP-40) was added, cells were scraped off and centrifuged ($10 000 \times g$, 10 min). Four volumes of blocking buffer (nine volumes of HEN buffer plus one volume 25% SDS, 20 mM MMTS (from a 2 M stock prepared in dimethylformamide)) were added to the one volume of the $10 000 \times g$ supernatant and incubated for 20 min at 50°C with frequent vortexing to block free SH groups. MMTS was then removed by protein precipitation with acetone. Biotin-HPDP (final concentration of 2 mM) and sodium ascorbate (1 μl to reach a final concentration of 1 mM) were added. After 1 h at 25°C, SDS–PAGE sample buffer was added and the samples were resolved by SDS–PAGE and transferred for immunoblotting. Considering that cysteine biotinylation is reversible, SDS–PAGE sample buffer was prepared without reducing agents. Furthermore, to prevent non-specific reactions of biotin-HPDP, samples were not boiled prior to electrophoresis. All steps preceding SDS–PAGE were carried out in the dark. Immunoblots were washed twice with TBS (140 mM NaCl, 50 mM Tris–HCl, pH 7.3) containing 0.1% Tween 20, blocked to avoid unspecific binding with TBS plus 5% skim milk for 1 h and incubated with FITC-labelled biotin antibody (1:500 in TBS plus 5% milk) for 1 h at room temperature. Nitrocellulose membrane was washed five times for 5 min each with TBS containing 0.1% Tween 20 prior to detection of S-nitrosated proteins. For this purpose the membrane was scanned with a FluorImager 595 (Molecular Dynamics). The amount of SNO groups in the protein was calculated using the program ImageQuant 5.0. As a standard we used bovine serum albumin (BSA), which has one reactive SH group per molecule, S-nitrosated in the presence of GSNO for 60 min.

2.9. Western blot of HIF-1α
HIF-1α was determined by Western blot analysis, as previously described [15]. Blots were stripped and reprobed with a polyclonal antibody against actin to confirm equal protein loading.

2.10. Glutathione determination
Following incubations, cells were scraped off, centrifuged, and lysed [33]. The amounts of GSH and GSSG in lysate were measured according to the method of Tietze [34], which is based on the reduction of DTNB (150 μM).

2.11. Protein quantification
The amount of protein was measured by the Bradford method [35].

2.12. 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*) expressed as *$P < 0.01$. The normal distribution of data was checked.

3. Results

3.1. S-Nitrosation of purified HIF-1α
In a first set of experiments we determined whether thiol groups of purified HIF-1α are subjected to post-translational modification by NO donors. HIF-1α protein was exposed to GSNO and SNAP at concentrations ranging from 50 to 2000 μM for 30 min. Thereafter, the content of S-nitrosothiol was determined as outlined in Section 2. HIF-1α was S-nitrosated and the amount of protein SNO groups increased proportionally to the concentration of NO donors up to roughly 1 mM (Fig. 1A). In the case of GSNO or SNAP up to 15 thiol groups of HIF-1α became S-nitrosated.

Determination of accessible free thiol groups with DTNB and sequence analysis of the human HIF-1α protein revealed that 15 cysteines are present in the protein. Therefore, we concluded that all thiol groups in HIF-1α are subjected to S-nitrosation in vitro.

In a next set of experiments we considered whether a NO radical generating donor such as spermine-NONOate would
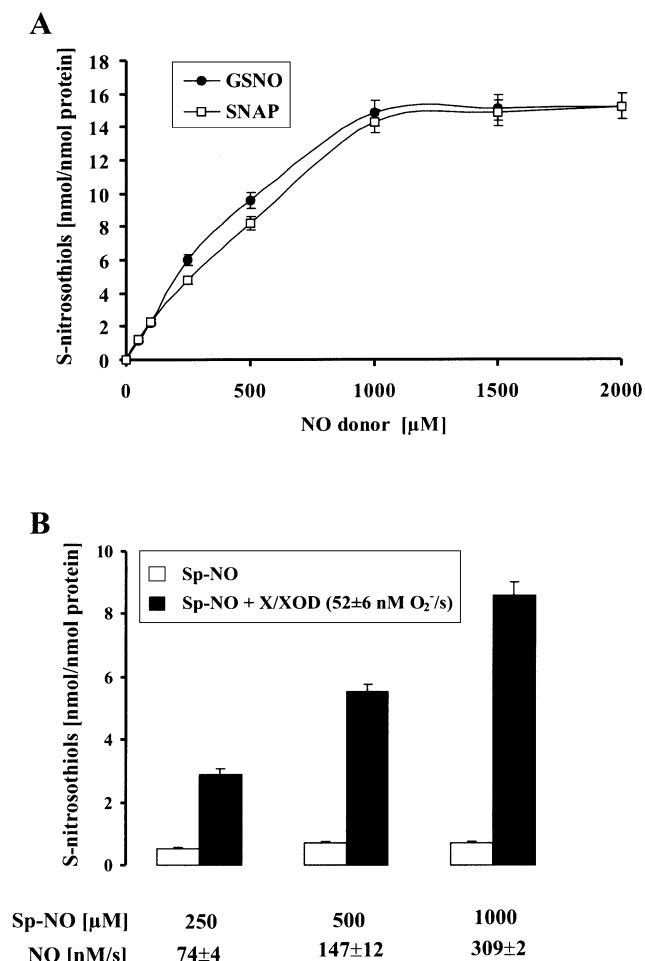
A

B

Sp-NO [μM]	250	500	1000
NO [nM/s]	74±4	147±12	309±2

Fig. 1. S-Nitrosation of HIF-1α. A: Purified HIF-1α (10 μg) was treated with GSNO and SNAP for 60 min followed by detection of S-nitrosothiols as outlined in Section 2. B: Purified HIF-1α (10 μg) was exposed to spermine-NONOate in the presence or absence of xanthine (1 mM) and xanthine oxidase (10 mU/ml) for 30 min followed by S-nitrosothiols detection. Results are expressed in nmol protein-SNO per nmol of protein. Rates of superoxide and NO production were measured as outlined in Section 2. Data are mean values ± S.D. of at least five individual experiments.

reproduce S-nitrosation. As seen in Fig. 1B, concentrations of 250–1000 μM of Sp-NO did not modify thiols of HIF-1α. However, addition of the superoxide generating systems composed of 1 mM xanthine and 10 mU/ml xanthine oxidase in combination with Sp-NO provoked substantial S-nitrosation. Rates of superoxide formation performed by xanthine/xanthine oxidase amounted to 52 ± 6 nM/s. Flax rates of NO from Sp-NO increased with the amount of NO donor employed (Fig. 1B) and raised from roughly 70 to 300 nM/s. These data suggest that a nitrosating species formed by the breakdown of either GSNO or SNAP as well as the formation of O_2^- in the presence of increasing amounts of NO provoked massive S-nitrosation of HIF-1α.

We went on to study the sensitivity of S-nitrosation and/or reversibility of that post-translational modification towards antioxidants. For this purpose we treated purified HIF-1α for 60 min with 1 mM GSNO either alone or in the presence of reduced glutathione versus sodium ascorbate (Fig. 2A). Antioxidants were present at concentrations of 0.1–1 mM.

Reduced GSH as well as ascorbate antagonized S-nitrosa-

tion of HIF-1α with the notion that ascorbate was more effective at low, i.e. 0.1 mM, concentrations. At higher doses (0.5 and 1 mM of the antioxidants) S-nitrosation was reduced to low levels of one to two NO molecules remaining bound to HIF-1α. In a modified version of the experimental set-up we provoked S-nitrosation of HIF-1α with 1 mM GSNO for 60 min followed by protein precipitation to remove GSNO (Fig. 2B). Precipitated protein was dissolved in HEN buffer, subsequently treated for 15 min with 1 mM sodium ascorbate or 1 mM GSH, followed by S-nitrosothiol determinations. In this case S-nitrosation of HIF-1α was completely reversible.

3.2. S-Nitrosation and stabilization of HIF-1α in RCC4 and HEK293 cells

Considering that purified HIF-1α is subjected to S-nitrosation we became interested to study this type of post-translational modification in cells. To achieve this, we used RCC4 cells, known to constitutively express HIF-1α due to a defective pVHL and thus impaired proteasomal degradation of the HIF-1α protein. When RCC4 cells were stimulated with GSNO or SNAP we noticed increased stabilization of HIF-1α compared to controls (Fig. 3A). However, NO donors not only achieved HIF-1α stabilization but also promoted S-nitrosation of HIF-1α as determined by Western immunofluorescence analysis.

Having demonstrated that HIF-1α is S-nitrosated by exogenous NO we wanted to know whether endogenously, i.e. inducible NO synthase, generated NO provoked these altera-
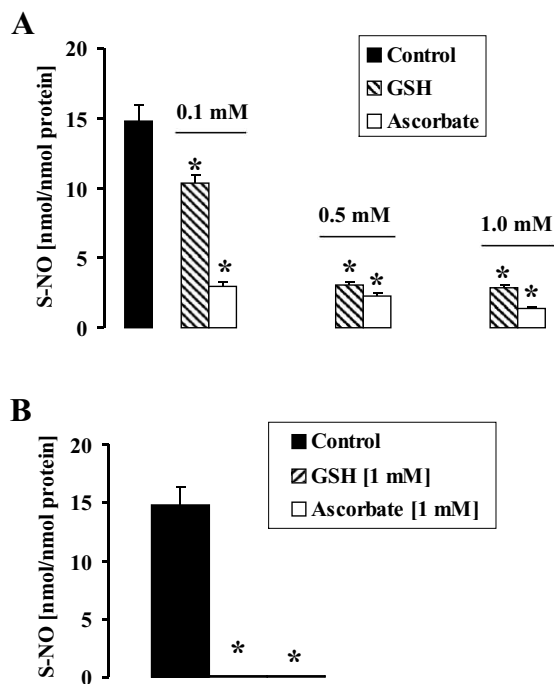
A

B

Fig. 2. S-Nitrosation of HIF-1α and antioxidants. A: HIF-1α protein (10 μg) was treated with 1 mM GSNO together with 0.1, 0.5, 1.0 mM GSH or equivalent concentrations of sodium ascorbate for 30 min followed by detection of S-nitrosothiols as described in Section 2. B: HIF-1α protein (10 μg) was treated with 1 mM GSNO for 30 min. HIF-1α was precipitated with 5-sulfosalicylic acid to remove GSNO, followed by treatment of the protein with 1 mM GSH or 1 mM sodium ascorbate for 15 min, prior to detection of the protein-SNO content. Data are mean values ± S.D. of at least five individual experiments. *$P < 0.01$ vs. control.
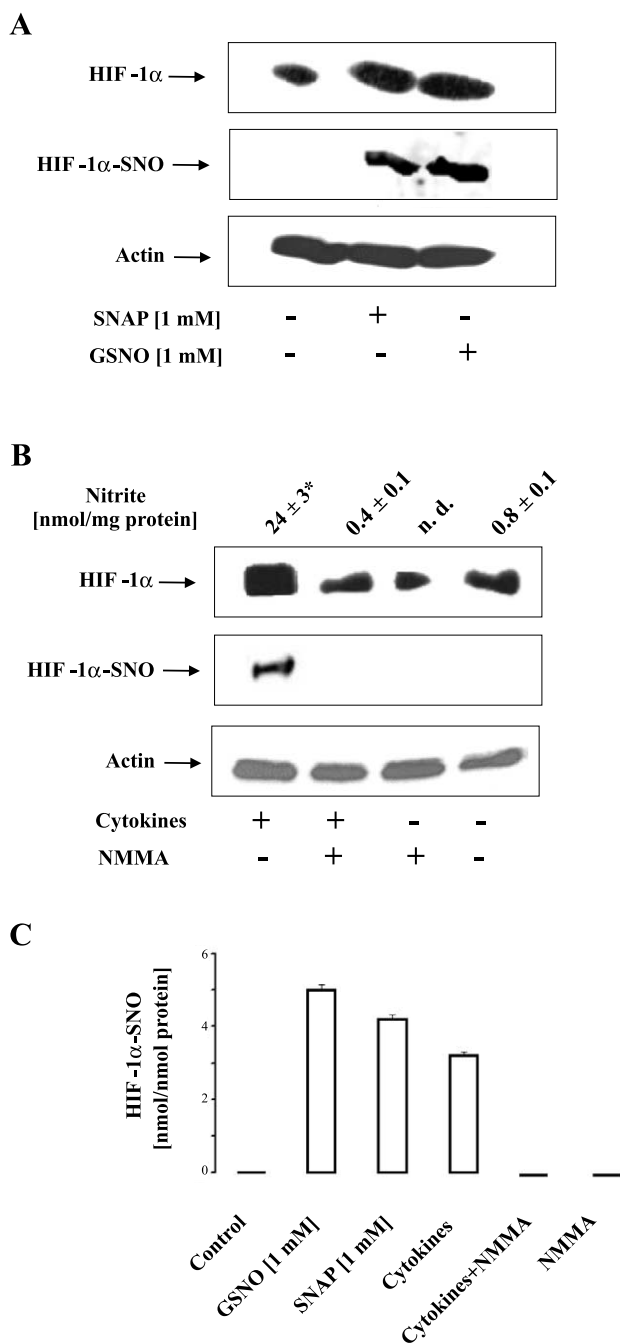
A

B

C

Fig. 3. S-Nitrosation of HIF-1α in RCC4 cells. A: Cells were stimulated with 1 mM GSNO or 1 mM SNAP for 3 h followed by Western analysis of HIF-1α and S-nitrosated HIF-1α. B: Cells were treated with 75 U/ml of IL-1β, 250 U/ml of IFN-γ and 25 μg/ml of LPS for 24 h. NO synthase activity was blocked with 1 mM NMMA. Nitrite was determined by the Griess assay. HIF-1α and S-nitrosated HIF-1α were determined by Western analysis as outlined under Section 2. Actin staining confirmed equal protein loading. C: Quantitation of HIF-1α-SNO was performed as described in Section 2. Results are expressed in nmol protein-SNO per nmol of protein. n.d. – not detectable. Data are mean values ± S.D. of at least five individual experiments.

tions as well. A cytokine mix (75 U/ml interleukin (IL)-1β, 250 U/ml interferon (IFN)-γ, 25 μg/ml lipopolysaccharide (LPS)) added for 24 h to RCC4 cells elicited nitrite formation in stimulated versus control cells. Blocking nitrite formation by N-monomethyl-L-arginine (NMMA) suggested the involve-

ment of a NO synthase, while NMMA by its own revealed no major impact (Fig. 3B). Endogenous NO formation was associated with HIF-1α protein stabilization in association with protein S-nitrosation as determined by Western analysis. Cytokines in the presence of NMMA left the amount of HIF-1α comparable to controls and blocked S-nitrosation of HIF-1α implying the participation of endogenously produced NO. When we calculated the amount of S-nitrosated HIF-1α in RRC4 cells after NO donor and/or cytokine treatment we obtained values around 3–5 nmol HIF-1α-SNO/nmol protein (Fig. 3C). Although these data imply S-nitrosation of HIF-1α in response to exogenous as well as endogenous NO formation in RCC4 cells it needs consideration that these values are lower compared to analysis performed with the purified protein (Fig. 1).

In order to confirm the impact of NO in a cell system not showing constitutive expression of HIF-1α we chose HEK293 cells (Fig. 4). In controls HIF-1α is absent while GSNO provoked a dose-dependent accumulation of HIF-1α as determined by Western blot analysis. In close correlation with stabilization of HIF-1α we quantitated increasing amounts of S-nitrosated protein.
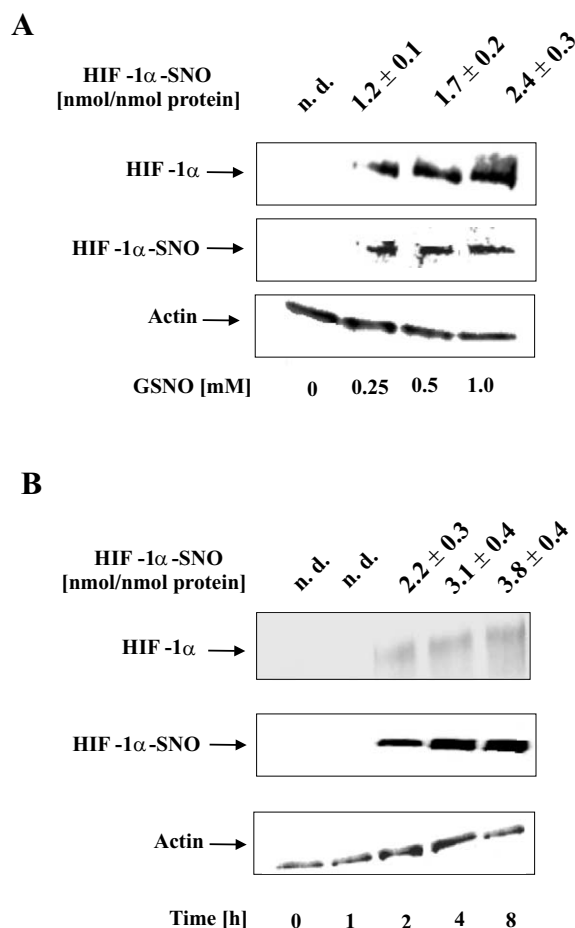
In extending examinations we studied the time response of

A

B

Fig. 4. S-Nitrosation of HIF-1α in HEK293. A: HEK293 cells were stimulated with 0.25, 0.5 and 1.0 mM GSNO for 4 h followed by HIF-1α and HIF-1α-SNO Western analysis as described in Section 2. HIF-1α-SNO was quantified as described in Fig. 3C. B: HEK293 cells were exposed to 1 mM GSNO for 1, 2, 4 and 8 h. Detection of proteins was done as described above. n.d. – not detectable.
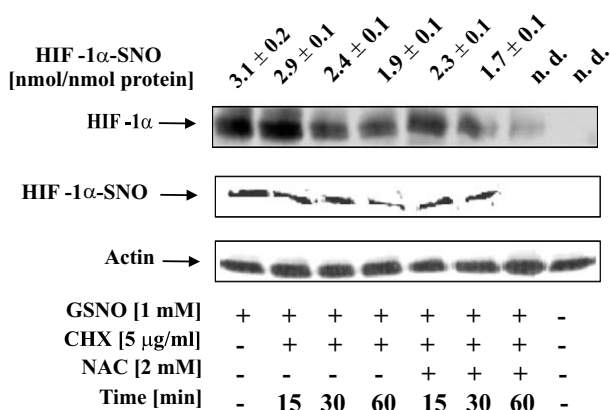
HIF-1α-SNO [nmol/nmol protein]: 3.1 ± 0.2 2.9 ± 0.1 2.4 ± 0.1 1.9 ± 0.1 2.3 ± 0.1 1.7 ± 0.1 n.d.   n.d.

HIF-1α →								
HIF-1α-SNO →								
Actin →								
GSNO [1 mM]	+	+	+	+	+	+	+	-
CHX [5 μg/ml]	-	+	+	+	+	+	+	-
NAC [2 mM]	-	-	-	-	+	+	+	-
Time [min]	-	15	30	60	15	30	60	-

Fig. 5. Impact of NAC on HIF-1α and HIF-1α-SNO. HEK293 cells were treated with 1 mM GSNO for 4 h. Thereafter, medium was removed and replaced by fresh medium supplemented with 5 μg/ml of CHX and 2 mM of NAC. Incubation went on for 15, 30 and 60 min followed by HIF-1α and HIF-1α-SNO detection as described in Section 2. For details see Fig. 4. n.d. – not detectable.

HIF-1α S-nitrosation (Fig. 4B). Under the impact of 1 mM GSNO stabilization of HIF-1α was noticed after 2 h, with increasing protein levels at 4 and 8 h. In parallel, we detected S-nitrosated HIF-1α and quantitation of the thiol-modified protein showed an increase between 2 and 8 h. We concluded that NO elicited stabilization of a S-nitrosated HIF-1α protein in HEK293 cells.

3.3. Destabilization of HIF-1α correlates with reduced protein S-nitrosation

This set of experiments was designed to explore whether destabilization of HIF-1α and decreased protein S-nitrosation are correlated. In HEK293 cells we achieved stabilization and S-nitrosation of HIF-1α during a 4 h lasting exposure to 1 mM GSNO (Fig. 5). Thereafter GSNO was removed by changing the medium and protein translation was attenuated by adding cycloheximide (CHX; 5 μg/ml). HIF-1α degradation was followed for 15, 30 and 60 min.

We noticed HIF-1α destabilization starting at 30 min with a more pronounced effect at 60 min. Protein destabilization was paralleled by decreased S-nitrosation of HIF-1α as determined by Western blot analysis. The addition of NAC in combination with CHX following HIF-1α stabilization with GSNO accelerated destabilization of the transcription factor. The protein amount of HIF-1α was significantly decreased at 30 min compared to the absence of NAC, a situation that was

even more appreciated at 60 min. In parallel to protein destabilization we noticed a decrease in the amount of S-nitrosated HIF-1α. We conclude that stabilization as well as destabilization of HIF-1α following the addition or removal of NO are paralleled by the appearance or disappearance of protein S-nitrosation.

To verify post-translational modification of HIF-1α with NO and to put this into the context of glutathione redox modifications we determined reduced GSH, oxidized GSH and the amount of protein S-nitrosothiols (Table 1). Application of GSNO decreased intracellular glutathione, provoked GSH oxidation and increased the amount of protein S-nitrosothiols. NAC prevented the drop in reduced GSH, attenuated GSH oxidation and lowered the level of protein S-nitrosothiols.

Thus, modification in the glutathione redox balance, especially the amount of protein S-nitrosothiol formation, mirrors changes seen in S-nitrosated HIF-1α protein as determined by Western analysis.

4. Discussion

Regulation of HIF-1α stability and HIF-1 activity under normoxic conditions is known for some time although detailed insights into molecular mechanisms remain unknown. More recently we and others noticed stabilization of HIF-1α in response to NO donors or under conditions of inducible NO synthase activity. Considering that NO-evoked signal transmission was cGMP-independent, it may be proposed that nitrosative, oxidative, or nitrative reactions are involved [36]. Here we asked whether HIF-1α itself would be a target for S-nitrosation. Indeed, using purified HIF-1α we have been able to modify cysteine thiols of HIF-1α to a maximum of 15 modifications due to S-nitrosation. Taking into account that HIF-1α harbors 15 free, i.e. DTNB accessible, thiol groups we must conclude that all of them are susceptible to S-nitrosation. Maximal thiol modification was achieved by employing NO^+ donating NO donors such as GSNO or SNAP. NO^+ confers strong electrophilicity and reactivity towards protein $R-S^-$ groups. This activity is not shared by a $NO^{\bullet}$ donating compound such as spermine-NONOate. Thus, in agreement with several other studies we excluded nitric oxide ($NO^{\bullet}$) itself as the primarily nitrosating species. Interestingly, formation of $NO^{\bullet}$ in the presence of O_2^- achieved substantial S-nitrosation most likely due to the generation of a nitrosating species, probably dinitrogen trioxide or a closely related species. Formation of a nitrosating species, most likely N_2O_3, can be rationalized if an excess of $NO^{\bullet}$ over O_2^- abolished the strong

Table 1
Reduced glutathione, oxidized glutathione and protein S-nitrosothiol content in HEK293 cells

Treatment	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	pSNO (nmol/mg protein)
Control	17.6 ± 0.8	0.68 ± 0.02	1.4 ± 0.2
GSNO (4 h)	$11.4 \pm 0.7^*$	$1.15 \pm 0.06^*$	$3.4 \pm 0.4^*$
CHX (15 min)	$12.6 \pm 0.9^*$	$1.1 \pm 0.03^*$	$2.7 \pm 0.2^*$
CHX (30 min)	$12.7 \pm 1.4^*$	$0.95 \pm 0.04^*$	$2.5 \pm 0.4^*$
CHX (60 min)	13.4 ± 1.8	$0.90 \pm 0.07^*$	2.1 ± 0.1
CHX/NAC (15 min)	17.5 ± 1.1	0.85 ± 0.03	1.6 ± 0.3
CHX/NAC (30 min)	$24.0 \pm 2.5^*$	$0.41 \pm 0.02^*$	1.4 ± 0.3
CHX/NAC (60 min)	$42.0 \pm 3.8^*$	$0.36 \pm 0.03^*$	1.2 ± 0.1

HEK293 cells were treated with 1 mM GSNO for 4 h. Thereafter, medium was removed and replaced by fresh medium supplemented with 5 μg/ml of CHX and 2 mM of NAC. Incubation went on for 15, 30 and 60 min followed by determination of GSH, GSSG and protein S-nitrosothiols (pSNO) as outlined in Section 2. Data are mean values $\pm$ S.D. of at least five separate experiments. $^*P < 0.01$ vs. control.

oxidations seen with peroxynitrite under equimolar generation of O_2^- and $NO^\bullet$ in favor for S-nitrosation reactions [37–40]. This is deduced from observations when studying nitrosation of 4,5-diaminofluorescein or modification of alcohol dehydrogenase [38,39]. This situation is comparable to our situation. A minor excess in the rate of NO versus O_2^- formation provokes little S-nitrosation. However, the amount of HIF-1α S-nitrosation increases when the rate of NO formation is roughly three–six-fold over the rate of O_2^- formation. S-Nitrosation of HIF-1α is efficiently antagonized by nucleophilic substances such as GSH and ascorbate. This may limit yields of nitrosation by competitive scavenging the nitrosative species or by reversing post-translational protein modification. However, when exposing RCC4 or HEK293 cells to NO donors we noticed S-nitrosation of HIF-1α by comparison with standard amounts of S-nitrosated BSA and we were able to quantify the amount of S-nitrosated HIF-1α. Despite intracellular antioxidants, nitrosation of HIF-1α was elicited with NO donors and more importantly by provoking NO formation from inducible NO synthase in RCC4 cells. The involvement of endogenously generated NO was proven by blocking S-nitrosation with the NO synthase inhibitor L-NMMA. However, the amount of modified HIF-1α was much lower compared to in vitro studies with purified protein but clearly detectable. Although cells can affect the rate of nitrosation, nitrosative reactions that function as redox switches may be sheltered from quenching by nucleophilic substances in compartments that exclude these agents. Our results are compatible with the notion that only a few, most likely highly reactive thiol groups that may harbor a motive for nitrosation [41], serve as prominent targets for S-nitrosation. In this respect HIF-1α shows substrate properties with many other proteins without any indication whether S-nitrosation accounts for altered protein function. However, investigations in both cell systems allowed to conclude that any existing HIF-1α protein is subjected to S-nitrosation and accumulation of HIF-1α is closely correlated with post-translational modification under conditions of NO formation. S-Nitrosation of HIF-1α was time- and concentration-dependently correlated with GSNO-evoked protein stabilization. Moreover, this correlation was extended to protein destabilization as well. Removing the NO donor and blocking new protein synthesis with CHX allowed a gradual decline of HIF-1α protein in its nitrosated form in HEK293 cells. The addition of NAC accelerated protein destabilization with similar kinetics of protein and nitrosothiol disappearance. The decrease in HIF-1α-SNO upon NAC addition can be correlated with a decreased protein S-nitrosothiol content in general and an increase in reduced GSH. These results show that NO-evoked HIF-1α stabilization and S-nitrosation are inversely correlated to intracellular reduced glutathione, which is in some analogy to effects seen in vitro with the addition of glutathione. An imbalance in the level of key endogenous scavenger substances for reactive nitrogen species may affect nitrosative reactions and thus HIF-1α stabilization by NO. In future experiments it needs to be established whether the intriguing correlation between NO-evoked HIF-1α stabilization and S-nitrosation accounts for a cause–effect relation.

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