In vitro formation of S-nitrosohemoglobin in red cells by inducible nitric oxide synthase

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Abstract The present study demonstrates that NO produced in vitro by inducible nitric oxide synthase in red cells can convert hemoglobin contained in the red cells to S-nitrosohemoglobin. Experiments carried out either in the absence or in the presence of a low molecular weight thiol, such as cysteine, showed that in the first case the target of NO is heme-Fe^{2+}. On the contrary, in the presence of cysteine, the first step is the formation of S-nitrosocysteine, followed by transfer of the NO group to a particular cysteine residue of β-globin, cysteine 93. These results confirm previous data indicating the preferential formation of S-nitrosohemoglobin at that site by chemical methods [Ferranti et al. (1997) FEBS Lett. 400, 17–24], and the existence of a physiological mechanism of inactivation for NO circulating in blood. The analysis of S-nitrosohemoglobin can also allow the quantification of the NO levels in blood to be applied for in vitro and in vivo measurements.

Key words: Nitric oxide; Hemoglobin; S-Nitrosothiol; S-Nitrosohemoglobin; Inducible nitric oxide synthase; Liquid chromatography-electrospray mass spectrometry

1. Introduction

The interaction of nitric oxide (NO) with proteins plays a critical role in several physiological systems extending from blood pressure regulation to neurotransmission [1–3]. In addition, NO produced in infected and inflamed tissue can contribute to the process of carcinogenesis [4,5]. The targets of NO on proteins are Cys and bound metals such as heme-Fe^{2+} (for a review see [6]), through which NO exerts its effects by covalently modifying or oxidizing critical thiols or transition metals in proteins. Although much work has been performed in characterizing the NO-Fe^{2+} interaction [7,8], there are considerably less data on the NO-Cys interactions. One reason is the labile nature of S-nitrosothiols (RSNOs) which are unstable in aqueous solution. It has been assumed that the lability of RSNOs is due to their propensity to undergo homolytic cleavage of the S–N bond with release of NO [9].

Recent studies have demonstrated the biological importance of the interaction of RSNOs, which can be considered as NO donors, with proteins at the level of Cys residues [10,11]. Low mass RSNOs are one class of endogenous compounds capable of protein S-nitrosylation as demonstrated in the case of hemoglobin (Hb) [12]. The β-chain of Hb contains a highly reactive thiol group at position 93 [13], which is conserved among mammalian species, although its function remains unknown. It has been recently proposed that its interaction with endogenous RSNOs to form S-nitrosohemoglobin (HbSNO) [12] may serve for regulation of blood pressure and facilitates efficient delivery of oxygen to tissues [14]. Recently, structural data have been produced to support the preferential in vitro formation of HbSNO at βCys-93 [15] at RSNO concentrations close to that in vivo, thus indicating that physiological HbSNO is S-nitrosylated at that site. Other forms of HbSNO, where nitrosylated cysteines are also βCys-112 and αCys-104, are produced in vitro at higher RSNO concentrations [15].

Current methods of RSNO colorimetric detection require large amount of sample. Other spectrophotometric methods exist including UV, IR and NMR techniques. Again sample amount, purity, and quantitation are serious difficulties associated with these methods. Electrospray ionization mass spectrometry (ES/MS) can detect RSNOs in very small amount and can determine the stoichiometry of substitution [15,16]. Furthermore, this method allows coupling to high performance liquid chromatography which enables on-line quantitative analysis and peptide mapping of sites of RSNO formation.

We have used this approach to demonstrate the formation of HbSNO either in hemolysate or in whole red cells by action of inducible nitric oxide synthase (iNOS) on the same Hb residue (βCys-93). At the same time, it was possible to quantify the amount of HbSNO. The approach can be extended to the characterization of S-nitrosoproteins from different sources. This makes possible structural studies on extremely labile species such as protein nitrosothiols.

2. Materials and methods

2.1. Materials

Blood samples were collected from non-pathologic subjects immediately prior to their use for in vitro incubation in anti-coagulation tubes containing 20 mM EDTA, and stored at 4°C. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant mouse macrophage iNOS, no. 60802, from Cayman Chemical Co. (Ann Arbor, MI, USA), was stored in HEPES, pH 7.4, contain-

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Abbreviations: Hb, hemoglobin; NO, nitric oxide; iNOS, inducible nitric oxide synthase; RSNO, S-nitrosothiol; HbSNO, S-nitrosohemoglobin; metHb, methemoglobin; ES/MS, electrospray mass spectrometry; LC/ES/MS, liquid chromatography-electrospray mass spectrometry; SIM, single ion monitoring

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ing 10% glycerol. HPLC-grade solvents were from Carlo Erba (Milan, Italy). 2.1 mm inner diameter columns, 25 cm long, for both analytical and micropreparative scale procedures were used. The columns were Vydac (Hesperia, CA, USA) C_4 (214TP52) for globin analysis, and Vydac C_{18} (218TP52) for peptide mapping. The HPLC apparatus was constituted by a Hewlett-Packard series 1100 modular system with an integrated diode array detector. The effluent from HPLC separation was on-line injected into a Platform (Micromass, UK) mass spectrometer equipped with a standard electrospray source via a 75 μm i.d. fused silica capillary. The Masslynx (Micromass, UK) software furnished by the instrument manufacturer was used for data processing.

2.2. Preparation of red cell samples

Aliquots of 1 ml of blood were centrifuged at 3000 rpm. After discarding plasma, erythrocytes were washed three times with 2 ml isotonic 0.9% NaCl solution by centrifugation at 3000 rpm for 15 min at 4°C. Two ml of packed erythrocytes were suspended in 2 ml of 20 mM sodium dihydrogen phosphate, pH 7.4. Samples were aerated with a gentle stream of air for 5 min to fully convert Hb in the oxy-form. This procedure resulted in a Hb concentration of about 2 mM.

2.3. Preparation of the hemolysate samples

Hemolysates were prepared by suspending 1 ml of washed erythrocytes in 1 ml of deionized water. After occurrence of hemolysis, sample was separated by centrifugation (10 000 rpm, 20 min at 4°C twice), and the supernatant fraction used at once or stored frozen (−20°C for up to a week or −70°C for up to 3 months) until required. This hemolysate had a Hb concentration of about 1 mM. The sample was aerated with a gentle stream of air for 5 min. This oxy-Hb was estimated by SDS-gel electrophoresis and by isoelectric focusing (IEF) to be about 95% pure and prepared immediately prior to its use [17].

2.4. Enzymatic synthesis of S-nitrosohemoglobin

Before its use, activity of iNOS was quantitated spectrophotometrically using the oxyhemoglobin assay [18]. This assay measures the reaction of nitric oxide produced with oxyhemoglobin to yield methemoglobin (metHb) ($\varepsilon = 60\,000\ M^{-1}\ cm^{-1}$ at 401 nm). One unit of enzyme produces 1 nmol of nitric oxide per min at 37°C in 50 mM HEPES (pH 7.4) containing 1 mM L-arginine, 0.1 mM magnesium acetate, 0.1 mM NADPH, 12 μM tetrahydrobiopterin, 12 μM FAD, 12 μM FMN and 170 μM DTT. These conditions were employed for checking the enzyme activity.

In a first experiment, 3 μl of freshly prepared 0.2 mM hemolysate (previously flushed with a gentle air stream) and 1 unit of iNOS were contemporary incubated for 30 min at 37°C in 1 ml 50 mM HEPES (pH 7.4) containing 1 mM L-arginine, 0.1 mM magnesium acetate, 0.1 mM NADPH, 12 μM tetrahydrobiopterin, 12 μM FAD, 12 μM FMN and 170 μM DTT (incubation buffer).

Two further incubations were then carried out. In the first of them, a solution of freshly prepared hemolysate, containing 0.2 mM Hb, was prepared in the incubation buffer (1 ml) containing also 1 mM Cys. The enzyme (1 unit) was added and the reaction allowed to take place for 30 min. In the second experiment, the incubation buffer (1 ml) also containing 1 mM Cys was added with the enzyme, and this reaction mixture was left at 37°C for 30 min, after which the hemolysate (3 μl, 0.2 mM) was added and the incubation carried out for 10 min. All the reactions were monitored by absorbance at 401 nm. To avoid HbSNO degradation, the reactions were stopped by directly adding 200 μl 5% TFA, after which liquid chromatography-electrospray mass spectrometry (LC/ES/MS) analysis was carried out. For any experiment a blank sample was prepared in which all the above components were present except the enzyme solution.

For the production of HbSNO in red cells, we followed the same procedure as in hemolysate, but the HEPES concentration was 100 mM (to obtain the isotonic condition) and the reaction was stopped by lysis of red cells with 1 ml distilled water and the sample filtered. After the spectrophotometric assay, the solution was added with 200 μl TFA 5% and analyzed by LC/ES/MS.

2.5. Liquid chromatography-mass spectrometry of S-nitrosohemoglobin

Globin chains from HbSNO were purified by RP-HPLC using the procedure developed by Shelton et al. [19] with the following modifications: liquid chromatography was carried out with a 2.1 mm i.d. × 250 mm, C4, 5 μm (Vydac) reverse-phase column, in the following buffer system: buffer A: 80:20:0.1 (v:v:v) water:acetonitrile:TFA and buffer B: 40:60:0.07 (v:v:v) water:acetonitrile:TFA. The flow rate was 0.1 ml/min. The column was equilibrated at 52% buffer B. After applying the sample (50 μg of freshly synthesized HbSNO dissolved in 20 μl volume) buffer B was raised to 66% over 50 min. The electrospray needle was set at a voltage of 3.6 kV, the cone voltage at 20 V, the sheath gas pressure (nitrogen 99.997%) at 75 psi, the source temperature at 100°C. The mass spectra were scanned from 1600 to 600 U at a scan cycle of 5 s/scan. Nitrosylated α- and β-globin and the derived peptides were identified by a +29 mass shift with respect to native chains. A rough estimate of the proportion of native and nitrosylated globins/peptides was obtained from the relative intensity of the ionization current of molecules through the reconstructed spectrum. Indeed, the ionization level is dependent on the nature of the compound. From the native and nitrosylated species, however, being very close in composition and structure, we have inferred that the comparison of the ionization level could give a valuable estimation of their relative amount. Therefore, quantitative analysis of components was carried out by integration of the multiply charged ions for the single species. For both α- and β-globin chains, the ions carrying 13–22 charges, which in the analytical conditions used were the most intense, were chosen. This quantitative analysis was carried out at low cone voltage (20 V) and temperature (100°C), which minimize nitrosothiol decomposition in the ion source, furnishing the best quantitative data [15].

2.6. LC/ES/MS peptide mapping

For peptide mapping, globins were digested with pepsin (porcine stomach mucosa, Sigma). Samples were dissolved in 100 μl of 5% formic acid and a substrate: enzyme ratio 15:1 (w/w) was used. After incubation at 37°C for 1 h, the reaction was stopped by freezing at −20°C. Liquid chromatography was performed using a 2.1 mm i.d. × 250 mm, C18 5 μm (Vydac) reverse-phase column with a flow rate of 0.1 ml/min. Solvent A was 0.03% TFA (v/v) in water; solvent B was 0.02% TFA in acetonitrile. The column was equilibrated at 5% solvent B. Separation of the peptides was effected with a gradient 5–40% solvent B over 50 min. The mass spectra were scanned from 1800 to 400 U at scan cycle of 5 s/scan. The components were identified by the use of a dedicated software program (Peptide Tools, Hewlett-Packard). For single ion monitoring (SIM) analysis, the doubly- and triply-charged ions (*m/z* 1041.7 and *m/z* 694.8 respectively) of the nitrosylated peptide β(89–105) were monitored.

3. Results

In Fig. 1 the spectrophotometric analysis of an hemolysate sample incubated with iNOS in different conditions is reported. The direct incubation of a hemolysate sample with iNOS (panel a) led to a rapid increase in the absorbance at 401 nm, which corresponded to the conversion of Hb to metHb. In panel b the same experiment was carried out in the presence of 1 mM Cys. The curve was much less sloping, indicating that the formation of metHb by reaction of Hb with NO was slower in the presence of Cys. When Cys was added to the reaction mixture containing iNOS 30 min before adding the hemolysate (panel c), no absorbance increase was observed, which demonstrated that the NO formed was not available for reaction with heme and no metHb was produced.

The three samples were then analyzed by LC/MS at the end of the reaction. In the first two samples, only native α- and β-globins were identified on the basis of the respective molecular weight [15]. In the third experiment the mass measured for the α-chain (15 126.5 ± 1.1) perfectly matched that of the native globin (15 126.4). On the contrary, of the two species present at the β-position (not shown), the first one was the native β-globin (15 868.5 ± 1.4, expected mass 15 867.2) whereas the second had a mass of 15 897.8 ± 1.1 with a shift of +29 units compared with that measured for the β-globin, indicating that a single NO moiety was bound to the globin.

The spectrophotometric and MS data, taken together, demonstrated that (i) when Hb was directly exposed to NO pro-

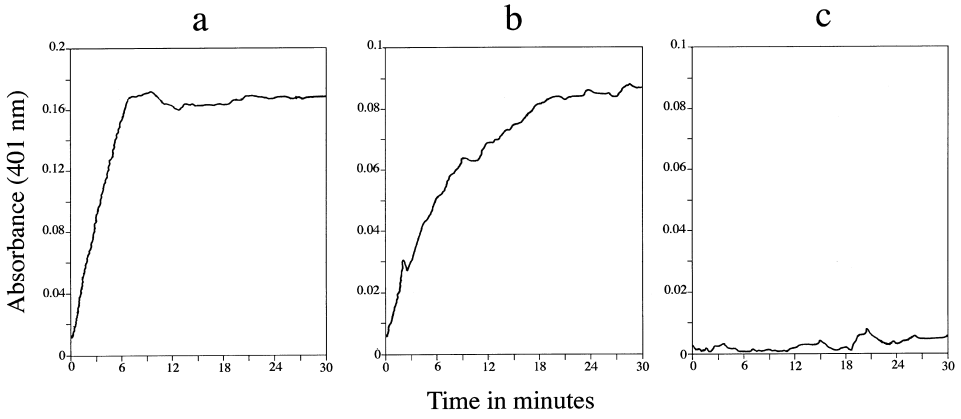

Fig. 1. Spectrophotometric analysis of the hemolysate incubated in the presence of iNOS. Panel a: hemolysate incubated directly with iNOS. The rapid conversion of Hb to metHb is observed. Panel b: hemolysate and Cys incubated simultaneously with iNOS. The much slower formation of metHb is observed. Panel c: hemolysate added 30 min after incubation of Cys with iNOS. No conversion of metHb is observed.

duced by iNOS, only formation of metHb was observed. When the same reaction was performed in the presence of a low mass thiol, such as Cys, the oxidation of Hb to metHb was slower because part of the NO was removed as S-nitrosocysteine. On the contrary, when preliminary incubation of Cys with iNOS was performed, no metHb and the contemporary synthesis of HbSNO was observed.

The same experiments were then carried out on a red cell sample. In this case only the LC/MS analysis was performed, because the presence of intact cells made not possible spectrophotometric analysis. The reaction pattern was comparable to that obtained using the hemolysate. In particular (Fig. 2), the formation of nitrosylated β-globin was observed only when preliminary incubation of iNOS was performed in the presence of Cys. The relative amount of the native and nitrosylated β-globin was 1:0.15.

For a full characterization of the nitrosylated species, the nitrosylated Hb samples were digested with pepsin in order to perform the peptide map of the modified globins. Pepsin was chosen as the proteolytic agent because it allowed us incubation in acidic buffer where nitrosylated species are more stable [9,15]. The LC/ES/MS separation of the peptic digest of the nitrosylated globin chains is shown in Fig. 3. HPLC peaks could be assigned to the peptides within the globin sequence on the basis of the measured molecular weight. It was possible to check out the entire sequence of the two globins in mixture by this procedure. The only nitrosylated peptide detected was peptide β(89–105).

The peak at 36.5 min in the chromatogram of Fig. 3 contained peptide β(89–105) (molecular weight 2052.3) containing βCys-93, with a molecular weight of 2052.6 ± 0.2 (identified by the doubly- and triply-charged ions at *m/z* 1027.2 and *m/z* 685.1 respectively). A novel peak was also detected at higher retention time (38.0 min) containing a peptide of mass 2081.3 ± 0.7 (identified by the doubly- and triply-charged ions at *m/z* 1041.7 and *m/z* 694.8 respectively). This value corresponded to the mass of peptide 89–105 increased by 29 mass units, due to nitrosylation at the level of βCys-93.

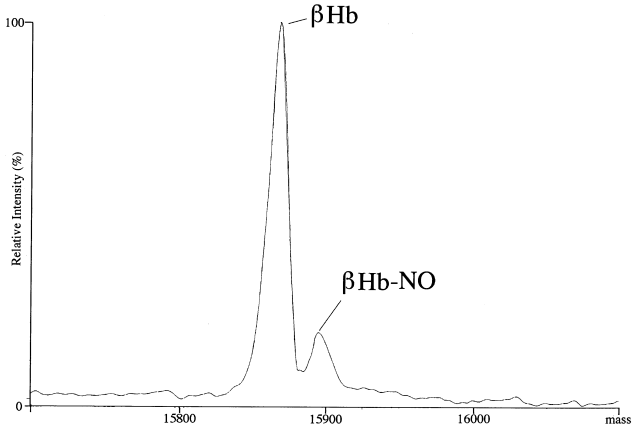

Fig. 2. ES/MS spectrum in the transformed mass scale of nitrosylated β-globin. Cys was incubated in the presence of iNOS for 30 min, then red cells were added and, after 10 min, the reaction products were analyzed by mass spectrometry.

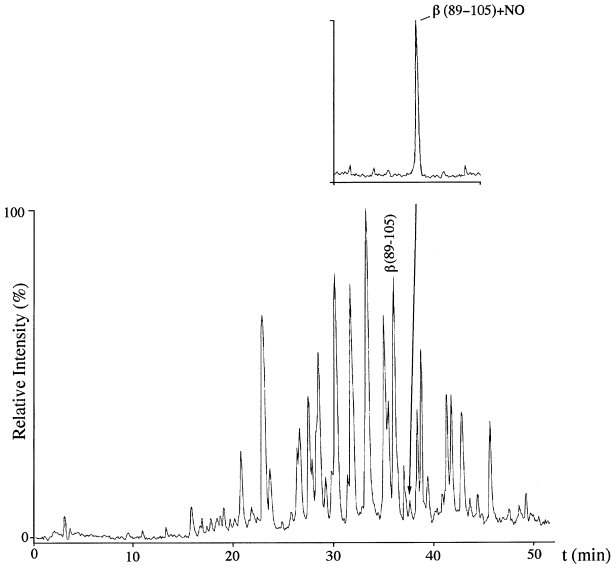

Fig. 3. LC/ES/MS analysis of the peptic digest of globin chains from a red cell sample incubated with iNOS. The peptide β(89–105), eluted at 36.5 min and with a molecular weight of 2052.6, is indicated. The nitrosylated peptide β(89–105), indicated by an arrow, is present at 38.0 min and has a molecular weight of 2081.3. In the inset the SIM chromatogram for *m/z* 1041.7 and *m/z* 694.8, corresponding to the doubly- and triply-charged ions of the nitrosylated peptide, is shown.

This was verified with the same procedure applied for the synthesis of HbSNO by chemical methods [15].

The same analysis was carried out in SIM mode (Fig. 3, inset). The peaks corresponding to the multicharged ions of the nitrosylated peptide β(89–105) were used for the acquisition. In this way it would be possible to obtain a better measure for the detection and the quantitative analysis of the nitrosylated species at lower levels, as expected in physiological conditions.

4. Discussion

This work demonstrates the formation of HbSNO in vitro either in hemolysate and in intact red cells by action of iNOS. This process takes place via low mass nitrosothiol intermediates, like S-nitrosocysteine. In this work we have analyzed a model system in order to determine whether the NO produced by iNOS in blood were able to convert Hb to HbSNO. To ascertain this, the experiments were first performed on an hemolysate sample, to monitor spectrophotometrically and mass spectrometrically the formation of metHb. The same experiments were then carried out on red cells to confirm that the reaction gave the same products when iNOS (and therefore the NO produced externally to the red cells) was not in close contact with Hb. In this last case the experiments were monitored only by mass spectrometry due to the high background in the spectrophotometric assay. Our data indicate that following the production of NO in the presence of a low-mass thiol system, such as Cys, HbSNO is formed in the red cells in vitro. It is possible that HbSNO is produced in vivo through a similar process. According to this hypothesis NO, once formed, can immediately react with nucleophiles such as the thiol group of Cys, either before or after entering the red cell membrane, and the resulting S-nitrosocysteine can act as an NO exchanger for Hb.

In the red cells several small molecular mass thiol systems are present, Cys and gluthatione [20,21] among others, which can be converted to nitrosothiols and consequently act as NO donors [22]. This means that the production of HbSNO can be mediated by a series of molecules via thiol exchange.

In the experiment we have carried out in the absence of Cys, the NO produced only metHb (Fig. 1, panel a). When the Cys was added to red cells in the course of the NO production, an intermediate situation was observed in which the formation of metHb was slower, because part of the NO had already reacted with Cys (Fig. 1, panel b). Mass spectrometric evidence, however, did not confirm the synthesis of HbSNO in this case. On the contrary, HbSNO was produced in detectable amounts only in the presence of a nitrosothiol system which was formed only when Cys was preliminary incubated with the NO produced by iNOS (Fig. 1, panel c). These data further support the existence of mechanisms involving endogenous thiols in the synthesis of HbSNO. In this model Hb would act as a reversible exchanger of NO in order to regulate several physiological systems as recently proposed [12].

iNOS is mainly expressed as a consequence of various inflammatory stimuli [23,24]. Under these circumstances, NO mediates anti-microbial and anti-tumoral activities of the immune system, but long term NO over production causes tissue damage and other serious diseases [25–27]. These two opposite effects imply that the organism must regulate NO synthesis precisely since it is thought that NO can not be stored or inactivated by conventional mechanisms after release [28] other than its entrapment by heme ferrous ion of Hb. However, our data seem to indicate for the first time the existence of an efficient mechanism of regulation of the NO level. The control of NO circulating in blood is essential for the organism, as it is now well documented the role of this messenger molecule in the regulation of many physiological mechanisms [29–34]. Therefore, the function of HbSNO is possibly that of subtracting the NO excess from the circulatory system in order to minimize the damage induced by NO overproduction. In this way the NO bound would be directed to lungs where it can be released in the different pH and pO_2 conditions [12].

The formation and stability of the HbSNO were confirmed by mass spectrometry. Mass spectrometric analysis showed that the half-life of HbSNO is about 24 h and also confirmed that the site of formation of HbSNO by iNOS (βCys-93) is the same as that involved in the chemical synthesis [15]. The different behavior exhibited by βCys-93 compared to the other cysteines was in agreement with the already reported in vivo reactivity of this residue in oxyhemoglobin, which readily undergoes oxidation to form mixed disulphides and other thioethers due to its position within the protein quaternary structure [13]. This different reactivity of Cys residues in Hb has been recently observed for another highly reactive electrophilic agent, methyl bromide [35–38].

Due to the close structural similarity of native and nitrosylated species, only differing by the presence of the NO group over the entire protein/peptide, it seemed reasonable to assume similar behavior in electrospray analysis, as already shown for methyl bromide [36,39]. Therefore, once the samples were enzymatically digested to peptides, the reactivity and the nitrosylation degree of βCys-93 could be measured by SIM analysis of nitrosylated peptides. By this measurement, the ratio of modified and native species could be derived.

In general agreement with our previous experience [15,36], the synthetic protocol used was not found to significantly interfere with the mass spectrometric detection of proteins and peptides. This analysis can be applied for quantitative evaluation of nitrosylated proteins. In fact, through peptide mapping, it is possible to use the methodology here described for the measurement of the NO level in blood in normal or pathological conditions.

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