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Electrochemical assay of human haemoglobin S-nitrosylation by nitrosocysteine

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Summary. Oxyhaemoglobin (oxyHb) and methaemoglobin (metHb) react with S-nitrosocysteine (CysNO) to form nitroso derivatives. We test this reaction with a new method for evaluating transnitrosation reaction. The assay exploits an amperometric sensor developed in our laboratory. The results we obtain are in good agreement with those reported by others, although at much higher sensitivity, indicating the suitability of the method for examining high-mass nitroso compounds.
The S-nitrosylation of oxyHb at a CysNO/haem ratio of 1:1 is about 5% in 60 min. In the same experimental conditions, the nitrosylation of met-Hb reaches 25%. OxyHb and metHb derivatize by 50% in 60 min upon using a CysNO/haem ratio of 10:1.
The oxidation of haem iron occurs at ratios of haem/CysNO of 1:5 or higher.
We conclude that CysNO transfers NO^+ both to metHb and oxyHb. We propose that NO transfer in RBC may occur through transnitrosation reactions between high and low-mass nitrosothiols.

Keywords: Cysteine – Nitrosocysteine – Haemoglobin – Methaemoglobin – Oxyhaemoglobin

Abbreviations: Hb: haemoglobin; SNO-Hb: S-nitrosohaemoglobin; CysNO: S-nitrosocysteine; metHb: methaemoglobin; oxyHb: oxyhaemoglobin; SNO-metHb: S-nitrosomethaemoglobin; SNO-oxyHb: S-nitrosooxyhaemoglobin

Introduction

NO can react with the haem group; NO^+ may form S-nitroso derivatives of human Hemoglobin (Hb) Cysβ93. A S-nitrosylation/denitrosylation cycle of Hb might be relevant for the physiology of oxygen transport and utilization (Gow and Stamler, 1998; Jia et al., 1996; Stamler et al., 1997).

In experimental systems NO donors are often used to simulate endogenous NO production. Nitrosocysteine (CysNO) is a low-mass nitrosothiol and may be used as NO donor. CysNO releases NO by splitting the S-N bond, as catalyzed by divalent ions (Cu^{2+}, Fe^{2+}). It also releases NO^+ in the presence of chelating agents. In its turn, NO^+ S-nitrosylates (at physiological pH values) peptides and proteins provided that free –SH groups are available. This property suggests potential roles for nitrosothiols in biological systems. MetHb and oxyHb are good acceptors of the NO^+ arising from low-mass nitrosothiols (Jia et al., 1996). SNO-Hb may, therefore, act as a NO reservoir in the RBC. It should also be noticed that SNO-Hb may influence the dilatation of blood vessels.

The determination of SNO-Hb may be performed through different methods. In this paper, we use a specific electrochemical assay (Palmerini et al., 1998), and find that it may also be used to determine high-mass nitroso compounds, because it gives results similar to those obtained with the displacement by $HgCl_2$ and the Griess reaction (Green et al., 1982; Saville, 1958), albeit at nmolar sensistivity.

Experimental procedures

Materials

Phosphate buffer was produced by Fluka (Buchs, Switzerland). Sephadex G-25 was obtained from Pharmacia fine chemicals (Upssala, Sweden). Other reagents were purchased from C. Erba, Milan, Italy and all of them were of reagent grade or better, unless stated otherwise.

Preparation of nitrosocysteine (CysNO)

CysNO was prepared by reacting equimolecular amounts of cysteine and nitrite for about 30 min in 0.1 M HCl (Williams, 1996).

The formation of CysNO was then assessed by measuring light absorbance at 340 nm.

S-nitrosylation of HbO_2 through the reaction with CysNO

Human Hb was obtained from venous blood samples and was purified as described (Kilbourn et al., 1994). Purified HbO_2 (200 μM) was then incubated in phosphate buffer, 0.1 M, pH 7.4, also containing 1 mM EDTA with various concentrations CysNO at 37°C from 10 to 60 min. SNO-Hb was determined after purification, performed through a passage on a Sephadex G-25 (60 × 2 cm) column. SNO-Hb was eluted with void volume and was assessed by using the electrochemical assay described below and for determining the concentration of Hb by light absorbance at 415 nm.

S-nitrosylation of metHb through the reaction with CysNO

Human Hb prepared as described above, was treated with $K_3Fe(CN)_6$ (10-fold molar excess for 10 min at room temperature) and subsequently purified on a Sephadex G-25 column (60 × 1.5 cm). The concentration of (FeIII)Hb was determined at 406 nm; a spectrum was made to check the complete oxidation to (FeIII).

MetHb (200 μM) was then incubated in phosphate buffer, 0.1 M, pH 7.4, also containing 1 mM EDTA with various concentrations CysNO at 37°C from 10 to 60 min. SNO-Hb was determined after purification, performed through a passage on a Sephadex G-25 (60 × 2 cm) column. SNO-Hb was eluted with void volume and was assessed by using the amperometric sensor described below and for determining the concentration of Hb by light absorbance at 406 nm.

Apparatus for determining SNO-Hb

The apparatus consisted of a reaction vessel (5 ml) equipped with an injector. The reaction mixture was maintained under a constant stirring at 25°C and supplied with a flow of 5 ml/min of nitrogen. The NO formed in the vessel was carried to the amperometric sensor described previously (Palmerini et al., 1998). The sensor detected, in different ranges of potential, both NO and NO_2, the latter with higher sensitivity. Therefore, NO was first transformed into NO_2 using a small trap filled with an acidic solution of permanganate (50 mM $KMnO_4$, 0.5 M $HClO_4$). The presence of NO in the reaction vessel was recorded versus time as a peak of electric current. The apparatus was calibrated by injecting known amounts of standard nitrite solution into the reaction vessel that also contained 50 mM cysteine dissolved in 50 mM HCl and 50 mM $CuCl_2$ (Palmerini et al., 2000). Samples of SNO-Hb, eluted from the G-25, were therefore injected into the reaction vessel. Liberated NO was carried to the permanganate trap and then to the sensor were it was determined as NO_2.

Results and discussion

Increasing evidence supports the possible role of S-nitrosylation of Hb in vascular (Ignarro et al., 1981) and respiratory physiology (Gaston et al., 1998). A S-nitrosylation/denitrosylation cycle of the Cysβ93 of human Hb has been claimed to help determining the deoxyHb/oxyHb ratio in red blood cells (Jia et al., 1996; Stamler et al., 1997).

NO reacts with the haem group of oxyHb to form metHb and nitrates (Olsson, 1981). In addition, it may be observed that NO does not react with thiols (unless in very acidic conditions and in the presence of reagents able to oxidize it to NO^+ (Kharitonov et al., 1995)). On the other hand, nitrosothiols may rapidly decompose into NO and –S-S-group in the presence of traces of metal ions, especially Cu^{2+} (Askew et al., 1995; Williams, 1996, 1999); whereas in the presence of chelating agents, such as EDTA, they may exchange NO^+ (Arnelle and Stamler, 1995). CysNO may easily transfer NO^+ to Hb (Cysβ93) at physiological pH values (Arnelle and Stamler, 1995; Jia et al., 1996). The formation of SNO-Hb may explain the transfer and the storage of NO in the RBC. The bioactivity of NO would so be preserved and kept in the RBC (McMahon et al., 2000). For these reasons, we study the S-nitrosylation of Hb by using CysNO as a donor of NO^+. SNO-metHb and SNO-oxyHb are produced by NO^+ transfer from low-mass nitrosothiols (CysNO) in 0.1 M phosphate buffer (pH 7.4), in the presence of 1 mM EDTA and under oxygen flux at 37°C. About 50% of either oxyHb or metHb were nitrosated in 60 min using a CysNO 10-fold excess (Fig. 1). On the other hand, if the haem/CysNO ratio was 1:1, the S-nitrosylation of metHb was 25% in 60 min, whereas that of oxyHb was 5%; in 10 min the S-nitrosylation of metHb was about 10%, and that of oxyHb was undetectable. Therefore, the S-nitrosylation extent of oxy-Hb is similar to that of met-Hb only using CysNO

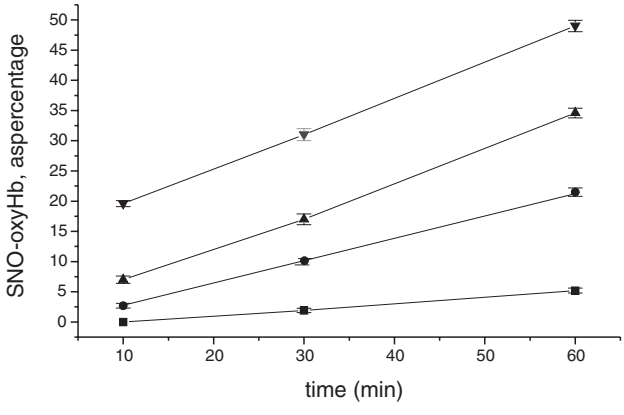

Fig. 1. Production of SNO-Hb from oxyHb and nitrosocysteine (CysNO) at different CysNO/haem molar ratios and at different times of incubation. OxyHb (200 μM of haem) was mixed to CysNO to obtain the ratios indicated in the figure, in PBS (0.1 M, pH 7.4) + 0.5 mM EDTA, in a final volume of 1 ml. The reaction was carried at 37°C for various incubation times. SNO-Hb was determined by electrochemical assay, after purification of the protein on a Sephadex G25 column. -■-: Haem to CysNO ratio 1:1; -●-: Haem to CysNO ratio 1:2; -▲-: Haem to CysNO ratio 1:5; -▼-: Haem to CysNO ratio 1:10

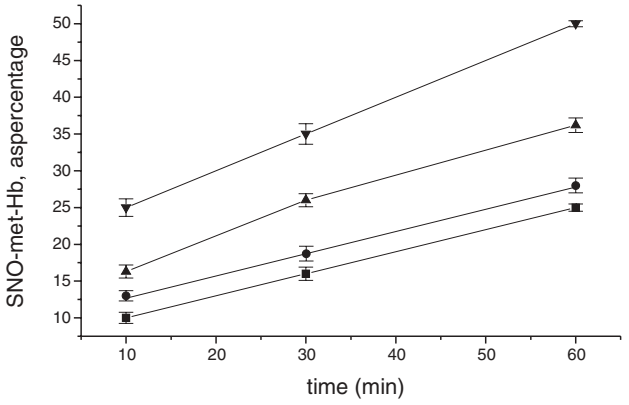

Fig. 2. Production of SNO-Hb from metHb and nitrosocysteine (CysNO) at different CysNO/haem molar ratios and at different times of incubation. MetHb (200 μM of haem) was mixed to CysNO to obtain the ratios indicated in the figure, in PBS (0.1 M, pH 7.4) + 0.5 mM EDTA, in a final volume of 1 ml. The reaction was carried at 37°C for various incubation times. SNO-Hb was determined by electrochemical assay, after purification of the protein on a Sephadex G25 column. -■-: Haem to CysNO ratio 1:1; -●-: Haem to CysNO ratio 1:2; -▲-: Haem to CysNO ratio 1:5; -▼-: Haem to CysNO ratio 1:10

to hem ratio 5:1 or greater. From these findings, we infer that the Cysβ93 of metHb is a better acceptor for NO^+ than the corresponding groups of oxyHb.

Assuming that hem iron in the blood is about 9 mM and that the amount of NO-bound to hem iron and to the –SH groups of Hb is about 900 nM, it follows that the concentration of bound NO is 1/10,000 the concentrations of oxygen (McMahon et al., 2000; Perutz, 1996). Taking into account that in RBC metHb is about 2–3% of total Hb (i.e. 170–270 μM haem iron in total blood), it follows that the amount of SNO-metHb is not irrelevant, in physiological conditions.

For CysNO to haem ratio of about 1, the haem centers of Hb are not oxidised. However, they are rapidly oxidised at ratios of 5 or more. Consequently, the prevailing form of SNO-Hb should be SNO-metHb. This observation further supports the idea that NO^+ reacts with OH^- to form nitrites, besides its reaction with thiol –SH groups (Gow and Stamler, 1998; Palmerini et al., 2002). Nitrite would then oxidize haem iron. This is in agreement with reported data claiming that SNO-metHb has a greater relaxing effects on vasal musculature than SNO-oxyHb.

SNO-Hb is usually determined as nitrite (Green et al., 1982) after $HgCl_2$ treatment (Saville, 1958). In this paper, we displace NO^+ from nitroso compounds by using a mixture of Cu^{2+} and cysteine before electrochemical determination.

Our finding supports the idea of a role of NO^+ transfer from nitrosothiols to –SH protein (and vice-versa). On the other hand, nitrosothiols have been found in blood plasma (Stamler et al., 1992), although the mechanism(s) through which they form remain(s) uncertain (Kharitonov et al., 1995). The allosteric transition towards the T form, favours the liberation of NO (from cysβ93) and its transfer to cytosolic protein thiols of the RBC ghosts (Gow et al., 1999; Pawloski et al., 2001).

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