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Redox thiol status plays a central role in the mobilization and metabolism of nitric oxide in human red blood cells

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Abstract

We assessed the redox thiol status influence on nitric oxide (NO) metabolism and efflux in erythrocytes stimulated with acetylcholinesterase substrate (acetylcholine, ACh) and inhibitor (velnacrine maleate, VM). Erythrocyte suspensions from healthy donors were incubated with increasing concentrations of dithiothreitol (1–50 μM), in the presence and absence of acetylcholine/velnacrine (10 μM). Levels of NO, nitrite/nitrate, S-nitrosohemoglobin, peroxynitrite and S-nitrosoglutathione were determined by spectrofluorimetric and spectrophotometric methods.

Dithiothreitol significantly mobilized NO toward nitrite/nitrate and S-nitrosoglutathione, and decreased the amount of NO efflux. Both ACh/VM induce changes on the levels of erythrocyte nitrite/nitrate dependent on the DTT concentration. Higher levels of peroxynitrite and S-nitrosoglutathione were seen with velnacrine in presence of DTT 1 and 50 μM.

We concluded that dithiothreitol-induced activation of erythrocyte thiol status decreases NO efflux and allows greater intracellular NO mobilization onto different derivative molecules, both in the absence and presence of acetylcholinesterase substrate and inhibitor.

Keywords: Acetylcholinesterase; Erythrocyte; Nitric oxide; Redox thiol status; S-nitrosothiols

1. Introduction

NO is a decisive modulator in microvascular homeostasis (Palmer et al., 1987). A central part of its bioactivity domain consists of the regulation of the microcirculation blood flow, aimed at controlling the balance between vasoconstriction and vasodilation. Classically, NO is a key component of the respiratory cycle endowed by its transport in erythrocytes, thereby having actions remote from its place of synthesis, the vascular endothelium (Pawloski et al., 2001; Pawloski and Stamler, 2002). Following its passage into red blood cells by diffusion, NO may either be stored or return to the bloodstream as an active S-nitrosothiol molecule. It interacts with several components of the erythrocyte, as well as with hemoglobin (Hb) (Gross and Lane, 1999). The major stable metabolites resulting from NO oxidation are represented by NO_x and include both nitrites (NO_2^-) and nitrates (NO_3^-). NO furthermore reacts with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), which may either yield nitrates or damage proteins, lipids and carbohydrates through oxidation and nitration reactions (Tarpey and Fridovich, 2001). The intraglobular NO binds to Hb through either to the heme group or to the β-chain cysteine 93. S-nitrosohemoglobin (SNO-Hb) is considered a central reservoir for NO (Gross and Lane, 1999; Dejam et al., 2005; Gladwin et al., 2004). Glutathione, an antioxidant molecule with a thiol group, binds to NO forming S-nitrosothiols, a secondary important storage.

We currently know that NO is selectively ferried throughout thiol groups of target proteins, thereby indicating

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this translocation to be on the basis of intracellular NO_x mobilization. Although the initial acceptor of NO from SNO-Hb appears to be the erythrocyte band 3 protein, namely a cysteine thiol within the cytoplasmic domain (Singel and Stamler, 2005; Rifkind et al., 2006), the identity of the species carrying NO bioactivity into the vessel wall (indicated as 'X-NO') is still unclear. Thiol groups are easily kept in the reduced state once a second thiol is added, such as cysteine or glutathione, since they rapidly exchange disulfide groups. Dithiothreitol (DTT) is an exogenous thiol reducing agent which owns thiol and non-thiol effects on humans, and functions as −SH donor.

Regarding to the setting of the Non-neuronal Cholinergic System (NNCS) components, it is well documented that blood circulating acetylcholine (ACh), is synthesized by endothelial cells and T-lymphocytes (Wessler et al., 2003). ACh can modulate erythrocyte hemorheological properties (aggregation and deformability) and membrane fluidity (Mesquita et al., 2002; Santos et al., 2003), and it modulates the red cell metabolism, plasma ions concentrations and induces NO translocation/metabolism (Carvalho et al., 2004b). Its effects on red cells have been questioned by the presence of other NNCS components, namely the membrane-bound acetylcholinesterase (AChE), known as a marker of membrane integrity. Recently, a signal transduction mechanism mediated by band 3 protein, associated with erythrocyte NO translocation enrolled by the active and less active AChE enzyme complexes and resulting from either substrate or inhibitor binding, has been observed (Carvalho et al., 2008; Saldanha, 1985).

According to these results, a redox status-dependent modulation of NO bioactivity, under the influence of the NNCS components, is hypothesized. Hence, this study was designed with the initial aim of assessing the *in vitro* influence of cellular redox status shift on the levels of erythrocyte-derived NO (and NO-derived molecules). A second aim was to follow the dependence of this mobilization onto derivative molecules in the presence of acetylcholine and the inhibitor, velnacrine maleate.

2. Methods

2.1. Preparation of erythrocyte suspensions

Human venous blood samples were collected from the forearm vein of 15 healthy caucasian men after informed consent. The blood container tubes were prepared with 10 IU ml^{-1} of sodium heparin (anticoagulant). The blood was centrifuged at 1040 g for 10 min in a Sorvall TC6 centrifuge. The plasma and buffy-coat (leucocytes and platelets) were discarded. Erythrocyte suspensions were made with the addition of sodium chloride (0.9% at pH 7,4, AnalaR, BDH Laboratory, Poole, UK) to reconstitute the initial hematocrit (45%).

Erythrocyte suspension aliquots were incubated for 30 min at room temperature in the presence or absence of dithiothreitol (1, 10 and 50 μM), acetylcholine (10 μM) and velnacrine maleate (10 μM). Twelve erythrocyte suspensions aliquots were treated as follows: control aliquots without DTT or AChE effectors; ACh 10 μM; VM 10 μM; DDT 1 μM; DDT 10 μM; DDT 50 μM; ACh 10 μM + DDT 1 μM; ACh 10 μM + DDT 10 μM; ACh 10 μM + DDT 50 μM; VM 10 μM + DDT 1 μM; VM 10 μM + DDT 10 μM; and VM 10 μM + DDT 50 μM.

2.2. Measurement of NO by an amperometric method

Following incubation, erythrocyte suspensions were centrifuged and sodium chloride 0.9 % at pH 7.0 was added in order to reach an Ht of 0.05%. The suspension was mixed by gently inversion of tubes.

For amperometric NO quantification, we used the *amino-IV* sensor (Innovative Instruments Inc. FL, USA), as previously described (Carvalho et al., 2004b). Briefly, after stabilization of the NO sensor immersed in erythrocyte suspensions ($n = 5$), the erythrocytes were stimulated with ACh 10 μM and changes in the electric current registered, the change being proportional to the amount of NO mobilized by ACh-stimulated erythrocytes.

2.3. Measurement of nitrite concentration in extra- and intra-erythrocyte compartments using the spectrophotometric Griess method

After incubation, erythrocytes suspensions as described above were centrifuged at 9600 g for 1 min using a Biofuge 15 Heraeus centrifuge. The supernatant was separated from the pellet to give packed erythrocytes.

Nitrite and nitrate levels in the intra-erythrocyte compartment were determined after submitting the pellet of each suspension to hemolysis and hemoglobin precipitation. Hemolysis was induced with distilled water and hemoglobin precipitation with ethanol and chloroform.

Nitrite concentration was measured with the spectrophotometric Griess reaction at 548 nm. For nitrate measurement, this compound was first reduced to nitrites in presence of nitrate reductase (Guevara et al., 1998).

2.4. SNO-Hb assay

Determination of SNO-Hb concentrations is based on a reaction where cleavage of S-nitrosothiols yields a nitrosant that activates 4,5-diaminofluorecein (DAF-2, Calbiochem, Darmstadt, Germany). After the incubation period, erythrocytes suspensions as described above were centrifuged at 9600 g for 1 min in a Biofuge 15 Heraeus centrifuge and the supernatants were separated from the pellet (packed erythrocytes). A volume of 500 μl H_2O (pH 7.0) was added to 500 μl of erythrocytes, to a final dilution of 1/200 with NaCl 0.9%. The solution was subsequently mixed with 2.11 μl DAF-2 (5 mM) (30 μmol/L final concentration). Half of this solution was reacted for 10 min with an equal volume of $HgCl_2$ (1.2 mmol/L). The reaction was terminated by adding a chloroform-ethanol mixture. Upon light excitation at 485 nm, DAF-2 associated light emission was read at 520 nm. SNOs were quantified as the difference in fluorescence signals