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Guest Editorial

NITRIC OXIDE – HEMOGLOBIN INTERACTIONS : ROLE IN OXYGEN UPTAKE AND DELIVERY

Nitric oxide (NO) is an endothelial cell derived endogenous vasodilator. The shear stress generated by flowing blood against the endothelial cell surface triggers the opening of calcium channels on endothelial cells, thereby leading to the calcium-dependent activation of endothelial NO synthase and increased local production of NO from the amino acid, L-arginine. Also calcium-independent activation of endothelial NO synthase can occur and lead to NO-mediated vasordaxation in response to shear stress. In 1980s, Ignarro et al postulated the mechanism by which NO activates the enzyme, soluble guanylate cyclase. NO interacts with heme iron to form NO-heme complex which remains as a five co-ordinate complex. The prophyrin binding site of guanylate cyclase undergoes conformational change when the heme iron is projected away from the enzyme protein and out of the plane of prophyrin ring. This conformational change exposes the catalytic site to the surface where GTP substrate and magnesium binds and guanylate cyclase is activated, resulting in increased formation of cyclic GMP (cGMP) which in turn activates cGMP – dependent protein kinase. The phosphorylation state of various proteins is then altered, and eventually, myosin light chain is dephosphorylated and relaxation occurs.

In the vascular system, various metabolic routes exist for the breakdown and/or conversion of NO. Plasma and red blood cells represent two potentially significant compartments for NO metabolism. NO can interact with molecular oxygen, thiols, and reduced hemoproteins. These reactions have considerable importance from the standpoint of NO bioactivity. In the human plasma NO is broken down to nitrite (NO_2^-), which in physiological concentrations has very little vasodilator activity (1). Studies in animals have revealed that NO reacts with redox-active thiols present in plasma to form bioactive S-nitrosothiols (RSNOs) (2). In the presence of oxygen, NO mainly reacts with plasma albumin to form S-nitrosoalbumin (SNOAlb) (3). There is no doubt about the existence of RSNOs and SNOAlb, but the mechanism of formation and subsequent release of NO from these compounds is poorly understood.

In red blood cells (RBCs), NO is rapidly deactivated by oxygenated hemoglobin (HbO_2) to form nitrate and methemoglobin. This means that the large amount of hemoglobin (Hb) present in the circulating erythrocytes of a normal adult should act as a potent sink for NO. Ignarro et al in 1987 demonstrated that endothelium dependent relaxation is sensitive to inhibition by HbO_2. In vascular relaxation experiments, HbO_2 produced concentration-

dependent inhibitory effects on NO mediated relaxation in isolated bovine intrapulmonary artery rings. HbO_2 (and also oxygenated myoglobin, MbO_2) lowers cGMP levels, increases tone in endothelium intact artery, and abolishes the marked accumulation of vascular cGMP caused by NO (4). Never the less, bioactivity of NO remains to exert control over vascular tone. In the natural set up, however, bioavailability of NO is preserved by the diffusional barriers between NO-producing endothelium and erythrocytes encapsulating Hb. These barriers include an RBC-free layer of plasma along the endothelium, RBC membrane, and cytoskeleton and associated NO-inert proteins. These barriers slow down the rate of reaction of NO with Hb within the erythrocytes as compared to that with free Hb.

The reactions of NO with Hb are linked to the allosteric properties of Hb and may lead to either conservation or consumption of NO. It may react with either HbO_2 to form nitrate, or with Hb to form nitrosylhemoglobin, or with the 93-cysteine residue of the β-subunit to form S-nitrosohemoglobin. Depending upon the partial pressure of oxygen, Hb exists in two alternative structures: R (relaxed with high O_2 affinity) and T (tense with low O_2 affinity). Hb assumes the T structure to efficiently release oxygen. The allosteric transition in Hb (from R- to T- state) controls the reactivity of NO with hemes and/or cysteine residues of Hb forming alternatively Hb(Fe(II)NO and SNO-Hb. Thus, iron nitrosylhemoglobin and SNO-Hb are in a dynamic, redox – dependent equilibrium [HS-Hb – (Fe(11) NO $(Fe(11)_3$ + $4O_2 \rightarrow$ SNO – Hb $[(Fe(11)\ O_2]4 + e^-$]. Thiol affinity for (S)NO is high in the R structure and low in the T structure, i.e. NO group is released from thiols of Hb in low PO_2 (5). A major function of (S)NO in the vasculature is to regulate blood flow, which is controlled by the resistance arterioles. On the other hand, NO is irreversibly consumed when it reacts with HbO_2 to produce methaemoglobin and nitrate: NO + Hb $(Fe2^+)$ $O_2 \rightarrow NO_3^-$ + Hb (Fe^{3+}).

However, this reaction is limited by the hydrophobic micro-environment of the RBC membrane. Deoxygenated Hb preferentially binds NO at the heme group to form nitrosyl (heme) hemoglobin [Hb(FeII)NO] (5). The affinity of deoxygenated hemoglobin for NO is greater than for either oxygen or carbon monoxide. Hb(FeII)NO is a six-coordinate species (iron binds four nitrogens, a proximal histidine, and NO). On deoxygenation of the other heme groups, the proximal histidine bond stretches or breaks, forming a five coordinate species with a lowered oxygen affinity. Hb(FeII)NO exerts an enhanced Bohr effect and promotes oxygen release (6). Therefore, Hb(FeII)NO may be more effective in delivering oxygen than pure Hb, especially in regions with low oxygen tension. Despite an apparent potential biological importance, the amount of Hb(FeII)NO formed in the body is too low to be detected by electron paramagnetic resonance, the primary method for measuring this species. Upon oxygenation NO group is transferred from Hb(FeII)NO to β-93 cys to form S-nitroso Hb (SNOHb). On deoxygenation in the tissues, the S-nitroso linkage is weakened and NO is released out of RBCs through the anion exchange protein, band 3, thereby resulting in vasodilation of the microvasculature (7). Functional and crystallographic studies have shown that the cys-β93 residues at which NO is bound as NO^+ in SNOHb are more accessible in the high affinity conformation of oxy (R-state) Hb than in deoxy (T-state) Hb (8). NO group release from SNOHb is accelerated in RBCs by glutathione (9). The

yields of various species of nitrosylated Hb are dependent on the NO/Hb ratio. Stamler and colleagues in 1998 have proposed a model in which the T-state hemes in the venous blood transfer their NO group to R-state thiols after oxygenation in the lungs (5). SNO-Hb then moves through the systemic circulation till it encounters low pO_2 in the resistance vessels that promotes the transition to T state and affects NO release. Some of this NO transferred to smooth muscles relaxes blood vessels and thereby promotes O_2 delivery. Thus, SNO-Hb acts as a reservoir for bioactive NO which is released during the oxygen-linked conformational shift of hemoglobin from its R-to-T state. Under physiological conditions rate of SNOHb formation is always greater than irreversible oxidation, and thus some NO is conserved.

The amount of SNO-Hb naturally occurring in the blood and its role in tissue oxygen delivery has been a point of controversy because of conflicting reports from different research groups. Stamler et al in 1996 reported that arterial – venous gradient exists for SNO – Hb and that delivery of O_2 and NO are allosterically coupled events (9). In yet another experiment carried out in 1999, Stamler et al showed that oxidation of nitric oxide to nitrate by HbO_2 under physiological conditions is of little clinical significance. They studied the reactions that occur on exposure of Hb to NO at physiological concentrations. It was found that the addition of NO to HbO_2 takes advantage of the co-operative effects of oxygen binding and thus effectively competes with the oxidation reaction. At high oxygen saturations, S-nitrosylate reaction to the protein occurs predominantly, and thus NO bioactivity is preserved (10). Regulation of blood flow is a function of arteriolar diameter to the fourth power, therefore requires low nanomolar SNO. Stamler et al realized that their finding of significant amount of SNO-Hb in arterial blood would not, however, be compatible with life or NO biology for two reasons. First, a large amount of SNO (from Hb) during each arterial venous cycle would cause life threatening hypotension and shunting of blood. Second, an organism that produces less NO would be loaded with it producing an insurmountable metabolic burden. They carried out further experiments in 2000 to address this issue and showed that (i) increased oxygen affinity of SNO-Hb restricts the hypoxia-induced allosteric transition that exchanges NO groups with ambient thiols for vasorelaxation, (ii) some NO groups released from cysteine β93 upon transition to T structure are autocaptured by the hemes, even in the presence of glutathione, and (iii) an oxygen – dependent equilibrium between SNO-Hb and iron nitrosylhemoglobin acts to conserve NO (11). Thus, by sequestering a significant fraction of NO liberated upon transition to T structure, Hb can conserve NO groups that would otherwise be released in an untimely or deleterious manner.

The SNO-Hb hypothesis however has been challenged by various research groups. In 2000 Gladwin et al quantified the reactions of NO with Hb under physiological conditions. Total nitrosylated haemoglobin as well as individual levels of Hb(FeII)NO and SNO-Hb were measured in the arterial and venous circulation of five normal volunteers before and during NO breathing. Because the basal levels of nitrosylated haemoglobin in the human circulation are very low, NO inhalation was used to augment NO binding to haemoglobin. Very low levels of SNO-Hb (0.26 μM heme) could be detected and without any significant

arterial – venous gradient suggesting that SNO-Hb has a minimal role in NO delivery under basal conditions (12). In 2002, Lancaster et al suggested that the large amount of SNO-Hb formed in the study of Stamler et al could be due to bolus addition of NO which raises its concentration to exceptionally high unphysiological levels (13). In a separate study, Gladwin et al in 2002 have demonstrated that the reductive intra-erymrocytic environment decomposed SNO-Hb and prevented its accumulation as a reservoir of bioactive NO (14). Thus, the predominant action of SNO-Hb appears to be similar to HbO_2, i.e. consumption rather than conservation of NO. The inactivation of NO is prevented primarily by diffusional barriers, and they reduce the reaction of NO with HbO_2 by a factor of 1000. However, the determining factor for vasodilatation is not the amount of SNO-Hb present in the erythrocyte, but the amount of free NO produced from mis SNO-Hb that is able to stimulate soluble guanylate cyclase in the smooth muscle.

It now appears that NO bioactivity plays an important role in cardio-respiratory cycle. Irrespective of pO_2, the total amount of NO bound to the hemoglobin remains constant, but is exchanged between hemes and thiols as a function of pO_2. Thioesteric effects brought about by S-nitrosation of cys- β93 thus act in opposition to the well known allosteric effectors of Hb function (protons, chloride, organic and inorganic phosphates, bicarbonate, and carbon dioxide) that typically decrease oxygen affinity and thereby enhance oxygen delivery to respiring tissues. It is probable that the cys-β93 residues do not assume their normal position in the deoxygenated molecule, and inhibit the formation of salt bridge between His-β146 and Asp-β94. This critical salt bridge is oxygen – linked, and its pH-dependent formation accounts for about half of the normal Bohr effect (8). Kinetic measurements and bioassay data indicate that heme iron functions to capture NO bioactivity whereas cysteine thiols function in NO delivery. HbCysβ93 acts as an allosterically controlled NO buffer, exchanging the NO group with ambient thiols, including glutathione, thereby regulating blood flow, the critical determinant of O_2 delivery. Studies in the rat brain have showed that interactions of NO with Hb can couple local blood flow to tissue oxygen tension by producing hypoxic vasodilation and hyperoxic vasoconstriction (15). Thus the respiratory cycle can be viewed as a "three gas ($NO/O_2/CO_2$) system" in which NO and O_2 are simultaneously taken up in the lung and then delivered to the tissue during the arterial/venous transit. Hb delivers to oxygen deficient vascular beds not only the oxygen required for sustained metabolism but also a vasodilator (NO).

It is now well established that the delivery of O_2 to the tissues depends primarily upon the flow of oxygenated blood in the microcirculation rather than the oxygen content of blood. Blood flow is controlled in part by NO/SNO and NO level in blood may provide an index of microcirculatory flow of importance in clinical decision making. The ability to monitor and manipulate levels of RBC – NO may prove useful in the assessment of blood gases, in the diagnosis and treatment of diseases of the heart, lung and blood, and in the rational development of therapeutics, including NO donors, erythropoietin therapy and blood substitutes. Systemic application of NO may compensate for the NO deficiency seen in various cardiovascular diseases associated with endothelial dysfunction. Increased understanding of the physiological and pathophysiological roles of NO in the

cardiovascular system may lead to novel opportunities for therapy in conditions such as hypertension, atherosclerosis, myocardial infarction, and shock.

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