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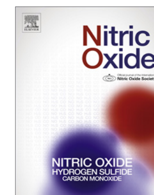

Hypoxic potentiation of nitrite effects in human vessels and platelets

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ABSTRACT

Previous studies in non-human blood vessels and in platelets have demonstrated that under hypoxic conditions release of NO from nitrite (NO_2^-) is potentiated by deoxyhaemoglobin. In the current study, we characterized hypoxic potentiation of NO_2^- effects in human vasculature and platelets *in vitro*, addressing underlying mechanisms.

The vasodilator efficacy of NO_2^-, in comparison with glyceryl trinitrate (GTN), was evaluated *in vitro*, using segments of human saphenous vein. Under hypoxic conditions, there was a leftward shift of the NO_2^- concentration–response curve (EC_{50}: 22 μM in hyperoxia vs 3.5 μM in hypoxia; $p < 0.01$), but no significant potentiation of GTN effect. In the presence of red blood cells, hypoxic potentiation of NO_2^- vasodilator effect was accentuated.

In whole blood samples and platelet-rich plasma (PRP) we assessed inhibition of platelet aggregation by NO_2^- (1 mM), in comparison with that of sodium nitroprusside (SNP, 10 μM). In individual subjects ($n = 37$), there was a strong correlation ($r = 0.75$, $p < 0.0001$) between anti-aggregatory effects of NO_2^- and SNP in whole blood, signifying that resultant sGC activation underlies biological effect and responses to NO_2^- are diminished in the presence of NO resistance. In PRP, the effects of NO_2^- were less pronounced than in whole blood ($p = 0.0001$), suggesting an important role of Hb (within RBCs) in the bioconversion of NO_2^- to NO. Inhibition of platelet aggregation by NO_2^- was almost 3-fold greater in venous than in arterial blood ($p < 0.0001$), and deoxyHb concentration directly correlated ($r = 0.69$, $p = 0.013$) with anti-aggregatory response. Incremental hypoxia applied to venous blood samples (in hypoxic chamber) caused a progressive increase in both deoxyHb level and anti-aggregatory effect of NO_2^-. When subjects inhaled a 12% O_2 mixture for 20 min, there was a 3-fold rise in blood deoxyHb fraction ($p < 0.01$). In PRP, response to NO_2^- also increased under hypoxia, and was further enhanced ($p < 0.01$) by deoxyHb. Furthermore, deoxyHb exerted significant anti-aggregatory effects even in the absence of added NO_2^-, suggesting a role for endogenous NO_2^-.

The results of this work provide further mechanistic insights into hypoxic potentiation of vasodilator and anti-aggregatory actions of NO_2^-. In human saphenous veins and blood, the balance of evidence suggests differential rates of NO release from NO_2^- (largely modulated by deoxyHb) as the fundamental mechanism.

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1. Introduction

The extensive physiological roles of nitric oxide (NO) in cardiovascular control and homeostasis of platelet function have been delineated over the past 30 years [1]. However, the physiological bases for NO generation are now understood to include not only bioconversion of arginine by NO synthases (NOS) [2] but also reduction of nitrite (NO_2^-), which was previously considered to be an inert product of NO metabolism [3–6]. In the early 1950's, Furchgott and co-workers demonstrated that high concentrations of sodium nitrite induced vasodilator responses [7]; the cellular mechanisms underlying this vasodilator effect have been shown to culminate in NO release. As NO can be generated from NO_2^- independently of NOS function, both the physiological and potential therapeutic roles of NO_2^- are of increasing interest (for review see [8]).

Abbreviations: NO_2^-, nitrite; SNP, sodium nitroprusside; CPTIO, carboxy-PTIO; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; Hb, haemoglobin; Mb, myoglobin; RBC, red blood cells.

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NO_2^- exhibits two potential advantages as a treatment for acute cardiovascular disorders. First, vasodilator responses of NO_2^- are potentiated in the presence of hypoxia [5,8]. In theory, this might facilitate selective vasomotor effect at sites of myocardial ischaemia, and might also potentiate NO_2^--mediated dilatation in the presence of acute heart failure [9]. Furthermore, NO_2^- vasomotor effects are not subject to tolerance induction and also do not exhibit cross-tolerance with organic nitrates [9,10]. Potentiation of NO generation from NO_2^- under hypoxia might also substantially augment the anti-aggregatory effects of NO_2^- [11,12], and this might be of particular relevance in myocardial ischaemia/heart failure. In fact, potentiation of NO generation from NO_2^- has been demonstrated to correlate with deoxyhaemoglobin concentrations in animal vascular studies [13] and also in platelet-rich plasma [12].

We have therefore undertaken a study in human subjects, in order to evaluate the phenomenon of 'hypoxic potentiation' of NO_2^- in human vasculature and platelets as regards its underlying mechanisms. We assessed vasodilator effects of NO_2^- in comparison to those of the NO donor glyceryl trinitrate (GTN) on human saphenous veins *in vitro* in hyperoxia and hypoxia. We also examined anti-aggregatory effects of NO_2^-, in comparison with those of the more direct NO donor sodium nitroprusside (SNP), in venous and arterial whole blood and platelet-rich plasma samples obtained from healthy subjects and patients with ischaemic heart disease (IHD). The results provide incremental insights into the therapeutic potential of NO_2^- as a treatment for acute cardiac illnesses.

2. Materials and methods

2.1. Subject selection

In order to study vascular reactivity *in vitro*, we recruited patients undergoing coronary artery bypass grafting surgery. During the surgery, discarded segments of saphenous vein were collected and immediately stored on ice-cold Krebs for transportation to the laboratory.

In order to evaluate a group of individuals with variable platelet responsiveness to NO, we studied both healthy subjects and patients with ischaemic heart disease, including individuals undergoing non-emergent cardiac catheterization, given that the latter have a high prevalence of NO resistance [14–16]. The only exclusion criterion was current therapy with ADP receptor antagonists, given that this would preclude assessment of reversal of ADP induced aggregation by NO donors and NO_2^-.

The protocols were approved by the Ethics of Research Committee of The Queen Elizabeth Hospital and informed consent was obtained prior to study entry.

2.2. Platelet aggregation studies

These studies were performed as described previously [15]. Blood samples were collected by venesection from an antecubital or femoral vein or a femoral artery into plastic tubes containing 1:10 volume of acid citrate anticoagulant (two parts of 0.1 M citric acid to three parts of 0.1 M trisodium citrate); acidified citrate was utilized to minimize deterioration of platelet function during experiments. Blood was centrifuged at 250*g* for 10 min at room temperature to obtain red blood cells (RBC) (used in vascular experiments, see below) and platelet-rich plasma (PRP). To assure a complete removal of RBCs, PRP was spun at 2500*g* for 10 s and the supernatant was collected. Absence of Hb was proved with Radiometer Copenhagen NPT 7 Series Blood Gas Analyser.

Platelet aggregation was examined utilizing impedance aggregometers (Models 560 and 700, Chrono-Log, PA, USA). In brief, tests were performed at 37 °C and stirring speed of 900 rpm. Samples of whole blood or PRP were diluted twofold with normal saline (final volume 1 ml) and pre-warmed for 5 min at 37 °C. Aggregation was induced with ADP (final concentration of 2.5 μM). Aggregation was monitored continually for 7 min, and responses were recorded for electrical impedance in Ohms. SNP and $NaNO_2$ (final concentration of 10 μM and 1 mM, respectively) were added to samples 1 min before ADP. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the anti-aggregatory agent studied. Throughout the platelet aggregation experiments, SNP was utilized as the comparator NO donor.

We utilized a custom-made Plexiglas hypoxic chamber (see schematic Fig. 1) so that aggregation could be evaluated under controlled hypoxia.

Oxygen concentration within the hypoxic chamber was monitored with an oxygen analyzer (Teledyne Brown Engineering, Inc., USA).

To check pH, Hb fractions and oxygen saturation of blood utilized in the platelet aggregation studies, the samples were collected into PICO 70 Radiometer blood gas syringes containing 60 IU of heparin and processed on the Radiometer Copenhagen NPT 7 Series Blood Gas Analyser after being incubated in the aggregometer as per protocol. The same gas syringes and the blood gas analyser were utilized to measure those parameters in organ baths when vessels were incubated with RBCs.

2.3. Vascular reactivity studies

During the operation, the discarded segments of the proximal saphenous vein (SV) were collected. The segments were placed in ice-cold Krebs solution, cleaned, and cut into 2 mm wide rings.

Vascular rings were suspended under tension in 15-mL organ baths containing Krebs solution at 37 °C. Resting tension was normalized for internal ring diameter, as previously described [17,18]. Mean resting tension was 1 g. SV ring resting tension was set at 1 g because this tension gave optimal contractions to KCl solution (120 mM) in preliminary experiments. The rings were equilibrated for 60 min before exposure to KCl solution; rings contracting <1 g were discarded. After a further 30 min of washout, the rings were contracted with increasing concentrations of phenylephrine (0.01–100 μM).

After a further 45 min of washout, the rings were preconstricted with phenylephrine to produce 70% of maximum tension in the SV. Throughout the vascular experiments, GTN was utilized as the comparator NO donor. Once the contractile response had reached

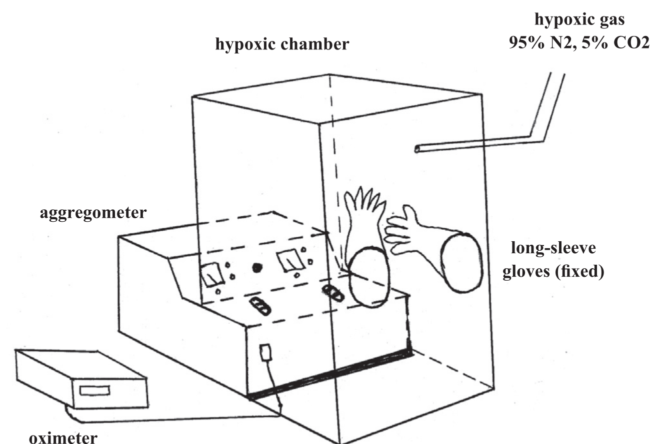

Fig. 1. Schematic: hypoxic chamber.

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