HAEMOGLOBIN SATURATION CONTROLS THE RED BLOOD CELL MEDIATED HYPOXIC VASORELAXATION

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Abstract: The vasorelaxant properties of red blood cells (RBCs) have been implicated in both the control of normal vascular tone and the protection of tissues from ischemic events. The identity of the vasorelaxant released from RBCs has yet to be elucidated, however growing evidence suggests that nitric oxide bound to the ß93 cysteine residue of haemoglobin (SNO-Hb) may be responsible. The vasorelaxant moiety is released during the transition of haemoglobin from its R (oxygenated) to T (deoxygenated) state. We subsequently chose to assess the significance of haemoglobin saturation on the capacity of RBCs to mediate hypoxic vasorelaxation.

Human RBC samples suspended in saline were manipulated in a thin film rotating tonometer, designed to rapidly change haemoglobin saturation within the time frame of circulatory transit. Various cycles of oxygenation and deoxygenation were performed. The vasorelaxant properties of the RBCs were analysed using an aortic ring bioactivity assay, wherein changes in isometric tension were recorded to study vessel relaxation. The rabbit aortic rings were preconstricted with phenylephrine under hypoxic conditions (~1% O_2) prior to RBC addition.

Highly saturated RBCs (98.22% ± 0.45 HbO_2) elicited significantly ($P<0.001$) more relaxation of hypoxic blood vessels compared to those partially saturated (20.40% ± 5.28 HbO_2). Upon re-oxygenation, previously de-oxygenated RBCs were also capable of eliciting vessel relaxation, which was not significantly different from that observed with the original oxygenated RBC relaxation response. Interestingly, the relaxant capability

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P. Liss et al. (eds.), *Oxygen Transport to Tissue XXX*, DOI 10.1007/978-0-387-85998-9_3,

was not simply returned from extracellular milieu upon re-oxygenation. This data provides further evidence that the conformational switch of haemoglobin from the R-state (oxygenated) to the T-state (deoxygenated) is essential for the release of the vasoactive moiety contained within red blood cells.

1 INTRODUCTION

Nitric oxide (NO) represents one of the most prominent, endogenous regulators of vascular tone and vessel health. A proportion of endothelial derived NO ultimately enters the vessel lumen where it can undergo a number of reactions with so-called 'scavenging' entities, perhaps the most noteworthy of which being haemoglobin. Haemoglobin contained with RBCs has the capacity to scavenge NO in both its deoxy and oxy conformations at a considerable rate, of approximately 10^7 M^{-1} s^{-1}[1, 2]. The products of these reactions are largely NO bound to the oxygen binding site of haemoglobin (HbNO) and the nitrate anion (NO_3^-) with met-haemoglobin respectively. These products alone are of little bioactive consequence, however HbNO is proposed to provide a source of NO for the formation of a second haemoglobin bound NO entity, SNO-Hb. Following the conformational switch in haemoglobin from its T-state (deoxy) to its R-state (oxy) across the lung, NO is belived to be passed from the oxygen binding site (HbNO) to the ß93 cysteine residue of haemoglobin (SNO-Hb)[3].

Evidence exists to suggest that an NO species released from SNO-Hb may represent the RBC vasorelaxant, whose effectiveness is proportionate to haemoglobin oxygen saturation[3, 4]. At present the precise identity of this vasorelaxant remains to be established, however the RBC induced relaxant is ODQ sensitive[4], demonstrating cGMP and thus NO dependence. In addition, the response is also independent of both endothelium and nitric oxide synthase (e.g. L-NMMA insensitive)[4] suggesting that the relaxant can directly stimulate smooth muscle cells. The release of an NO species from SNO-Hb is understood to occur via a mechanism that is essentially opposite to its formation. When oxygenated RBCs (R-state) encounter an oxygen demand, oxygen is liberated causing their haemoglobin to switch from the R-state to the T-state resulting in the simultaneous release of an NO moiety. Here we provide *ex vivo* evidence to support this *in vivo* theory in addition to data that may help to confirm the identity of this elusive species, utilising the well established organ chamber bioassay. This system provides a highly sensitive and reproducible investigation tool, allowing the relaxant capacity of RBCs (following different interventions) to be accurately determined.

2 MATERIALS AND METHODS

2.1 Aortic Ring preparation

Male New Zealand white rabbits (2 to 2.5 Kg) were terminally anaesthetised by intravenous injection of sodium pentobarbitone (0.75ml/Kg). The aorta was harvested and endothelium intact rings of thoracic aorta were prepared for isometric tension recordings. Aortic rings were mounted at 2g resting tension, 37°C in 5ml Krebs buffer (NaCl 109.0 nM, KCl 2.7 nM, KH_2PO_4 1.2nM, $MgSO_4.7H_2O$ 1.2 nM, $NaHCO_3$ 25.0 nM,

$C_6H_{12}O_6$ 11.0 nM, $CaCl_2.2H_2O$ 1.5 nM, all Fisher Scientific). Appropriate gas mixes were used to bubble gas into the bottom of the baths to achieve desired O_2 concentrations as previously described[4]. Tissue was pre-conditioned at 95% O_2 with phenylephrine (PE) 10^{-6}mol/L and Acetylcholine (Ach) 10^{-5}mol/L (both from Sigma UK, Poole). At ~1% O_2 PE $3x10^{-6}$mol/L was used to induce constriction before 20µL of RBC pellet was injected into each bath.

2.2 Blood Collection and Preparation

Fresh blood samples were collected by venepucture of the antecubital vein from normal male and female subjects immediately prior to requirement. Samples were centrifuged (1200g, 5mins, 4°C) and the plasma and buffy coat were removed. RBCs were then diluted (1 in 10 to give approx. 3g/dL haemoglobin) in isotonic saline (0.9% w/v, Fresenius Kabi) before loading into the thin film rotating tonometer[5] (Figure 1) maintained at 37°C. The rotating tonometer (which is constantly purged with gas) creates a thin film of RBC in solution, providing optimum conditions for rapid gas exchange. This devise allows the manipulation of oxygen gradients between ~ 70-100% HbO_2 within a physiological circulatory transit time. A RBC sample can be fully deoxygenated (100% to ~ 20% HbO_2) within minutes. Different gas mixes were used (medical grade 95% O_2/5% CO_2 and 95%N_2/5% CO_2) along with different oxygenation-deoxygenation cycles (Figure 2). HbO_2% and haemoglobin content were measured using a blood gas analyser (OSM 3 Hemoximer, Radiometer, Copenhagen).

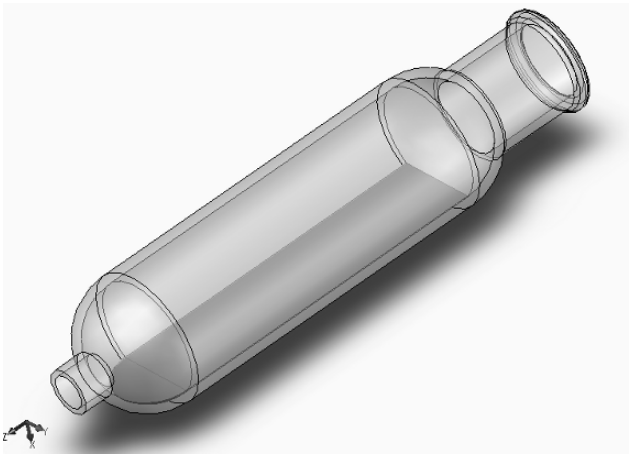

Figure 1. Diagram of the thin film rotating tonometer.

To identify whether the vasorelaxant moiety was simply passed between the RBC and the extracellular milieu upon de-oxygenation and re-oxygenation, RBC samples taken to low saturation had the extracellular milieu removed prior to re-oxygenation by

repeated washing with fresh oxygenated bottled saline, prior to having saturation and haemoglobin content retested (Figure 2). Finally all samples were centrifuged (1200g, 3mins 4°C) and the saline removed before addition of RBC pellet to the aortic ring preparation (see section 3.1).

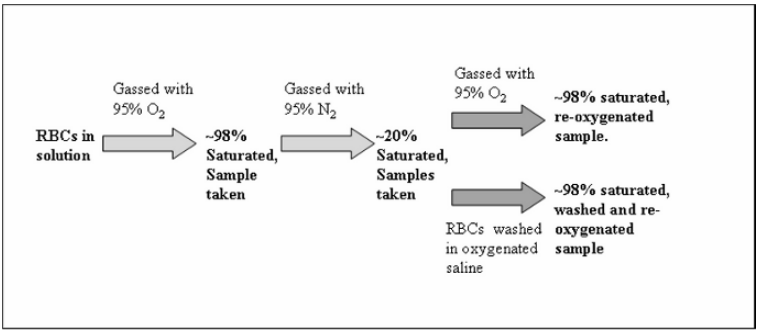

Figure 2. Flow chart of experimental RBC preparation.

2.3 Data Collection and Analysis

Isometric tension was recorded from transducers linked to a Powerlab 8Sp/octal bridge (AD Instruments) connected to a PC running 'Chart for Windows'. For each experiment, eight aortic ring preparations were run in parallel, n = 1 represents data averaged from 2 individual aortic ring preparations given identical treatment. % relaxation is the induced decrease in gram tension as a percentage of the maximum contraction. Groups were compared with a standard *t* test or a one-way analysis of variance using a Tukey's multiple comparison *post hoc* test. Assessment of correlations was made using Pearson's correlation coefficient. All data are reported as mean ± standard deviation, in all cases $p < 0.05$ was considered significant.

3 RESULTS

3.1 The Effect of Saturation on RBC Induced Relaxation

Highly saturated RBCs (~ 98% HbO_2) elicited significantly greater relaxation than highly saturated RBC rapidly deoxygenated to ~ 50% and ~ 20% HbO_2 ($p < 0.01$ and $p < 0.001$, respectively; Figure 3). RBC relaxation was found to directly correlate with HbO_2 % (Figure 4), with relaxation increasing with HbO_2 ($r = 0.815$, $P<0.0001$).

3.2 The Effect of Saturation Cycling on RBC Induced Relaxation

Figure 5 demonstrates the effect of saturation cycling on RBC induced relaxation. RBCs immediately taken to a high saturation (98.22% ± 0.45 HbO_2) induced a mean relaxation of 12.00% ± 3.60. Following the de-saturation of these RBCs to ~ 20% and

their return to a high saturation (97.70% ± 0.39 HbO_2) a mean relaxation of 8.28% ± 2.97 was observed. Although the mean relaxation induced by re-oxygenated RBCs was lower than that of RBCs with a high saturation, the trend was not statistically significant.

3.3 Potential Cross-Membrane Transfer of the Vasorelaxant

RBCs taken from a high saturation to a low saturation level, followed by removal of the extracellular milieu and re-oxygenation (washed in oxygenated saline) induced a mean relaxation of 12.97% ± 1.89 compared to RBCs taken immediately to a high saturation 12.0% ± 3.60 (Figure 6). This demonstrates that the removal of the initial extracellular milieu and replenishment with fresh saline has no significant effect on RBC induced relaxation. In addition, preliminary data suggest that the presence of NEM (a thiol blocker which prevents transnitrosation) reduces RBC induced relaxations when incubated with highly saturated RBCs (data not shown).

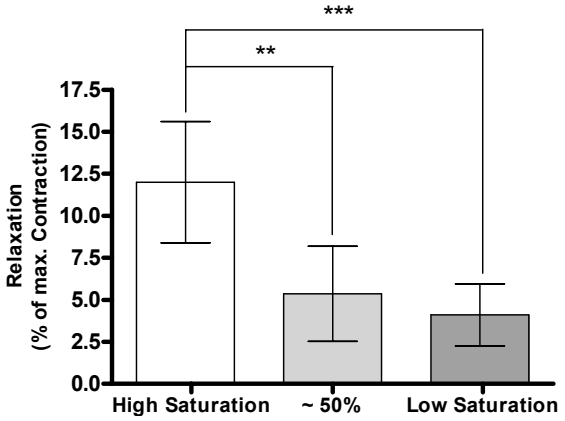

Figure 3. Red blood cell induced relaxations produced by highly saturated red cells (average saturation 98.22% ± 0.45, n=13) compared to red cells approximately 50% saturated (average saturation 51.43% ± 6.16, n=4) and to red cells of low saturation (average saturation 20.40% ± 5.28, n=13). Both the 50% and low saturation RBCs elicited a significantly lower level of relaxation than the highly saturated RBCs **P<0.01, ***P<0.001. Groups were compared by one way ANOVA and Tukey's multiple comparison *post hoc* test, bars represent standard deviation.

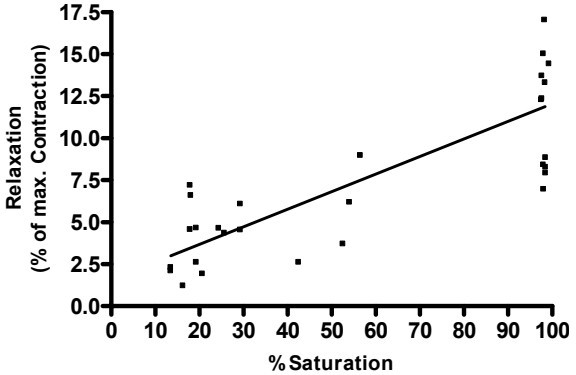

Figure 4.Plot of RBC induced relaxations vs. % RBC saturation A significant positive correlation (R=0.815, p<0.0001, n = 30, between he two variables.

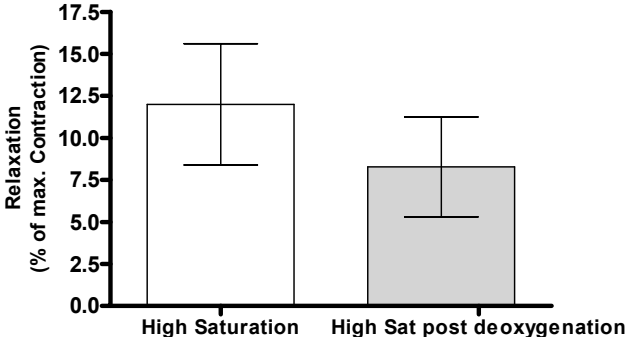

Figure 5. Red blood cell induced relaxations produced by highly saturated red cells (average saturation 98.22% ± 0.45, n=13) compared to highly saturated red cells which were previously taken down to a deoxygenated saturation of ~ 20% (average saturation 97.70% ± 0.39, n=4). No significant difference between groups, bars represent standard deviation.

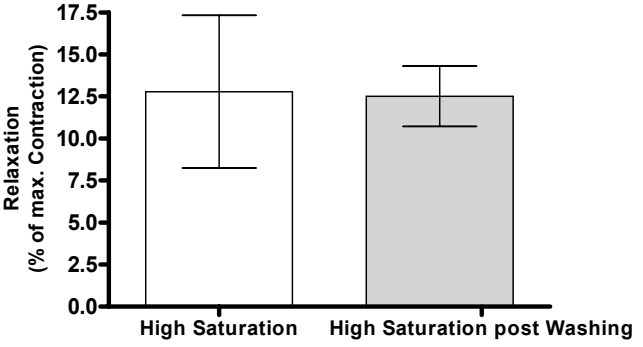

Figure 6. Red blood cell induced relaxations produced by highly saturated red cells (average saturation 98.22% ± 0.45, n=13) compared to Highly saturated red cells which were previously taken down to a deoxygenated saturation of ~ 20% then centrifuged at 1200g and had the extracellular solution removed and were then washed 3 times in oxygenated saline to bring the saturation up (average saturation 98.05% ± 0.65, n=4). No significant difference between groups, bars represent standard deviation.

4 DISCUSSION

The ability of fully oxygenated native human RBCs to induce relaxation in the hypoxic organ chamber bioassay is well known. This *ex vivo* response, demonstrated to be at least in part mediated by NO, has been attributed to several mediators within the RBC, including SNO-Hb[6], ATP[7] and/or nitrite [8, 9]. More recently however increasing evidence has supported SNO-Hb as the RBC vasorelaxant.

SNO-Hb is proposed to be relatively stable under oxygenated conditions. Only upon the switch in haemoglobin from the R-state (oxygenated) to the T-state (deoxygenated) conformation is the NO moiety purportedly made available for release. We therefore examined the effect of RBC haemoglobin saturation on the capacity of RBCs to induce relaxation in hypoxic vessels. We now demonstrate:

- RBC induced vasorelaxation is dependent on (and directly proportional to) haemoglobin oxygen saturation. The release of oxygen must occur to allow the conformational switch to take place, releasing the vasoactive moiety contained within RBCs. The presence and liberation of oxygen from haemoglobin purely acting as a 'release switch'.
- RBCs contain a considerable (potentially able to be replenished) store of vasorelaxant which is not released in its entirety during an individual hypoxic exposure/event. RBCs deoxygenated and subsequently re-oxygenated were in fact still able to elicit a notable relaxation. This raises the question as to why only a proportion of vasorelaxant is released upon each hypoxic exposure? We are currently investigating this further.
- The RBC vasorelaxant is not simply passed back and forth between the RBC supernatant and the RBC following a deoxygenation-re-oxygenation cycle. Replacing the extracellular milieu (saline) had no effect upon the capacity of the RBC to relax the vessels.
- The relaxant species released from the RBC must be capable of avoiding re-capture by or reaction with haemoglobin in the local region of release. The proposed mechanism of exit in the case of an NO moiety released from SNO-Hb is via transnitrosation reactions (thiol to thiol transfer) across the RBC membrane[10]. In this bound state the NO moiety is effectively protected from haemoglobin re-capture/reaction. We have carried out some preliminary work that supports this hypothesis. RBCs transiently incubated with the thiol blocker NEM did not appear to generate relaxations of the same magnitude as those that were not exposed. It is possible that NEM is blocking potential 'carrier' thiols on proteins located in the RBC membrane, reducing the amount of vasorelaxant reaching the tissue. At the very least this demonstrates that thiols are involved in the release mechanism of the NO moiety.

This study is the first of its kind to investigate the oxygen dependency of ex vivo blood vessel relaxation responses utilising intact RBCs. In conclusion, we provide further evidence that the degree of haemoglobin saturation is a crucial factor in the hypoxic RBC vasorelaxant response, supporting the idea that the vasorelaxant response is associated with a change from the R to the T-state conformation of the haemoglobin molecule.

5 ACKNOWLEDGEMENTS

We would like to thank Ross Pinder for his help creating figure 1 and Dr Doctor of the Department of Paediatrics and Biochemistry & Molecular Biophysics, Washington University in St Louis for the use of the thin film rotating tonometer. We also thank the British Heart Foundation for their continued support.

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