

# Oxygen Mediates Vascular Smooth Muscle Relaxation in Hypoxia

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## Abstract

The activation of soluble guanylate cyclase (sGC) by nitric oxide (NO) and other ligands has been extensively investigated for many years. In the present study we considered the effect of molecular oxygen (O<sub>2</sub>) on sGC both as a direct ligand and its affect on other ligands by measuring cyclic guanosine monophosphate (cGMP) production, as an index of activity, as well as investigating smooth muscle relaxation under hypoxic conditions. Our isolated enzyme studies confirm the function of sGC is impaired under hypoxic conditions and produces cGMP in the presence of O<sub>2</sub>, importantly in the absence of NO. We also show that while O<sub>2</sub> could partially affect the magnitude of sGC stimulation by NO when the latter was present in excess, activation by the NO independent, haem-dependent sGC stimulator 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) was unaffected. Our *in vitro* investigation of smooth muscle relaxation confirmed that O<sub>2</sub> alone in the form of a buffer bolus (equilibrated at 95% O<sub>2</sub>/5% CO<sub>2</sub>) had the ability to dilate vessels under hypoxic conditions and that this was dependent upon sGC and independent of eNOS. Our studies confirm that O<sub>2</sub> can be a direct and important mediator of vasodilation through an increase in cGMP production. In the wider context, these observations are key to understanding the relative roles of O<sub>2</sub> versus NO-induced sGC activation.

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## Introduction

Soluble guanylate cyclase (sGC) is well known as the “receptor” for nitric oxide (NO). Binding of this gaseous diatomic molecule to the haem moiety of the enzyme stimulates the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), a nucleotide that is involved in several vital intracellular signalling cascades and physiological processes [1]. Although NO is the preferred ligand for sGC, activating the enzyme several hundred fold over its basal level, other gaseous and synthetic activators, such as carbon monoxide (CO) and the benzylindazole derivative 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) respectively, have been identified. While only a moderate activator, CO would seem to mediate its action via the same mechanism as NO. YC-1 on the other hand employs a totally different strategy, binding to an allosteric site on the enzyme thereby increasing the maximal catalytic rate. Such an action produces a 10-fold increase in basal sGC activity independently of other ligands, while also potentiating the responses to subsequent exposure to such agents [2]. Given the above it is evident that there are more ways than one to activate sGC. Importantly the physiological relevance of actions that modulate the response of sGC to other ligands remains in question.

Perhaps one of the most significant roles for the NO/sGC axis is in the control of vascular tone [3–5], a function that is essential to maintaining blood flow and oxygen/nutrient delivery to tissues. As such, in response to NO produced by adjacent endothelial cells,

sGC located within vascular smooth muscle is activated and the subsequent production of cGMP mediates vasorelaxation. Since CO can also induce this cGMP-dependent response, it is perhaps a little surprising, given that NO, CO, and molecular oxygen (O<sub>2</sub>) differ by only one valence electron, that a role for O<sub>2</sub> in this process has not been identified.

Conventional understanding would suggest that O<sub>2</sub> simply does not bind to sGC [6–8], at least not in the same way as NO and CO. However changes in O<sub>2</sub> tension are widely recognised to influence vascular tone. For instance, via the regulation of specific potassium channel activity, O<sub>2</sub> plays a major role in the control of pulmonary vascular tone [9,10]. Perhaps of more relevance to an interaction with sGC is our previous demonstration that the relaxation response to endogenous NO stimulation or exogenous NO addition is enhanced the lower the O<sub>2</sub> tension [11], implying an inverse relation between NO effectiveness and O<sub>2</sub>. These factors contribute to the concept of “hypoxic vasodilation”, an innate physiological response designed to maintain tissue perfusion in the face of falling O<sub>2</sub> concentrations [12–16]. While the exact mechanisms that underlie this response have been the subject of active research and debate for many years, it is now widely accepted that activation of sGC is intrinsically involved.

The introduction of oxygenated red blood cells (RBCs) to hypoxic tissues is now well recognised to immediately induce vasorelaxation. However, while these cells would certainly, and very quickly, release O<sub>2</sub> under such conditions, the latter has been overlooked as a potential mediator of the relaxation response in

favour of more conventional activators of sGC. To this end, oxygenated haemoglobin-derived nitrosothiol (HbSNO) [17], nitrite (NO<sub>2</sub><sup>-</sup>)-derived NO as a consequence of deoxyhaemoglobin nitrite reductase activity [15,20], and RBC-derived adenosine triphosphate (ATP) stimulation of endothelium-dependent release of NO and prostacyclin [21,22] have all been postulated to mediate “hypoxic vasodilation”.

While all or any of the above could contribute to RBC-induced vasodilation in the acute setting, it would seem that none provides a totally clear mechanism. The data described in this manuscript advocates an alternative and more straight-forward candidate, molecular O<sub>2</sub>. Therefore, the aim of the present study was to investigate whether O<sub>2</sub> could act as a possible direct ligand for sGC and/or a modulator of the actions of other preferred ligands. To accomplish these aims two model systems were used; an isolated sGC enzyme system and an isolated blood vessel system, both of which allowed for tight control of the local O<sub>2</sub> environment. Importantly, these models not only allowed us to probe O<sub>2</sub>-dependent mechanisms but also to investigate how these align with the known features of the putative sGC-dependent relaxants described above.

## Materials and Methods

### Ethics Statement

**Animal.** In accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986, this study did not require a Home Office project license because no regulated procedures were carried out. White, male New Zealand rabbits were humanely killed at a designated establishment by sodium pentobarbitone, which is an appropriate method under Schedule 1 of the Act.

**Human.** Methods requiring human blood samples were fully approved by the local research ethics committee (South East Wales Research Ethics Committee) and the joint NHS/University Research and Development Office (Cardiff and Vale University Health Board). All healthy volunteers gave written informed consent.

### Materials

L-N<sup>G</sup>-monomethyl Arginine (L-NMMA) and 1H-[1,2,4] oxadiazolo-[4, 3-a] quinoxalin-1-one (ODQ) were from Alexis Biochemicals. Soluble guanylate cyclase (sGC) purified enzyme was from Axxora as ≥90% pure α<sub>1</sub>β<sub>1</sub>. Methylamine hexamethylene methylamine (MAHMA) NONOate (NOC-9) was from Enzo Life Sciences. 2, 3-diphosphoglycerate (2, 3-DPG), YC-1, PEG-SOD (polyethylene glycol superoxide dismutase) and PEG-CAT (catalase) were from Sigma Aldrich.

### Isolated sGC Study

All experiments were performed in an Invivo<sub>2</sub> Hypoxia Workstation 400 (Ruskin). Normoxic experiments were carried out at 37°C and 20% O<sub>2</sub>/5% CO<sub>2</sub> via a 25% O<sub>2</sub>/5% CO<sub>2</sub> gas cylinder (BOC). Hypoxic experiments were maintained at 37°C and ~0% O<sub>2</sub>/5% CO<sub>2</sub>. Reagents were allowed to equilibrate for 1 hour before all tests were completed. Precise buffer O<sub>2</sub> concentrations for these experiments are also shown in Table 1.

The pure sGC enzyme was reconstituted in buffer (Tris 50 mmol/L, DTT 1 mmol/L, 0.5% BSA, pH 7.4) and diluted 1 in 200 in assay buffer (Tris 50 mmol/L, EGTA 100 μmol/L, MgCl<sub>2</sub> 0.3 mmol/L, 0.045% BSA, pH 7.4. GTP, dissolved in equimolar MgCl<sub>2</sub>, was then added to a final concentration of 1 mmol/L to start the reaction. As appropriate, NOC-9 (to give final concentrations of 0.118, 1.118, 11.8 and 118 μmol/L) or YC-1 (100 μmol/L) were added at the same time as the GTP. In

experiments utilising superoxide dismutase (SOD) and catalase (CAT), agents were added 60 minutes prior to GTP. Hyperoxic samples were prepared by perfusing the buffer-enzyme mix with 95% O<sub>2</sub>/5% CO<sub>2</sub>. All reactions were incubated for 10 minutes at 37°C, after which boiling inactivation buffer (Tris 50 mmol/L, EDTA 4 mmol/L, pH 7.5) was added in 4 times excess. Samples were then heated to boiling point before storage at -20°C for further analysis.

### cGMP ELISA

Sample cGMP content was measured by commercial ELISA (R&D Systems) as directed in the kit manual.

### Isolated Vessel Preparation

Male New Zealand White rabbits (2–2.5 kg) were killed by a lethal dose of sodium pentobarbitone (120 mg/kg, i.v) under Schedule 1 guidelines (see ‘Ethics statement’). Subsequently the thoracic aorta was carefully removed and placed in fresh Krebs buffer (composition (mmol/L): NaCl 109.2, KCl 2.7, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, Glucose 11, CaCl<sub>2</sub> 1.5) on ice. Excess adipose tissue was removed from the aorta and 8 rings, 2 mm in width, were prepared.

For isometric tension recording, rings were suspended in tissue baths containing 5 ml of Krebs at 37°C and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Resting tension was set to 2 g. Signals from the transducer (AD Instruments, Chalgrove, UK) were amplified and visualised on the Powerlab/Chart 4 for Windows software. All rings were allowed to equilibrate for 60 minutes prior to experimentation.

Constriction-relaxation exercises to 1 μmol/L phenylephrine (PE) and 10 μmol/L acetylcholine (ACh) (Sigma Aldrich), respectively, were performed in order to establish both smooth muscle and endothelial integrity.

In order to equilibrate the rings in defined levels of hypoxia, the gas supply was switched to the appropriate O<sub>2</sub> content (ensuring balance of N<sub>2</sub> and 5% CO<sub>2</sub>) for 10 minutes. Constriction to PE was tested across a broad range of concentrations and the appropriate PE concentration to achieve 80% of maximal attainable constriction used in all studies. For hypoxic studies (0% O<sub>2</sub>), rings were typically exposed to 3 μmol/L PE in order to achieve a similar sub-maximal constriction as observed in normoxic rings. The O<sub>2</sub> concentration measured in the tissue bath equilibrated under hypoxic conditions was 0.9% (equivalent to 9 μmol/L O<sub>2</sub>) as we have detailed previously [11].

### Krebs Samples

Krebs solution was gassed at 95% O<sub>2</sub>/5% CO<sub>2</sub> in order to achieve 95% O<sub>2</sub> samples. 21% O<sub>2</sub> samples were allowed to equilibrate with the atmosphere. 0% O<sub>2</sub> samples were gassed with 95% N<sub>2</sub>/5% CO<sub>2</sub>. In order to accurately measure O<sub>2</sub> concentration within these equilibrated Krebs buffer samples, electron paramagnetic resonance (EPR) oximetry was undertaken utilising N<sup>15</sup> per-deuterated tempone (2,2,6,6-tetra-methyl-4-piperidone; PDT) as the O<sub>2</sub>-sensitive probe. In brief, the spectral line width obtained from PDT (~5 μmol/L) shows a linear relationship with O<sub>2</sub> concentration and sample readings can be measured against known standards. This technique measures O<sub>2</sub> with an accuracy of ±0.2% O<sub>2</sub> in our hands as we have described in detail previously [23]. The precise O<sub>2</sub> concentrations within “95%”, “21%” and “0%” buffer are shown in Table 1.

**Table 1.** Example O<sub>2</sub> contents of replica buffer samples for both the myograph and hypoxic chamber, read from a PDT-probe standard curve (EPR oximetry).

Sample	Stock Buffer O <sub>2</sub> concentration (μmol/L)	Final Tissue Bath concentration (μmol/L) <sup>1</sup>
0%	49	1.96
21%	175	7.0
95%	772	30.9
Hypoxic chamber 0% +1 hour	38	
Hypoxic chamber 20% +1 hour	197	

<sup>1</sup>represents the effective O<sub>2</sub> concentration added to each tissue bath. The existing O<sub>2</sub> in the "hypoxic" bath perfused with N<sub>2</sub>/CO<sub>2</sub> was 0.9% (equivalent to 9 μmol/L O<sub>2</sub>). doi:10.1371/journal.pone.0057162.t001

### Haemoglobin (Hb) Samples

Venous RBCs were lysed and diluted 1 in 3 in PBS (0.9% sodium chloride w/v), of which 250 μl was then loaded on to a Sephadex G25 M column (GE Healthcare). Samples of pure Hb were collected in fractions for use in the myograph studies. The HbO<sub>2</sub> content was verified by blood gas analysis as described (see 'Venous RBC samples'). The volume used in the myograph studies gave a final O<sub>2</sub> concentration in the tissue bath of 30.9 μmol/L which was equal to the O<sub>2</sub> content of the tissue bath following addition of either buffer or RBC samples.

### Venous RBC Samples

Blood was drawn from the antecubital vein of healthy volunteers and immediately centrifuged at 1200×g for 5 minutes. The plasma layer and buffy coat were removed and replaced with an equivalent volume of gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) phosphate buffered saline (PBS). Following re-centrifugation at 1200×g for 5 minutes the PBS layer was removed. In order to achieve RBCs of varying O<sub>2</sub> saturation, the cells were firstly diluted ~1:3 with PBS and loaded on to a thin film rotating tonometer, which was purged with O<sub>2</sub>/CO<sub>2</sub> to achieve higher saturations or N<sub>2</sub>/CO<sub>2</sub> for lower saturations as we have described previously [24]. The O<sub>2</sub> saturation of representative RBC samples was measured on an OSM3 Hemoximeter (Radiometer, Copenhagen).

### In vitro NO Analysis

Phosphate buffered saline (PBS), pH 7.4, was kept at 37°C in a reaction vessel purged with a flow of nitrogen gas feeding into a Nitric Oxide Analyser (NOA) 280i (Analytix) for on-line ozone-based chemiluminescence (OBC) detection of NO. NOC-9 was reconstituted in 0.1M sodium hydroxide (NaOH) to give a final stock concentration of 24 mmol/L and kept on ice in the dark. For the experimental samples, the NOC-9 was further diluted in 1 ml of PBS (protected from light in a sealed vessel) to give the following concentrations (in μmol/L): 2.4; 1.2; 0.24; 0.12 and 0.024. In order to compare NO release under different conditions, the PBS was either pre-equilibrated to 0% or 95% O<sub>2</sub> by vigorous bubbling. Samples were incubated for 10 minutes at 37°C, after which 200 μl of the gas layer was drawn up using a Hamilton syringe and injected immediately into the purge vessel for OBC analysis. After the signal trace returned to baseline values, 200 μl of the corresponding PBS sample was drawn up and injected into the purge vessel. Data were recorded in real time and presented as area under the curve (Liquid software).

### Statistical Analysis

All data were analysed by one-way ANOVA plus Tukey's or Dunnett's *post hoc* test or Student's *t* test as appropriate. Pearson's correlations were assessed and coefficients are shown where appropriate. All data are expressed as mean ± standard error (SEM). Differences were considered significant where *p*<0.05.

### Results

#### Isolated Pure sGC Activity in Normoxia/Hypoxia

A striking observation of these experiments was the difference in basal sGC activity in normoxia (~100 pmol/ml) compared to hypoxia (~30–40 pmol/ml) (Figure 1a).

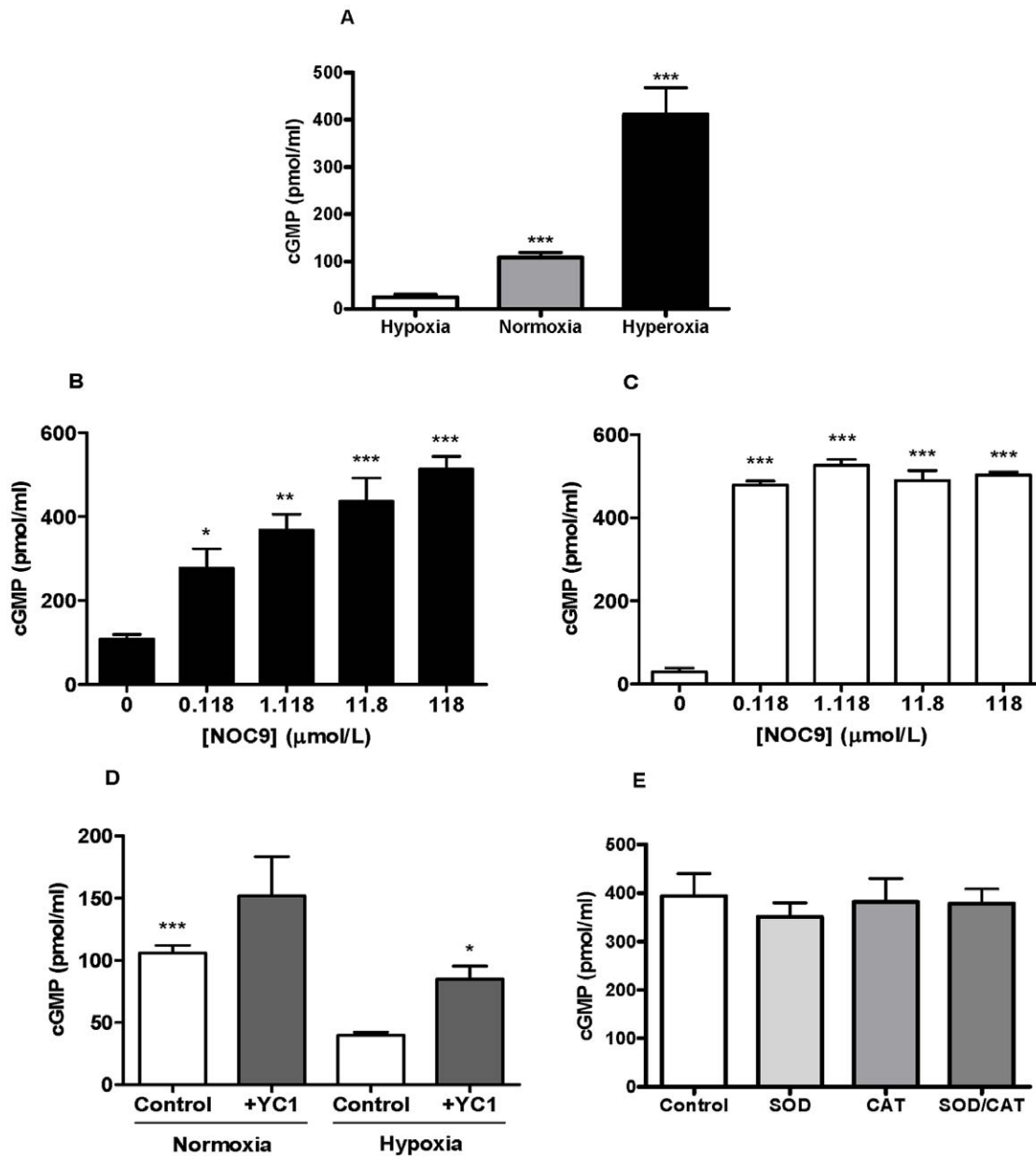
Exposure to the NO donor, NOC-9, at various concentrations (0, 0.118, 1.118, 11.8 and 118 μmol/L), stimulated cGMP production in normoxia in a concentration dependent manner (108.4±10.78 pmol/ml to 513.7±30.12 pmol/ml) (Figure 1b). In hypoxia, cGMP production was similar across all concentrations of NOC9 compared with the control (~500 pmol/ml vs. ~30 pmol/ml) (Figure 1c).

In separate experiments, YC-1 (100 μmol/L), a compound known to stimulate sGC without direct interaction with the sGC haem group [2], was added to sGC under hypoxic and normoxic conditions as above. While the addition of YC-1 enhanced the amount of cGMP produced by the enzyme, importantly the increase from baseline was similar in both normoxia and hypoxia (~46.20 pmol/ml & ~44.86 pmol/ml, respectively) (Figure 1d).

To deduce whether superoxide (O<sub>2</sub><sup>-</sup>) was having a direct influence on sGC activity in normoxia, SOD and CAT were incubated with the enzyme to observe any change in cGMP levels produced. Compared to control levels, SOD and CAT did not significantly affect the activity of the isolated enzyme (Figure 1e).

#### Effects of Buffer Containing Increasing O<sub>2</sub> on Vascular Tone in Hypoxia

Tetrameric Hb has the ability to carry four O<sub>2</sub> molecules [25]. The O<sub>2</sub> content of a RBC suspension is therefore much greater than buffer equilibrated with the same O<sub>2</sub> concentration. In order to attain a comparable O<sub>2</sub> delivery, Krebs buffer was gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>, either in a sealed glass bottle (high O<sub>2</sub> saturation) or an open container allowed to equilibrate with atmospheric air (21% O<sub>2</sub>), or with 95% N<sub>2</sub>/5% CO<sub>2</sub> (0% O<sub>2</sub>). 200 μl of the appropriate Krebs buffer samples were then added to tissue baths containing hypoxic PE-pre-constricted aortic rings as described above. A 3 fold greater (*p*<0.01) relaxation was

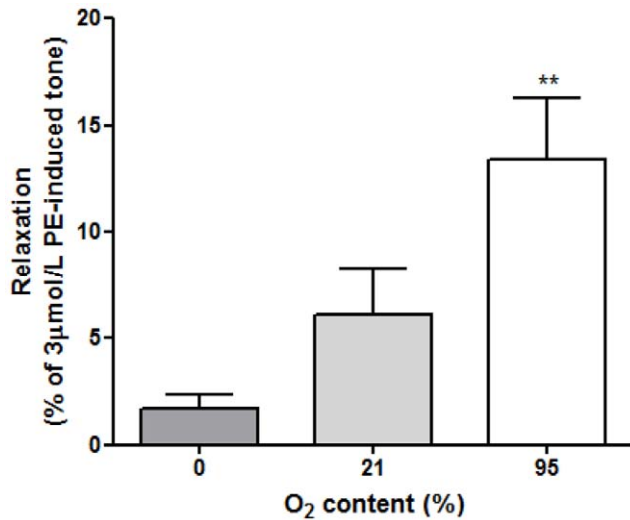


**Figure 1. Measurement of cGMP as an index of sGC activity in normoxic and hypoxic conditions.** a. Comparing cGMP production in hypoxia (0% O<sub>2</sub>), normoxia (~21% O<sub>2</sub>) and hyperoxia (95% O<sub>2</sub>). Normoxia and hyperoxia stimulated an increased production of cGMP compared with hypoxia. (\*\*\*)*p*<0.001 cf. hypoxia, *n*=4–6). b. Concentration response curve to NOC9 under normoxic conditions. Significant differences were observed at each concentration level compared to the control containing no NOC9, (\*\*\*)*p*<0.001 cf. 0 μmol/L, (\*\*)*p*<0.01 cf. 0 μmol/L and (\**p*<0.05 cf. 0 μmol/L (*n*=3 to 6 experiments). c. Concentration response curve to NOC9 under hypoxic conditions. Significant differences were observed at each concentration level compared to the control containing no NOC9, (\*\*\*)*p*<0.001 cf. 0 μmol/L (*n*=3 to 6 experiments). cGMP detected for 0.118 and 1.118 μmol/L NOC9 were much greater than the levels in normoxia initiated by the same concentrations. d. Exposure of sGC to the NO-independent activator YC-1 (100 μmol/L). Paired analysis of cGMP levels catalysed by sGC revealed a significant increase in the normoxic control versus hypoxic control, (\*\*\*)*p*<0.001 cf. hypoxia control. The addition of YC-1 in normoxia induced an increase in cGMP compared to control but this was not significant. A similar trend was observed in hypoxia, however the addition of YC-1 induced a significant rise in cGMP, (\**p*<0.05 cf. control. (*n*=4). e. SOD (100 U/ml) and CAT (250 U/ml) incubated with sGC in normoxia stimulated a similar level of cGMP compared to the control, *p*>0.05 cf. control. (*n*=4).  
doi:10.1371/journal.pone.0057162.g001

observed following addition of highly oxygenated versus minimally oxygenated samples (Figure 2).

### Effects of Administering a Constant O<sub>2</sub> Supply to Hypoxic Tissue

In separate experiments, aortic tissue was incubated in hypoxia and pre-constricted with PE as described above. The gas supply was then switched to 95% O<sub>2</sub>/5% CO<sub>2</sub> permanently. An instantaneous and transient relaxation response, akin to that



**Figure 2. Krebs buffer samples introduced to hypoxic aortic rings.** Maximum relaxation was demonstrated with 95% oxygenated buffer samples compared with 0% O<sub>2</sub> samples (all n=6, \*\*p<0.01 cf. 0%).

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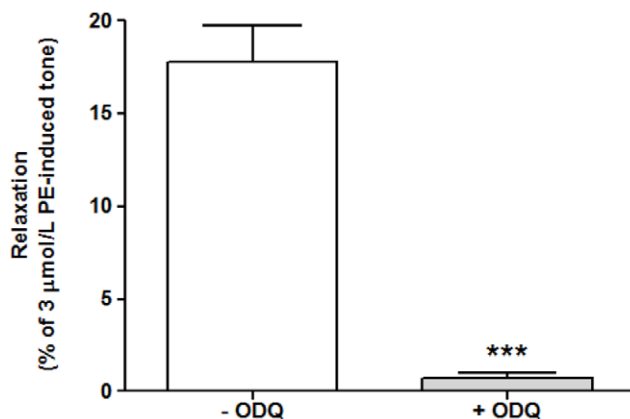
following addition of O<sub>2</sub> buffer solution, was observed (data not shown).

#### Effects of sGC Inhibition on O<sub>2</sub>-mediated Vasorelaxation

To confirm that O<sub>2</sub>-mediated relaxation of hypoxic vessels is mediated via the sGC pathway, the sGC inhibitor, 1H-[1,2,4]oxadiazolo-[4, 3-a] quinoxalin-1-one (ODQ) which binds to the haem moiety of sGC, was introduced 30 minutes prior to PE constriction. The presence of ODQ caused a significant inhibition of the relaxation induced by a bolus of 95% O<sub>2</sub>/5% CO<sub>2</sub>-gassed Krebs buffer ( $0.72 \pm 0.28\%$  vs.  $17.81 \pm 1.90\%$  in the absence of ODQ, n = 7, \*\*\*p = 0.001) (Figure 3).

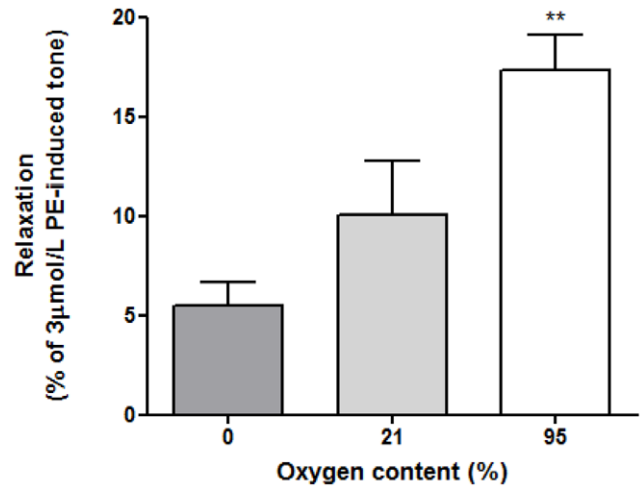
#### Influence of Endothelial NO Synthase (eNOS) on O<sub>2</sub>-mediated Vasorelaxation

Experiments were repeated in the presence of the eNOS inhibitor L-N<sup>G</sup>-monomethyl arginine (LNMMA, 300 μmol/L for



**Figure 3. The effect of sGC inhibitor, ODQ, upon O<sub>2</sub>-mediated relaxation.** In the presence of ODQ (10 μmol/L) the relaxation response to 95% oxygenated buffer was almost completely inhibited (both n=7, \*\*\*p<0.001 cf. control).

doi:10.1371/journal.pone.0057162.g003



**Figure 4. The influence of eNOS inhibitor, LNMMA, upon O<sub>2</sub>-mediated relaxation.** LNMMA had no effect on the relaxation responses. Similarly to Figure 3, 95% oxygenated buffer samples generated the greatest relaxation compared with 0% O<sub>2</sub> samples (all n=4, \*\*p<0.01 cf. 0% O<sub>2</sub>).

doi:10.1371/journal.pone.0057162.g004

30 minutes). Figure 4 demonstrates that this agent has no effect on the oxygenated buffer-induced relaxations (n = 4, \*\*p<0.01 cf. 0% O<sub>2</sub>).

#### Vascular Effects of Superoxide and Hydrogen Peroxide

It is well acknowledged that reperfusion of O<sub>2</sub> to hypoxic vessels causes the generation of O<sub>2</sub> radical species [26]. Therefore, it was necessary to ascertain whether O<sub>2</sub><sup>-</sup> had a role in the myography experiments conducted here. SOD and CAT linked to polyethylene glycol (PEG) were therefore used to abrogate any effects of intracellular O<sub>2</sub><sup>-</sup> and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) generation, respectively. Neither PEG-SOD, PEG-CAT, nor both together had a significant effect on the O<sub>2</sub>-induced relaxation of vessels in hypoxic conditions (Figure 5).

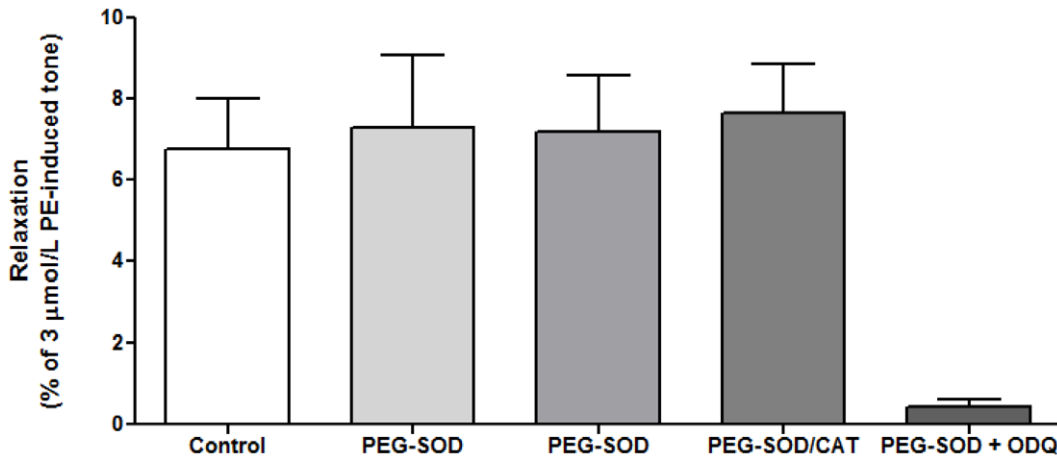
#### Effects of Oxygenated RBCs on Vascular Tone in Hypoxia

RBCs equilibrated at a higher O<sub>2</sub> saturation ( $98.22 \pm 0.45\%$  O<sub>2</sub>, n = 13) caused significantly (p<0.001) more relaxation than those of partial saturation ( $51.43 \pm 6.16\%$  O<sub>2</sub>, n = 4) or low saturation ( $20.40 \pm 5.28\%$  O<sub>2</sub>, n = 13) when administered to a hypoxic tissue bath (Figure 6). These data confirm a positive relationship between RBC O<sub>2</sub> saturation and the relaxation induced in hypoxic aortic tissue (Pearson's correlation coefficient r = 0.815, p<0.0001).

#### Effects of RBC O<sub>2</sub> Cycling upon Hypoxic Vasorelaxation

Highly oxygenated RBC ( $98.22 \pm 0.45\%$  O<sub>2</sub>, n = 13) were administered to hypoxic aortic rings to induce relaxation. In a parallel sample, highly oxygenated RBC were first deoxygenated to ~20% O<sub>2</sub> ( $20.68 \pm 1.66\%$  O<sub>2</sub>) before reoxygenation ( $97.70 \pm 0.39\%$  O<sub>2</sub>, n = 4) and subsequent addition to hypoxic rings. There was no significant difference in the relaxation induced post deoxy-oxy cycling of RBCs compared with controls ( $8.28 \pm 2.97\%$  vs.  $12.00 \pm 3.60\%$ ).

The RBC O<sub>2</sub> cycling prompted further investigation into the movement of the relaxing moiety in and out of the cell. RBC were deoxygenated to a low saturation ( $22.38 \pm 1.69\%$  O<sub>2</sub>) before re-suspension in fresh oxygenated buffer to give a saturation of ~98% ( $98.05 \pm 0.65\%$  O<sub>2</sub>, n = 4). As above, there was no significant difference between the relaxations induced by buffer-



**Figure 5. Effect of  $O_2^-$  and  $H_2O_2$  on vessel relaxation in hypoxia.** The inhibition of  $O_2^-$  radicals (100 U/ml PEG-SOD) and  $H_2O_2$  (250 U/ml PEG-CAT) within the vessel system did not affect the magnitude of the relaxation to a bolus of 95% oxygenated buffer. PEG-SOD (100 U/ml) plus ODQ (10 μmol/L) abolished most of the relaxation (\* $p < 0.05$  cf. control). All  $n = 3$ . doi:10.1371/journal.pone.0057162.g005

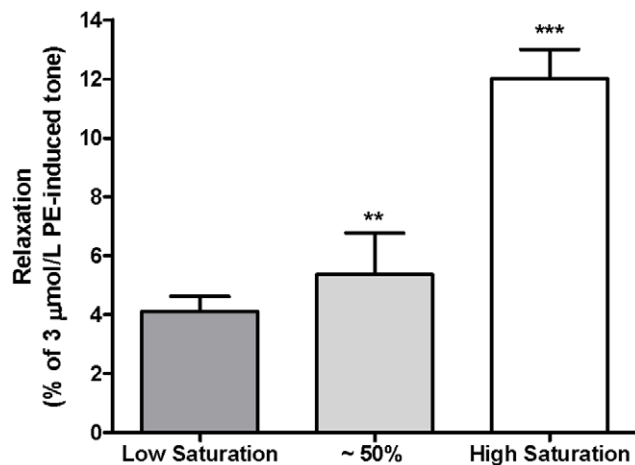
replaced RBCs compared with controls ( $12.97 \pm 1.89\%$  vs.  $12.00 \pm 3.60\%$ ).

#### Influence of 2, 3-diphosphoglycerate on RBC-mediated Hypoxic Vasorelaxation

2, 3-diphosphoglycerate (2, 3-DPG), (5 mmol/L) discourages  $O_2$  from binding to Hb, facilitating diffusion into respiring tissue [27]. In the presence of 2, 3-DPG, hypoxic PE-pre-constricted aortic rings relaxed significantly more ( $10.75 \pm 1.11\%$  vs.  $8.25 \pm 1.36\%$ ,  $n = 4$ , \* $p < 0.05$ ) to addition of oxygenated RBCs than in its absence, supporting the role of this intermediate in the regulation of  $O_2$  delivery.

#### Comparison of $O_2$ -mediated Vasorelaxation by RBCs, Buffer or Isolated Hb

In additional experiments, purified oxygenated Hb was prepared from RBCs and applied to tissue baths containing

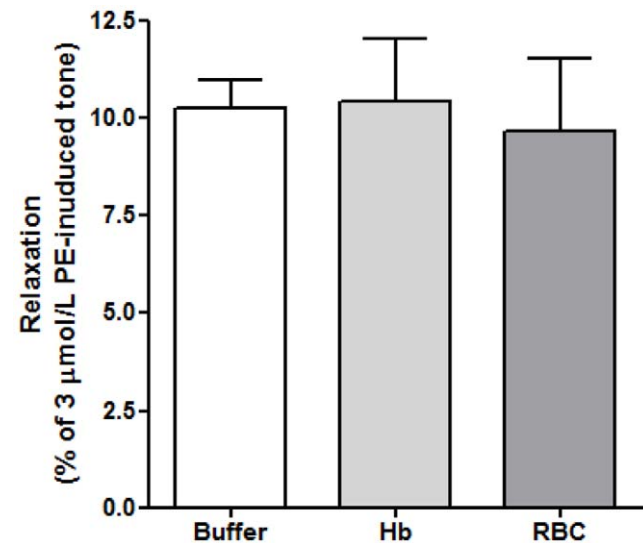


**Figure 6. Varying  $O_2$  saturations of RBC administered to hypoxic rabbit aortic rings.** Partially saturated RBC (~50%) and low saturated RBC displayed a significantly lower relaxation than RBC of high saturation, \*\* $p < 0.01$  and \*\*\* $p < 0.001$  respectively. (High saturation,  $n = 13$ ; partial saturation,  $n = 4$ ; low saturation,  $n = 13$ ). doi:10.1371/journal.pone.0057162.g006

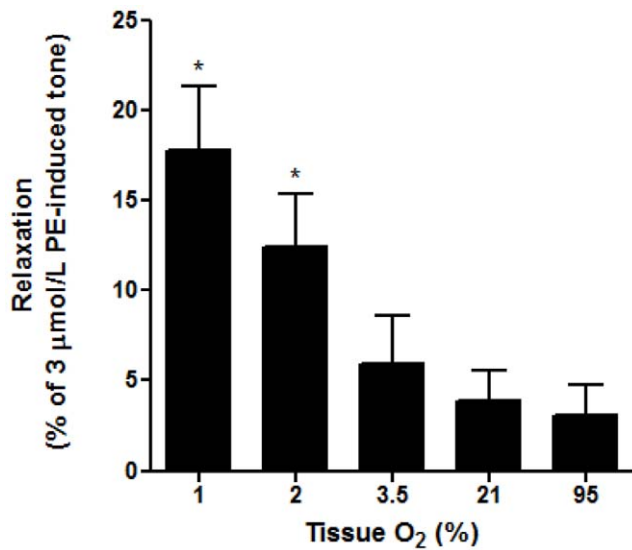
hypoxic PE-pre-constricted aortic rings as described above. Samples of RBC, buffer or Hb were prepared such that addition to the tissue bath resulted in final  $O_2$  concentrations of  $\sim 30$  μmol/L. Figure 7 shows grouped results confirming very similar relaxation responses for RBCs, Hb and oxygenated buffer ( $n = 4$ ,  $p > 0.05$ ).

#### Effects of Oxygenated RBCs on Vascular Tone at Different Tissue $pO_2$

In separate experiments, aortic tissue was perfused for 10 minutes with 1%, 2%, 5%, 21% or 95%  $O_2/5\%CO_2$ . Constriction to PE was normalised to 80% of maximum at each  $O_2$  tension, then fully oxygenated RBCs were administered. This is in contrast to most of the experiment described above in which varying  $O_2$  was introduced to hypoxic tissue or isolated sGC.



**Figure 7. Effects of various oxygenated samples on relaxation responses of hypoxic vascular tissue.** Krebs buffer, Hb and RBC samples of equal  $O_2$  content ( $\sim 30$  μmol/L) administered to hypoxic rabbit aortic rings. There was no difference in the magnitude of the relaxations produced ( $p > 0.05$ ) ( $n = 4$ ). doi:10.1371/journal.pone.0057162.g007



**Figure 8. Relaxations observed after addition of RBC samples to aortic rings equilibrated at various O<sub>2</sub> saturations.** Tissues held at a lower O<sub>2</sub> (1 and 2%) relaxed significantly more to oxygenated RBC's, \*\*\* $p < 0.001$  cf. 95% and \* $p < 0.05$  cf. 95%, respectively. doi:10.1371/journal.pone.0057162.g008

Significantly greater relaxation was induced at tissue incubated in 1% (\*\* $p < 0.001$  cf. 95% O<sub>2</sub>) and 2% O<sub>2</sub> (\* $p < 0.05$  cf. 95% O<sub>2</sub>) ( $n = 6$ ) (Figure 8).

#### Effects of O<sub>2</sub> on the Liberation of NO from NOC9

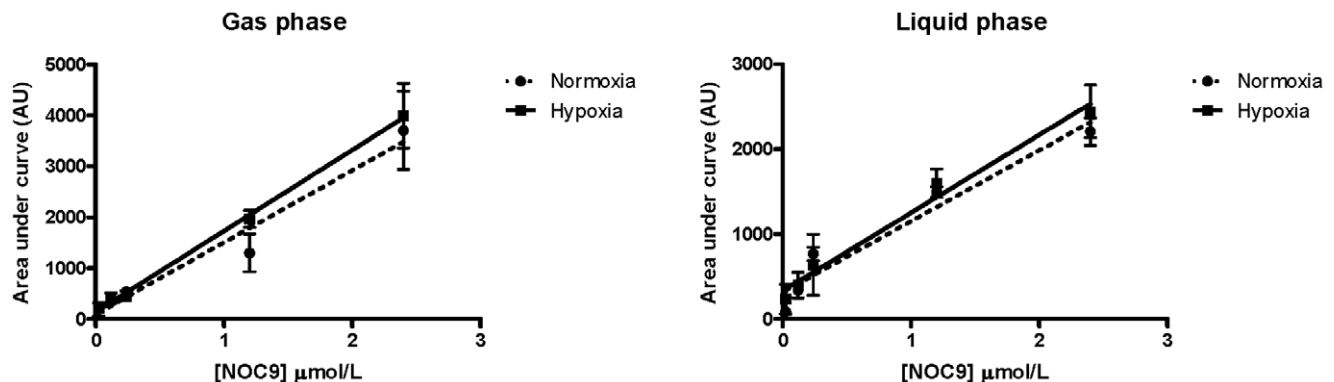
In order to test whether production of NO from NOC-9 was affected under normoxic or hypoxic conditions, we measured NO released from NOC-9 into both the gaseous and liquid phases of a PBS sample across a range of NOC-9 concentrations. Our data confirms that the quantity of NO detected in both the gas and liquid phases (quantified as area under the curve), are not significantly different under normoxic versus hypoxic conditions ( $n = 3$ ,  $p < 0.05$ ) (Figure 9).

#### Discussion

The aim of the present study was to investigate the effects of O<sub>2</sub> on sGC function, and in particular, on responses of the enzyme to

known ligands and stimulators/activators. Our data conclusively shows that molecular O<sub>2</sub> has the ability to increase sGC activity in the absence of NO. Moreover, exposure of sGC to hypoxic conditions revealed a novel modulatory effect of O<sub>2</sub> on NO-stimulated sGC activity. Our isolated vessel model confirms that the sGC-mediated transient relaxation of tissues in hypoxic conditions can occur simply by injecting a bolus of oxygenated buffer. As such, the data presents an important and hitherto undescribed role for O<sub>2</sub> in the production of cGMP.

As previously described, and when compared to NO, sGC can be activated to a lesser extent by other diatomic species such as CO [28]. However, despite the obvious structural similarity, the ability of O<sub>2</sub> to interact with sGC, and specifically with the haem moiety to form a stable complex, has been overlooked [6–8,29,30]. Importantly, the data described herein goes against conventional thinking and supports a role for O<sub>2</sub> in upregulating baseline sGC activity as evidenced by greater cGMP production in our isolated enzyme system under normoxic compared to hypoxic conditions. Moreover, the presence of O<sub>2</sub> is also shown to have a modulatory effect on the subsequent activation of sGC by NO. Thus following addition of NO to hypoxic sGC, similar large increases in cGMP were observed irrespective of NO concentration. Conversely, a concentration dependent response to NO was revealed for normoxic sGC, the largest response to 23.6 μmol/L NO being similar to that produced by 100-fold less NO under hypoxic conditions. As such, the removal of O<sub>2</sub> from sGC both decreases baseline enzyme activity and makes it more sensitive to activation by lower concentrations of the NO. That the NO and haem-independent sGC activator, YC-1, produced a different response, similar increases in cGMP above baseline being observed in both normoxia and hypoxia, would support a possible role for the interaction of O<sub>2</sub> with the sGC in a way that modulates the haem group. sGC has been reported to function optimally at intracellular O<sub>2</sub> concentrations between 20 and 40 μmol/L [31] indicating the ability of sGC to distinguish between ligands. Importantly, we confirmed that the amount of NO released by the NO donor NOC-9 was similar under both hypoxic/normoxic experimental conditions and thus cannot be accountable for the results achieved. While a stable isolatable complex of O<sub>2</sub> and sGC has not been identified, it is possible that intracellular concentrations of O<sub>2</sub> could have an influence on enzyme activity. The majority of studies conducted with sGC are under anaerobic conditions [8,32,33] however Ullrich and colleagues [34] observed a decrease in cGMP produced by platelet sGC following N<sub>2</sub>



**Figure 9. NO liberation from NOC-9 under normoxic versus hypoxic conditions.** Gas and liquid samples drawn up from reaction vessels incubated with varying concentrations of NOC-9, were injected into the NOA. The solutions were either equilibrated at 0 or 95% O<sub>2</sub>/5% CO<sub>2</sub> prior to the addition of NOC-9. There was no difference in the total NO detected in either gas or liquid samples under ( $n = 3$ ,  $p < 0.05$ ). doi:10.1371/journal.pone.0057162.g009

addition compared to basal conditions. In addition, Soret band analysis by UV spectrophotometry show subtle changes in sGC binding under oxygenated conditions [35].

In order to substantiate our isolated enzyme data, we next developed an *in vitro* vascular model. Isolated rabbit aortic rings pre-constricted with PE under hypoxic conditions were exposed to single bolus additions of buffer equilibrated with varying  $O_2$  concentrations. In such experiments, transient and concentration-dependent relaxation responses to  $O_2$  were produced. The involvement of sGC and classic cGMP-dependent downstream signalling in smooth muscle to induce relaxation was confirmed by the fact that the  $O_2$ -dependent responses were inhibited in the presence of ODQ, the irreversible haem-site inhibitor of sGC [36,37]. Since eNOS function is close to normal even under hypoxic conditions in this vascular preparation [38] a role for endothelium-dependent NO in the responses described above was also investigated. In that the relaxation induced by  $O_2$  was insensitive to eNOS inhibition by LNMMA, such an involvement was ruled out. Together these observations support the role of  $O_2$  in sGC activation already described above.

The *in vitro* isolated vascular system used in the present study relates closely to ischaemia reperfusion, where a vessel of low oxygenation quickly becomes reperfused with  $O_2$ , for example following removal of an occlusion [39]. Under such circumstances many studies have shown evidence for the generation of free radical species [26,40–43]. Taken with the recently observed inhibitory effect of  $O_2^-$  on sGC [44] it was important to confirm that  $O_2^-/H_2O_2$  were not involved in the sGC-driven effects we observe following addition of  $O_2$  to the tissues. To this end experiments were repeated in the presence of the appropriate cell permeable inhibitors (PEG-SOD and PEG-CAT). That the relaxation responses to  $O_2$  were again insensitive to these agents ruled out this possibility.

In order to further translate our study, we repeated our *in vitro* studies, substituting the  $O_2$  buffer bolus for highly  $O_2$ -saturated human RBCs. When oxygenated, RBCs have the ability to change Hb conformation into the R state. Indeed, in this state Hb has the capacity to offload  $O_2$  and *in vivo* this is conveyed by the transfer of  $O_2$  from the blood to respiring tissue beds. Importantly, such a mechanism is crucial for RBCs to give up  $O_2$  when encountering a hypoxic environment. Therefore, by altering the saturation of RBCs, we could change the proportions of R and T state Hb and therefore potentially show the preference for the RBCs to give up  $O_2$  in the R state. The highly saturated ( $98.22 \pm 0.45\%$   $O_2$ ) RBCs induced a significantly greater relaxation of hypoxic rabbit aortic rings compared with RBCs of low saturation ( $20.40 \pm 5.28\%$   $O_2$ )/mainly T state Hb. Consequently, purified Hb was used in further experiments to confirm the relaxant species was released directly from Hb without the need for RBCs *per se*. Interestingly, RBCs, Hb and buffer boluses of comparable  $O_2$  content induced very similar relaxations of the hypoxic vessel tested in the present study. Additional experimentation demonstrated that the relaxant species is not depleted by repeat deoxygenation/re-oxygenation cycles, confirming the species is readily replenishable and is essentially delivered by simply replacing the Krebs buffer surrounding the hypoxic RBCs with adequately oxygenated buffer.

For some time it has been suggested that RBC-induced vasorelaxation is linked to Hb allostery, although a particular interest has focussed on the now well established vasodilatory capacity of HbSNO. We ourselves followed this line of investigation demonstrating greater RBC-induced relaxations with increasing HbSNO [45], and an altered HbSNO to relaxation response in highly glycosylated RBCs that we linked to an effect on the Hb  $O_2$  saturation curve [11]. Concurrently, we also measured the

transient release of  $O_2$  into the tissue bath but at the time took this to be a consequence of Hb transition rather than the relaxant species. Subsequent studies have later confirmed the potential for  $O_2$  in buffer alone to induce relaxation, but considered this control considerably less than the relaxation induced by RBCs [15]. Importantly, the samples and the buffer controls were perfused with the same gas, but crucially were not equalised in terms of  $O_2$  content in the latter study.

Further studies have confirmed the importance of Hb allostery in RBC-mediated hypoxic vasorelaxation [20,46] although they contend partially oxygenated Hb acts as a  $NO_2^-$  reductase that converts readily available  $NO_2^-$  to free NO to exhibit vasodilatation. Whilst both HbSNO and  $NO_2^-$  exhibit properties that suggest they could contribute to RBC-induced relaxation [15,18–20,45], neither completely satisfies the characteristics of a readily replenishable, freely diffusing relaxant species that is present in simply oxygenated buffer as we show here.

A potential criticism of our model system is that we induce an artificial condition in tissue whereby a defined unit of  $O_2$  (either buffer or RBCs) is introduced to an essentially hypoxic environment. To address this, we conducted studies under conditions where either fully oxygenated RBCs or buffer samples were administered to baths containing tissue equilibrated at various  $O_2$  concentrations. Of physiological significance,  $O_2$ -mediated relaxation was inversely related to the extent of tissue oxygenation, in complete concurrence with established Hb $O_2$  delivery and matching the need for dilation across the whole  $O_2$  range. Furthermore, we conducted studies in which  $O_2$  was confirmed as a relaxant of blood vessel segments since the first response to administration of a constant supply of  $O_2$  being re-introduced into a hypoxic setting was transient relaxation.

In previous work we had demonstrated how vessel relaxation response to exogenous addition of NO is enhanced with lower  $O_2$  tension [11]. This had been ascribed to the influence of  $O_2$  on competing mechanisms, including direct effects on constriction. However, taken together with the data we show herein, would indicate a more direct interplay between the effects of NO and  $O_2$  on sGC. Clearly, NO is the preferred ligand as shown by its ability to fully activate isolated sGC in our model system at a concentration as low as  $10 \mu\text{mol/L}$  even in the presence of  $210 \mu\text{mol/L}$   $O_2$  (“normoxia”). Under conditions where NO is relatively low,  $O_2$  may influence sGC in two ways; the influence of weak sGC activation by  $O_2$  may accrue greater importance, and the effect of NO will be enhanced.

In conclusion, our data has revealed the novel finding that in the presence of  $O_2$ , sGC can stimulate an increased production of cGMP from GTP compared to under hypoxic conditions. The use of YC-1 in our isolated enzyme model clearly enhanced cGMP production however the compound was not affected by the presence of  $O_2$ , perhaps suggesting the effects of  $O_2$  on the enzyme may be occurring at a site independent to that of YC-1. Furthermore, we show that  $O_2$  can modulate the effect of NO (concentration-dependent) upon the enzyme, indicative of an interaction of  $O_2$  with the haem of sGC or an indirect involvement with haem via an allosteric site. Armed with the knowledge that  $\sim 6\%$  of sGC within vascular tissue is stimulated by NO under normal conditions [47], our findings may have important consequences in the wider context of vascular relaxation via stimulation of sGC across the physiological and pathological  $O_2$  range.

## Author Contributions

Manuscript structure: DL PEJ. Conceived and designed the experiments: JD AGP DL PEJ. Performed the experiments: JD AGP. Analyzed the data:



JD AGP. Contributed reagents/materials/analysis tools: JD AGP DL PEJ.  
Wrote the paper: JD AGP.

## References

- Stone J, Marletta M (1995) The ferrous heme of soluble guanylate cyclase: formation of hexacoordinate complexes with carbon monoxide and nitrosomethane. *Biochemistry* 34: 16397–16403.
- Friebe A, Koesling D (1998) Mechanism of YC-1-induced activation of soluble guanylyl cyclase. *Molecular pharmacology* 53: 123.
- Galle J, Zabel U, Hübner U, Hatzelmann A, Wagner B, et al. (1999) Effects of the soluble guanylyl cyclase activator, YC-1, on vascular tone, cyclic GMP levels and phosphodiesterase activity. *British journal of pharmacology* 127: 195.
- Poulos T (2006) Soluble guanylate cyclase. *Current opinion in structural biology* 16: 736–743.
- Chen K, Pittman RN, Popel AS (2008) Nitric oxide in the vasculature: where does it come from and where does it go? A quantitative perspective. *Antioxid Redox Signal* 10: 1185–1198.
- Martin E, Berka V, Bogatenkova E, Murad F, Tsai AL (2006) Ligand selectivity of soluble guanylyl cyclase: effect of the hydrogen-bonding tyrosine in the distal heme pocket on binding of oxygen, nitric oxide, and carbon monoxide. *J Biol Chem* 281: 27836–27845.
- Ma X, Sayed N, Beuve A, van den Akker F (2007) NO and CO differentially activate soluble guanylyl cyclase via a heme pivot-bend mechanism. *The EMBO Journal* 26: 578.
- Derbyshire ER, Deng S, Marletta MA (2010) Incorporation of tyrosine and glutamine residues into the soluble guanylate cyclase heme distal pocket alters NO and O<sub>2</sub> binding. *J Biol Chem* 285: 17471–17478.
- Post JM, Hume JR, Archer SL, Weir EK (1992) Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. *Am J Physiol* 262: C882–890.
- Patel AJ, Honoré E (2001) Molecular physiology of oxygen-sensitive potassium channels. *Eur Respir J* 18: 221–227.
- James P, Lang D, Tufnell-Barret T, Milsom A, Frenneaux M (2004) Vasorelaxation by red blood cells and impairment in diabetes: reduced nitric oxide and oxygen delivery by glycated hemoglobin. *Circulation research* 94: 976.
- McMahon TJ, Moon RE, Luschinger BP, Carraway MS, Stone AE, et al. (2002) Nitric oxide in the human respiratory cycle. *Nat Med* 8: 711–717.
- Kim-Shapiro DB, Gladwin MT, Patel RP, Hogg N (2005) The reaction between nitrite and hemoglobin: the role of nitrite in hemoglobin-mediated hypoxic vasodilation. *J Inorg Biochem* 99: 237–246.
- Allen B, Piantadosi C (2006) How do red blood cells cause hypoxic vasodilation? The SNO-hemoglobin paradigm. *American Journal of Physiology- Heart and Circulatory Physiology* 291: H1507.
- Crawford J, Isbell T, Huang Z, Shiva S, Chacko B, et al. (2006) Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. *Blood* 107: 566.
- Diesen D, Hess D, Stamler J (2008) Hypoxic vasodilation by red blood cells: evidence for an S-nitrosothiol-based signal. *Circulation research* 103: 545.
- Stamler J, Jaraki O, Osborne J, Simon D, Keaney J, et al. (1992) Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proceedings of the National Academy of Sciences* 89: 7674.
- Huang Z, Shiva S, Kim-Shapiro D, Patel R, Ringwood L, et al. (2005) Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *Journal of Clinical Investigation* 115: 2099–2107.
- Cosby K, Partovi K, Crawford J, Patel R, Reiter C, et al. (2003) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature medicine* 9: 1498–1505.
- Gladwin M, Crawford J, Patel R (2004) The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation\* 1. *Free Radical Biology and Medicine* 36: 707–717.
- Bergfeld G, Forrester T (1992) Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. *Cardiovascular research* 26: 40.
- Ellsworth M, Forrester T, Ellis C, Dietrich H (1995) The erythrocyte as a regulator of vascular tone. *American Journal of Physiology- Heart and Circulatory Physiology* 269: H2155.
- James PE, Jackson SK, Grinberg OY, Swartz HM (1995) The effects of endotoxin on oxygen consumption of various cell types in vitro: an EPR oximetry study. *Free Radical Biology and Medicine* 18: 641–647.
- Pinder AG, Rogers SC, Morris K, James PE (2009) Haemoglobin saturation controls the red blood cell mediated hypoxic vasorelaxation. *Oxygen Transport to Tissue XXX*: 13–20.
- Brunori M, Taylor J, Antonini E, Wyman J, Rossi-Fanelli A (1967) Studies on the oxidation-reduction potentials of heme proteins. *Journal of Biological Chemistry* 242: 2295.
- Li C, Jackson RM (2002) Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *American Journal of Physiology-Cell Physiology* 282: C227–C241.
- Berg JM, Tymoczko JL, Stryer L (2002) *Biochemistry*; Freeman WH, editor. New York.
- Friebe A, Schultz G, Koesling D (1996) Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. *The EMBO Journal* 15: 6863.
- Boon E, Huang S, Marletta M (2005) A molecular basis for NO selectivity in soluble guanylate cyclase. *Nature chemical biology* 1: 53–59.
- Derbyshire ER, Marletta MA (2009) *Biochemistry of soluble guanylate cyclase*. *Handb Exp Pharmacol*: 17–31.
- Boon E, Marletta M (2005) Ligand specificity of H-NOX domains: from sGC to bacterial NO sensors. *Journal of inorganic biochemistry* 99: 892–902.
- Martin E, Sharina I, Kots A, Murad F (2003) A constitutively activated mutant of human soluble guanylyl cyclase (sGC): implication for the mechanism of sGC activation. *Proceedings of the National Academy of Sciences of the United States of America* 100: 9208.
- Makino R, Park SY, Obayashi E, Izuka T, Hori H, et al. (2011) Oxygen binding and redox properties of the heme in soluble guanylate cyclase: implications for the mechanism of ligand discrimination. *J Biol Chem* 286: 15678–15687.
- Brüne B, Schmidt K, Ullrich V (1990) Activation of soluble guanylate cyclase by carbon monoxide and inhibition by superoxide anion. *Eur J Biochem* 192: 683–688.
- Gerzer R, Böhme E, Hofmann F, Schultz G (1981) Soluble guanylate cyclase purified from bovine lung contains heme and copper. *FEBS letters* 132: 71.
- Garthwaite J, Southam E, Boulton CL, Nielsen EB, Schmidt K, et al. (1995) Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol Pharmacol* 48: 184–188.
- Schrammel A, Behrends S, Schmidt K, Koesling D, Mayer B (1996) Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Mol Pharmacol* 50: 1–5.
- Pinder AG, James PE (2009) When does low oxygen become hypoxia? Implications for nitrite reduction. *Circ Res* 104: e25–26.
- Ferdinandy P, Schulz R (2003) Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. *British journal of pharmacology* 138: 532–543.
- Zweier JL, Talukder MA (2006) The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res* 70: 181–190.
- Yasmin W, Strynadka KD, Schulz R (1997) Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc Res* 33: 422–432.
- Zweier JL, Flaherty JT, Weisfeldt ML (1987) Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci U S A* 84: 1404–1407.
- Angelos MG, Kutala VK, Torres CA, He G, Stoner JD, et al. (2006) Hypoxic reperfusion of the ischemic heart and oxygen radical generation. *Am J Physiol Heart Circ Physiol* 290: H341–347.
- Friebe A, Schultz G, Koesling D (1998) Stimulation of soluble guanylate cyclase by superoxide dismutase is mediated by NO. *Biochemical Journal* 335: 527.
- Gladwin M, Raat N, Shiva S, Dezfoulian C, Hogg N, et al. (2006) Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *American Journal of Physiology- Heart and Circulatory Physiology* 291: H2026.
- Patel RP, Hogg N, Kim-Shapiro DB (2011) The potential role of the red blood cell in nitrite-dependent regulation of blood flow. *Cardiovasc Res* 89: 507–515.
- Mergia E, Friebe A, Dangel O, Russwurm M, Koesling D (2006) Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *Journal of Clinical Investigation* 116: 1731.