Oxygen Mediates Vascular Smooth Muscle Relaxation in Hypoxia

Jessica Dada*, Andrew G. Pinder*, Derek Lang, Philip E. James*

Institute of Molecular and Experimental Medicine, Wales Heart Research Institute, Cardiff University School of Medicine, Heath Park, Cardiff, United Kingdom

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 (O2) on sGC both as a direct ligand and its effect 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 O2, importantly in the absence of NO. We also show that while O2 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 O2 alone in the form of a buffer bolus (equilibrated at 95% O2/5% CO2) had the ability to dilate vessels under hypoxic conditions and that this was dependent upon sGC and independent of eNOS. Our studies confirm that O2 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 O2 versus NO-induced sGC activation.

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 (O2) differ by only one valence electron, that a role for O2 in this process has not been identified.

Conventional understanding would suggest that O2 simply does not bind to sGC [6–8], at least not in the same way as NO and CO. However changes in O2 tension are widely recognised to influence vascular tone. For instance, via the regulation of specific potassium channel activity, O2 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 O2 tension [11], implying an inverse relation between NO effectiveness and O2. These factors contribute to the concept of "hypoxic vasodilation", an innate physiological response designed to maintain tissue perfusion in the face of falling O2 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 O2 under such conditions, the latter has been overlooked as a potential mediator of the relaxation response in...
favour of more conventional activators of s'GC. To this end, oxygenated haemoglobin-derived nitrosothiol (HbSNO) [17], nitrite (NO$_{2}^{-}$)-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 prostanycin [21,22] have all been postulated to mediate "hypoxic vasodilatation".

While all or any of the above could contribute to RBC-induced vasodilatation 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 straightforward candidate, molecular O$_2$. Therefore, the aim of the present study was to investigate whether O$_2$ could act as a possible direct ligand for s'GC and/or a modulator of the actions of other preferred ligands. To accomplish these aims two model systems were used; an isolated s'GC enzyme system and an isolated blood vessel system, both of which allowed for tight control of the local O$_2$ environment. Importantly, these models not only allowed us to probe O$_2$-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$^N$-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 ε$\beta$$_{1}$. Methylamine hexamethylenemethylene methylamine (MAHMA) NONOate (NOC-9) was from Enzo Life Sciences. 2, 3-diphosphoglycerate (2, 3-DPG), YC-1, PEG-CAT (polyethylene glycol superoxide dismutase) and PEG-ME (methylethylamine hexamethylenemethylene methylamine (MAHMA) NONOate) were from Sigma Aldrich.

**Isolated sGC Study**

All experiments were performed in an Invivo2 Hypoxia Workstation 400 (Ruskin). Normoxic experiments were carried out at 37°C and 20% O$_2$/5% CO$_2$ via a 25% O$_2$/5% CO$_2$ gas cylinder (BOC). Hypoxic experiments were maintained at 37°C and ~0% O$_2$/5% CO$_2$. Reagents were allowed to equilibrate for 1 hour before all tests were completed. Precise buffer O$_2$ 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$_2$ 0.3 mmol/L, 0.045% BSA, pH 7.4. GTP, dissolved in equimolar MgCl$_2$, 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. Hypoxic samples were prepared by perfusing the buffer-enzyme mix with 95% O$_2$/5% CO$_2$. 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$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 25, Glucose 11, CaCl$_2$ 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$_2$/5% CO$_2$. 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$_2$ content (ensuring balance of N$_2$ and 5% CO$_2$) 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$_2$), 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$_2$ concentration measured in the tissue bath equilibrated under hypoxic conditions was 0.9% (equivalent to 9 μmol/L O$_2$) as we have detailed previously [11].

**Krebs Samples**

Krebs solution was gassed at 95% O$_2$/5% CO$_2$ in order to achieve 95% O$_2$ samples. 21% O$_2$ samples were allowed to equilibrate with the atmosphere. 0% O$_2$ samples were gassed with 95% N$_2$/5% CO$_2$. In order to accurately measure O$_2$ concentration within these equilibrated Krebs buffer samples, electron paramagnetic resonance (EPR) oximetry was undertaken utilising N$^{15}$-per-deuterated tempone (2,2,6,6-tetra-methyl-4-piperideine; PDT) as the O$_2$-sensitive probe. In brief, the spectral line width obtained from PDT (−5 μmol/L) shows a linear relationship with O$_2$ concentration and sample readings can be measured against known standards. This technique measures O$_2$ with an accuracy of ±0.2% O$_2$ in our hands as we have described in detail previously [23]. The precise O$_2$ concentrations within "95%", "21%" and "0%" buffer are shown in Table 1.
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 HbO₂ content was verified by blood gas analysis as described (see ‘Venous RBC samples’). The volume used in the myograph studies gave a final O₂ concentration in the tissue bath of 30.9 µmol/L, which was equal to the O₂ 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 x g for 5 minutes. The plasma layer and buffy coat were removed and replaced with an equivalent volume of gassed (95% O₂/5% CO₂) phosphate buffered saline (PBS). Following re-centrifugation at 1200 x g for 5 minutes the PBS layer was removed. In order to achieve RBCs of varying O₂ saturation, the cells were firstly diluted 1:3 with PBS and loaded on to a thin film rotating tonometer, which was purged with O₂/CO₂ to achieve higher saturations or N₂/CO₂ for lower saturations as we have described previously [24]. The O₂ 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 200i; 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 (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% O₂ 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).

| Sample                  | Stock Buffer O₂ concentration (µmol/L) | Final Tissue Bath concentration (µmol/L) |
|-------------------------|----------------------------------------|-----------------------------------------|
| 0%                      | 49                                     | 1.96                                    |
| 21%                     | 175                                    | 7.0                                     |
| 95%                     | 772                                    | 30.9                                    |
| Hypoxic chamber 0% +1 hour | 38                                     |                                         |
| Hypoxic chamber 20% +1 hour | 197                                    |                                         |

1represents the effective O₂ concentration added to each tissue bath. The existing O₂ in the “hypoxic” bath perfused with N₂/CO₂ was 0.9% (equivalent to 9 µmol/L O₂).

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).

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 (O₂⁻) 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 O₂ on Vascular Tone in Hypoxia
Tetrameric Hb has the ability to carry four O₂ molecules [25]. The O₂ content of a RBC suspension is therefore much greater than buffer equilibrated with the same O₂ concentration. In order to attain a comparable O₂ delivery, Krebs buffer was gassed with 95% O₂/5% CO₂, either in a sealed glass bottle (high O₂ saturation) or an open container allowed to equilibrate with atmospheric air (21% O₂), or with 95% N₂/5% CO₂ (0% O₂). 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
observed following addition of highly oxygenated versus minimally oxygenated samples (Figure 2).

Effects of Administering a Constant O₂ 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% O₂/5% CO₂ permanently. An instantaneous and transient relaxation response, akin to that...
following addition of O₂ buffer solution, was observed (data not shown).

Effects of sGC Inhibition on O₂-mediated Vasorelaxation

To confirm that O₂-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% O₂/5% CO₂-gassed Krebs buffer (0.72 ± 0.28% vs. 17.81 ± 1.90% in the absence of ODQ, n = 7, ***p = 0.001) (Figure 3).

Influence of Endothelial NO Synthase (eNOS) on O₂-mediated Vasorelaxation

Experiments were repeated in the presence of the eNOS inhibitor L-NG-monomethyl arginine (LNMMA, 300 μmol/L for 30 minutes). Figure 4 demonstrates that this agent has no effect on the oxygenated buffer-induced relaxations (n = 4, **p<0.01 cf. 0% O₂).

Vascular Effects of Superoxide and Hydrogen Peroxide

It is well acknowledged that reperfusion of O₂ to hypoxic vessels causes the generation of O₂ radical species [26]. Therefore, it was necessary to ascertain whether O₂⁻ 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 O₂⁻ and hydrogen peroxide (H₂O₂) generation, respectively. Neither PEG-SOD, PEG-CAT, nor both together had a significant effect on the O₂-induced relaxation of vessels in hypoxic conditions (Figure 5).

Effects of Oxygenated RBCs on Vascular Tone in Hypoxia

RBCs equilibrated at a higher O₂ saturation (98.22 ± 0.45% O₂, n = 13) caused significantly (p<0.001) more relaxation than those of partial saturation (51.43 ± 6.16% O₂, n = 4) or low saturation (20.40 ± 5.28% O₂, n = 13) when administered to a hypoxic tissue bath (Figure 6). These data confirm a positive relationship between RBC O₂ saturation and the relaxation induced in hypoxic aortic tissue (Pearson’s correlation coefficient r = 0.815, p<0.0001).

Effects of RBC O₂ Cycling upon Hypoxic Vasorelaxation

Highly oxygenated RBC (98.22 ± 0.45% O₂, n = 13) were administered to hypoxic aortic rings to induce relaxation. In a parallel sample, highly oxygenated RBC were first deoxygenated to ~20% O₂ (20.68 ± 1.66% O₂) before reoxygenation (97.70 ± 0.39% O₂, 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 ± 2.97% vs. 12.00 ± 3.60%).

The RBC O₂ 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 ± 1.69% O₂) before re-suspension in fresh oxygenated buffer to give a saturation of ~98% (98.05 ± 0.65% O₂, n = 4). As above, there was no significant difference between the relaxations induced by buffer-

Figure 2. Krebs buffer samples introduced to hypoxic aortic rings. Maximum relaxation was demonstrated with 95% oxygenated buffer samples compared with 0% O₂ samples (all n = 6, **p<0.01 cf. 0%).
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Figure 3. The effect of sGC inhibitor, ODQ, upon O₂-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).
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Figure 4. The influence of eNOS inhibitor, LNMA, upon O₂-mediated relaxation. LNMA had no effect on the relaxation responses. Similarly to Figure 3, 95% oxygenated buffer samples generated the greatest relaxation compared with 0% O₂ samples (all n = 4, **p<0.01 cf. 0% O₂).
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replaced RBCs compared with controls (12.97±1.89% vs. 12.00±3.60%).

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

2, 3-diphosphoglycerate (2, 3-DPG), (5 mmol/L) discourages O₂ 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±1.11% vs. 8.25±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 O₂ delivery.

Comparison of O₂-mediated Vasorelaxation by RBCs, Buffer or Isolated Hb

In additional experiments, purified oxygenated Hb was prepared from RBCs and applied to tissue baths containing 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 O₂ concentrations of ~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 pO₂

In separate experiments, aortic tissue was perfused for 10 minutes with 1%, 2%, 5%, 21% or 95% O₂/5%CO₂. Constriction to PE was normalised to 80% of maximum at each O₂ tension, then fully oxygenated RBCs were administered. This is in contrast to most of the experiment described above in which varying O₂ was introduced to hypoxic tissue or isolated sGC.

**Figure 5. Effect of O₂⁻ and H₂O₂ on vessel relaxation in hypoxia.** The inhibition of O₂⁻ radicals (100 U/ml PEG-SOD) and H₂O₂ (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.

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**Figure 6. Varying O₂ 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).

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**Figure 7. Effects of various oxygenated samples on relaxation responses of hypoxic vascular tissue.** Krebs buffer, Hb and RBC samples of equal O₂ content (~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).

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Significantly greater relaxation was induced at tissue incubated in 1% (**p < 0.001 cf. 95% O2) and 2% O2 (*p < 0.05 cf. 95%) (n = 6) (Figure 8).

Effects of O2 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 O2 on sGC function, and in particular, on responses of the enzyme to known ligands and stimulators/activators. Our data conclusively shows that molecular O2 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 O2 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 O2 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 O2 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 O2 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 O2 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 O2 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 O2 with the sGC in a way that modulates the haem group. sGC has been reported to function optimally at intracellular O2 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 O2 and sGC has not been identified, it is possible that intracellular concentrations of O2 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 N2.
addition compared to basal conditions. In addition, Soret band analysis by UV spectrophotometry show subtle changes in sGC binding under oxygenated conditions [33].

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 O2 concentrations. In such experiments, transient and concentration-dependent relaxation responses to O2 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 O2-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 O2 was insensitive to eNOS inhibition by LNMMA, such an involvement was ruled out. Together these observations support the role of O2 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 O2, 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 O2− on sGC [44] it was important to confirm that O2−/H2O2 were not involved in the sGC-driven effects we observe following addition of O2 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 O2 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 O2 buffer bolus for highly O2-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 O2 and HbSNO [45], and an altered HbSNO to relaxation response [36,37]. Since an involvement of sGC and classic cGMP-dependent downstream mechanisms are involved in sGC activation already described above.

Furthermore, we show that O2 can modulate the effect of NO in reducing responses. For some time it has been suggested that RBC-induced vasorelaxation is linked to Hb allosteric effects. Indeed, in this study we show that RBC-induced vasorelaxation is linked to Hb allosteric effects. This hypothesis was supported by the use of the allosteric modifier YC-1. Our data demonstrate that YC-1 in our isolated enzyme model clearly enhanced 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 O2, perhaps suggesting the effects of O2 on the enzyme may be occurring at a site independent to that of YC-1. Furthermore, we show that O2 can modulate the effect of NO (concentration-dependent) upon the enzyme, indicative of an interaction of O2 with the haem of sGC or an indirect involvement with haem via an allosteric site. Armed with the knowledge that ~6% of sGC within vascular tissue is stimulated by NO under normal conditions our findings may have important consequences in the wider context of vasomotor relaxation via stimulation of sGC across the physiological and pathological O2 range.

**Author Contributions**

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