NO contributes to EDHF-like responses in rat small arteries: a role for NO stores

Sharmila Chauhan\textsuperscript{a,*}, Awahan Rahman\textsuperscript{b}, Holger Nilsson\textsuperscript{b}, Lucie Clapp\textsuperscript{a}, Raymond MacAllister\textsuperscript{a}, Amrita Ahluwalia\textsuperscript{c}

\textsuperscript{a}Centre for Clinical Pharmacology, University College London, The Rayne Institute, 5 University Street, London WC1E 6JJ, UK
\textsuperscript{b}Department of Physiology, Aarhus University, Universitetsparken 160, DK 8000 Aarhus C, Denmark
\textsuperscript{c}Department of Clinical Pharmacology, Barts and The London, Charterhouse Square, London EC1M 6BQ, UK

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Abstract

Objectives: Responses to EDHF are usually characterised in the presence of nitric oxide synthase (NOS) and cyclooxygenase (COX) inhibitors. The contribution of NO to endothelium-dependent relaxation in the presence of NOS inhibitors was assessed using NO scavengers with the objective of testing (i) whether any residual NO produces endothelium-dependent relaxation in a manner similar to EDHF and (ii) to identify the source of the residual NO.

Methods: Small rat hepatic and mesenteric arteries were mounted in a tension myograph for either isometric or membrane potential measurements.

Results: Relaxation to ACh was unaffected by pre-treatment with \textsuperscript{L}-nitro-arginine methyl ester (L-NAME, 300 \textmu M), and indomethacin (Indo, 5 \textmu M) in the absence or presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ, 1 \textmu M), nitro-arginine (300 \textmu M) or \textsuperscript{L}-nitro-mono-methyl-arginine (L-NMMA, 300 \textmu M). Addition of OxyHb (20 \textmu M) or carboxy-PTIO (300 \textmu M) produced a significant suppression of ACh-induced relaxations (\textsim 40\%). In L-NAME\textsuperscript{1}Indo treated arteries ACh-induced hyperpolarisation (D16.3\pm2.1 mV, n=8) was significantly suppressed with the addition of OxyHb (D10.2\pm1.6 mV, n=12). ACh-induced relaxation, in the presence of L-NAME+Indo+OxyHb, was abolished by raised extracellular K\textsuperscript{+}, or the combination of charybdotoxin (CTX, 100 nM)+apamin (100 nM). In contrast whilst L-NAME+indo+barium+ouabain suppressed ACh-induced relaxation, the presence of OxyHb had no additional effect. Ultraviolet light induced a relaxation in arteries treated with L-NMMA+Indo (37.0\pm5.2\%, n=9) which was sensitive to OxyHb (15.2\pm10.9\%, n=4), and barium+ouabain (6.39\pm2.7\%, n=4), but not CTX+apamin (37.8\pm2.4\%, n=4). Conclusions: These findings suggest that NO contributes significantly to the “EDHF-like” response seen in rat small arteries and that the source of this NO may be preformed vascular stores.

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Keywords: Endothelial factors; K-channel; Membrane potential; Nitric oxide

1. Introduction

Regulation of vasodilatation by the endothelium is determined by three main components; nitric oxide (NO), prostacyclin and endothelium derived hyperpolarizing factor (EDHF). The role that each of these factors plays in endothelium-dependent relaxation has been extensively studied. In particular it appears that, whilst EDHF plays little role in vasoactive responses of conduit vessels, it mediates a major component of the response to endothelium-dependent vasodilators in resistance arteries [1–3]. However, the identity of EDHF remains elusive with proposed candidates including potassium ions and epoxycosatrienoic acids [4,5].

EDHF causes hyperpolarization of vascular smooth muscle cells, thereby causing vasorelaxation via inhibition of voltage-gated calcium channels [6]. This change in...
membrane potential has been attributed to modulation of K$^+$ flux since raising extracellular K$^+$ concentration (25–40 mM) [7,8] or inhibiting specific K$^+$ channels, blocks EDHF responses. A role for large (BK$_{Ca}$), intermediate (IK$_{Ca}$) and small (SK$_{Ca}$) conductance calcium-activated K$^+$ channels has been implicated since, EDHF responses are sensitive to the toxins charybotoxin (CTX), which blocks both BK$_{Ca}$ and IK$_{Ca}$, and apamin which blocks the SK$_{Ca}$ [8,9]. Additionally, activation of the inward rectifier K$^+$ channel (K$_{IR}$) and Na$^+$/K$^+$-ATPase by extracellular K$^+$ may contribute to EDHF responses; inhibition of their activity using barium (Ba$^{2+}$) and ouabain, respectively attenuates EDHF responses (for review, see Edwards and Weston [10]). However, this theory is controversial, not least because K$^+$ ions fail to induce hyperpolarization or relaxation in some tissues that exhibit EDHF activity [11,12].

To facilitate investigation of EDHF responses, it is necessary to eliminate the activity of the other endothelium-derived mediators; primarily prostacyclin and NO. The complete removal of these mediators is essential since both NO and prostacyclin act, in part, by hyperpolarizing smooth muscle [13,14] and both appear to interfere with EDHF release and activity [15,16]. Classically inhibition of NO and prostacyclin is achieved using a combination of the NO synthase (NOS) inhibitor, N$^\text{G}$-nitro-L-arginine methyl ester (L-NAME), and the cyclo-oxygenase (COX) inhibitor, indomethacin (Indo). Relaxations in the presence of these two agents are attributed to an EDHF response [11,15,17,18]. However, whilst Indo is reliably effective at inhibiting prostacyclin synthesis [19,20], recent studies suggest that NOS inhibitors alone are insufficient to abolish endothelium-dependent responses accredited to NO [21,22]. Thus, despite the presence of NOS inhibitors it is possible that NO may contribute to responses that have previously been attributed to EDHF.

In light of the above findings, we have investigated the contribution of NO to the classical EDHF response in the presence of NOS inhibitors to determine whether some of the activity attributed to EDHF is due to NO activity not eliminated by NOS inhibition. In addition we have conducted studies to identify the source of this residual NO. Experiments were conducted in rat mesenteric and hepatic arteries; both preparations in which a significant component of endothelium-dependent relaxation is thought to be due to EDHF activity [4,11,24]. In addition to NOS inhibitors we have used pharmacological tools affecting the NO pathway further downstream, namely the soluble guanylate cyclase (sGC) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ) [25] and the NO scavengers oxy-haemoglobin (OxyHb) [26] and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) [27–29]. Some of these results have been presented in a preliminary form to the British Pharmacological Society [30].

2. Methods

Male Sprague Dawley rats (180–240 g) were stunned and killed by cervical dislocation. All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, United Kingdom. The mesentery or liver was removed and immediately placed into physiological salt solution (PSS (mM); NaCl 118, KCl 4.7, NaHCO$_3$ 25, CaCl$_2$ 2.5, glucose 11, MgSO$_4$ 1.2 and KH$_2$PO$_4$ 1.2] gassed with 5% CO$_2$–95% O$_2$. Third- or fourth-order mesenteric or hepatic arteries were cleared of extraneous tissue and approximately 2 mm lengths cut and mounted in a tension myograph for isometric tension measurements [31]. Vessels were equilibrated for 1 h at 37°C in PSS bubbled with 5% CO$_2$–95% O$_2$. After this time, arteries were normalised as previously described [35]. Vessels were then repeatedly contracted with the thromboxane A$_2$ analogue U46619 (1 µM) until two successive responses were within 10% of each other. Endothelial integrity was determined using acetylcholine (ACh, 1 µM); arteries were deemed intact if the percentage reversal by ACh of U-46619-induced precontraction was greater than 50%. The endothelium was removed by inserting a human hair into the lumen of the vessel, using a previously described protocol [32].

2.1. Functional reactivity studies

Following equilibration, arteries were precontracted with a concentration of U46619 (1–100 nM) giving 75–90% of the response to 1 µM U46619. On generation of a stable baseline, a concentration–response curve to ACh (1–10,000 nM) was constructed. To dissect the mechanisms involved in ACh-induced relaxation, vessels were pre-treated with one of a range of inhibitor combinations described below or maintained as a timed control. In all experiments the concentration of U-46619 required to precontract the arteries was titrated to produce a similar level of precontraction.

2.2. Determination of optimal NO blockade

To determine the role that NO plays in the classical EDHF response, mesenteric arteries were pre-treated with L-NAME (300 µM, 30 min) and Indo (5 µM, 30 min) in the presence or absence of either the sGC inhibitor, ODQ (1 µM, 30 min) or NO scavengers OxyHb (10 µM given 15 min prior to precontraction and then a second 10 µM applied just prior to application of ACh to give a total of 20 µM) or carboxy-PTIO (300 µM, 30 min). To ascertain whether maximal NOS inhibition was achieved using L-NAME, two other NOS inhibitors were also tested: nitro-L-arginine (L-NOLA, 300 µM) or L-nitro-mono-methyl-arginine (L-NMMA, 300 µM) in combination with L-NAME (30 µM)+Indo (5 µM). These combinations were chosen
since previous studies suggest that these particular combinations produce greater NOS inhibition than any of the individual NOS inhibitors at the maximally effective concentration (300 \( \mu \)M) [21].

2.3. Characterisation of relaxation to ACh in the presence of optimal NO blockade

2.3.1. Mesenteric arteries

To determine whether the remaining response in the presence of l-NAME+Indo+OxyHb was due to EDHF activity, mesenteric arteries were treated with l-NAME+Indo+OxyHb in the absence or presence of raised extracellular \( K^+ \) (KCl 30 mM, 15 min). To determine whether \( BK_{ca} \), \( IK_{ca} \) and \( SK_{ca} \) channels were involved in the response to ACh in the presence of optimal NO and prostacyclin inhibition, a combination of CTX (100 nM, 15 min)+apamin (100 nM, 15 min) was added to the l-NAME+Indo+OxyHb inhibitor combination. Finally, to assess whether the addition of OxyHb altered the sensitivity of EDHF responses to inhibition of \( K_{IR} \) and \( Na^+/K^+\)-ATPase, arteries were treated with l-NAME+Indo+OxyHb in the presence or absence of \( Ba^{2+} \) (30 \( \mu \)M, 15 min)+ouabain (1 mM, 15 min).

2.3.2. Hepatic arteries

The experiments with \( Ba^{2+} \)+ouabain were repeated in rat hepatic arteries; a preparation in which a significant component of the EDHF response has previously been shown to be sensitive to these agents [21]. To determine whether \( Ba^{2+} \)+ouabain had a direct effect on NO mediated relaxation the response to the NO donor sodium nitroprusside (SNP, 1-10,000 nM) was determined in the absence or presence of these inhibitors.

2.4. Effect of OxyHb and inhibitors of potassium flux on photorelaxation

To determine whether preformed NO stores were present in rat mesenteric arteries, vessels were subjected to ultraviolet illumination, a procedure thought to result in the release of NO from NO-thiol stores [33,34]. In brief, arteries were pre-contracted with U46619 (10-100 nM), and then exposed to ultraviolet light (15 exposures at wavelength of 366 nm, over a 20 s period). The mechanisms by which NO, from this source, caused vasorelaxation were investigated using selective inhibitors and blockers as described above. Arteries were treated with l-NMMA (300 \( \mu \)M)+Indo (5 \( \mu \)M), in the presence and absence of one of the following combinations: OxyHb, CTX+apamin, or \( Ba^{2+} \)+ouabain (concentrations as above). Finally, to determine the location of these stores, experiments were conducted in endothelium-denuded vessels treated with l-NMMA+Indo. l-NAME was not used in this study since it contains a nitro group, which releases NO when exposed to ultraviolet light [35].

2.5. Membrane potential studies

For electrophysiological measurements, mesenteric arteries were equilibrated as described above. Membrane potential was measured using aluminium silicate electrodes which had resistances between 70 and 135 \( \Omega \) when filled with 3 M KCl. Following measurement of a stable resting potential, vessels were treated with either l-NAME (300 \( \mu \)M, 30 min)+Indo (5 \( \mu \)M, 30 min) or l-NAME+Indo+OxyHb (20 \( \mu \)M, 15 min). The maximum response to ACh infusion (10 \( \mu \)M) was determined when a plateau was attained, and calculated as peak hyperpolarization. Electrode entry into a vascular smooth muscle cell was determined by an abrupt drop in voltage, followed by a sharp return to baseline on exit. All recordings produced a stable measurement for a minimum of 2 min with a minimal change in electrode resistance (maximum 10% change) [35].

2.6. Data and statistical analysis

ACh, SNP and ultraviolet light induced relaxations are expressed as percentage reversal of U46619-induced tone. The potency of ACh is expressed as the negative logarithm of the EC\(_{50}\) (pEC\(_{50}\)), which was calculated using either Prism Graphpad or SPSS software. All data is shown as mean±S.E.M. Tests of significance between curves were conducted using either two-way analysis of variance (ANOVA) for multiple comparisons or a general linear model for three or more curves. Students t-test were used to compare the differences between two data groups, where \( P<0.05 \) was considered significant. The \( n \) values quoted similarly indicate the number of experiments and animals used.

2.7. Drugs and material

All drugs were obtained from Sigma (Poole, UK), except U46619 (Biomol), charybdotoxin and apamin (Tocris Laboratories), carboxy-PTIO (Calbiochem Novabiochem) and ODQ which was a kind gift from Professor Garthwaite, The Wolfson Institute for Biomedical Research, London. Human haemoglobin was reduced using sodium dithionate [36].

Stock solutions of U-46619 were made in 100% ethanol and kept at \(-20^\circ C\). On the day of experimentation the first dilution of U-46619 was made in ethanol–saline (50:50), and successive dilutions in 100% saline. Stock solutions of ODQ and ouabain in 100% dimethylsulphoxide (DMSO), CTX and apamin in water were stored at \(-20^\circ C\) until day of use. Indo was made freshly each day in 1% sodium
Fig. 1. Effect of NO scavenging [oxy-haemoglobin (OxyHb, 20 μM) or carboxy-PTIO (300 μM) or soluble guanylate cyclase inhibition (ODQ, 1 μM)] on ACh-induced relaxation in rat mesenteric arteries. Concentration–response curves to ACh in the absence or presence of L-NAME (300 μM) and indomethacin (Indo, 5 μM), L-NAME+Indo+ODQ, L-NAME+Indo+OxyHb or L-NAME+Indo+carboxy-PTIO. Values shown are mean±S.E.M. Statistical analysis depicted as *P<0.001 compared to the untreated vessels using general linear regression model (n=11).

bicarbonate in saline. All other drugs were dissolved in saline. All dilutions were made in saline on day of use.

3. Results

The normalised diameter of the vessels studied was (RMA = 285.3±7.9 μm; RHA = 221.9±9.2 μm). ACh caused concentration-dependent relaxation that was abolished in endothelium-denuded vessels (maximum relaxation 9.0±5.8%; n=6; P>0.05). Basal tension was unaffected by drug treatments with the exceptions of OxyHb, which caused a transient contraction of 1.3±0.5 mN (n=37), i.e., 4.2±0.3% of maximal contraction to 1 μM U46619, that returned to baseline levels after a few minutes, and CTX+apamin which caused a sustained contraction of 8.1±2.4 mN; n=11 (36.7±12.2%).

3.1. Effect of oxy-haemoglobin, ODQ and carboxy-PTIO on EDHF responses

ACh caused concentration-dependent relaxation of rat mesenteric arteries that was unaffected by pre-treatment with L-NAME+Indo (Fig. 1, Table 1). Addition of ODQ to the cocktail of inhibitors had no additional effect on ACh-induced relaxation response curves (Fig. 1, Table 1). In contrast, addition of OxyHb significantly depressed the maximal relaxation to ACh but had no significant effect on potency (Fig. 1, Table 1). Similarly addition of carboxy-PTIO significantly (P<0.001) suppressed ACh induced relaxation. The presence of L-NAME in addition to OxyHb and Indo was necessary and sufficient for optimal NO blockade; OxyHb and Indo alone reduced maximum relaxation to only 78.5±12.4% of control (n=7), and addition of other NOS inhibitors; L-NOLA or L-NMMA to the L-NAME (30 μM)+Indo cocktail had no further inhibitory effect on ACh-induced relaxation (max. relaxations 89.8±2.1 and 74.5±7.8%, respectively; n=5; P>0.05).

3.2. Effect of inhibition of K⁺ flux on EDHF responses

In rat mesenteric arteries, elevation of extracellular K⁺ to 30 mM raised basal tension by 30.7±4.4% (% of contraction to 1 μM U46619); 7.52±1.3 mN (n=11). U-46619 (1–10 nM) was then titrated to produce a comparable level of precontraction to that used in the previous experiments, i.e., 75–90% of maximum contraction to 1 μM U-46619. In the presence of L-NAME+Indo+OxyHb, elevation of K⁺ abolished the remnant ACh-induced relaxation and exposed concentration-dependent contraction to ACh (pEC₅₀=7.0±0.3, n=11; Fig. 2A). Similarly, CTX+apamin treatment abolished the remnant relaxation to ACh, and unmasked a contractile response (pEC₅₀=6.8±0.7, n=11; Fig. 2B). In the presence of L-NAME+Indo (pEC₅₀=7.3±0.1, max.=89.7±1.2%, n=5), addition of either Ba²⁺+ouabain

Table 1

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Mean pEC₅₀</th>
<th>Maximum relaxation (%)</th>
<th>n value</th>
</tr>
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<td>Control</td>
<td>6.7±0.1</td>
<td>90.7±0.5</td>
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<td>L-NAME (300 μM)+Indo (5 μM)</td>
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<td>83.1±8.2</td>
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<td>L-NAME+Indo+ODQ (1 μM)</td>
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<td>L-NAME+Indo+OxyHb (20 μM)</td>
<td>6.7±0.2</td>
<td>58.0±15.0**</td>
<td>14</td>
</tr>
<tr>
<td>L-NAME+Indo+carboxy-PTIO (300 μM)</td>
<td>7.0±0.2</td>
<td>40.4±19.4**</td>
<td>6</td>
</tr>
</tbody>
</table>

Data are shown as mean±S.E.M. L-NAME = l-Nitro-arginine methyl ester. Indo = indomethacin. OxyHb = oxy-haemoglobin, ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. Carboxy-PTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Statistical analysis depicted as **P<0.01 compared to L-NAME+Indo treated vessels using one-way ANOVA followed by Bonferroni post-hoc tests.
The rat hepatic artery showed similar sensitivity to the drug cocktails as the mesenteric arteries. Pre-treatment of vessels with L-NAME + Indo + OxyHb suppressed ACh-induced relaxation (Fig. 4A, Table 2). Treatment of hepatic arteries with L-NAME + Indo + Ba\(^{2+}\) + ouabain also significantly suppressed ACh-induced relaxation (Fig. 4A, Table 2). However, addition of OxyHb to this cocktail, had no additional effect (Fig. 4A, Table 2). SNP produced concentration-dependent relaxation of rat hepatic arteries (pEC_{50} = 6.6 ± 0.3, max. = 51.9 ± 14.8, n = 5), which was significantly (P < 0.001) attenuated in the presence of Ba\(^{2+}\) + ouabain (pEC_{50} = 7.0 ± 0.3, max. = 55.0 ± 14.5%, n = 4) (Fig. 4B).

### 3.3. Effect of OxyHb and potassium channel inhibitors on photorelaxation

Ultraviolet light exposure caused a relaxation in mesenteric arteries treated with L-NMMA + Indo (37.5 ± 4.5%, n = 10) that was absent in endothelium denuded arteries (11.5 ± 9.8%, n = 7, P < 0.001). This relaxation was significantly reduced in arteries treated with OxyHb (11.9 ± 7.9%, n = 6, P < 0.01) or Ba\(^{2+}\) + ouabain (6.39 ± 2.7%, n = 4, P < 0.01). Treatment with CTX + apamin had no effect on ultraviolet light-induced relaxation (37.8 ± 2.4%, n = 4) (Fig. 5).
vessels with l-NAME+Indo had no significant effect on resting membrane potential (−56.7±2.7 mV, n=8). Under these conditions ACh (10 μM) caused a 16.3±2.1 mV (n=8) hyperpolarization. Addition of OxyHb, to these l-NAME+Indo treated arteries, had no effect on membrane potential per se (−53.5±3.0 mV, n=12) but significantly reduced (P<0.05) the hyperpolarization response to ACh (10.2±1.6 mV, n=12) (see Fig. 6A and B for typical responses).

4. Discussion

It is generally accepted that EDHF is responsible for the hyperpolarizing component of endothelium-dependent relaxation in the presence of NOS and COX inhibition. However, the current study suggests that, despite blockade of NOS and COX activity, NO still accounts for a major component of ACh-induced relaxation in rat mesenteric and hepatic small arteries attributed to EDHF. It is only upon further removal of NO, achieved using NO scavengers, that an environment in which responses to “classical” EDHF can then be clearly dissected. In addition this NOS inhibitor-resistant NO may be derived from endothelial NO stores and causes relaxation via the activation of potassium channels.

The arteries used for this study were chosen since a significant component of ACh-induced relaxation (95%) in both rat mesenteric [2] and hepatic arteries [24] is believed to be mediated by EDHF. Consistent with these observations, inhibition of NOS and COX, with concentrations of l-NAME and Indo thought to maximally inhibit enzyme activity, had little effect on ACh responses in both types of artery as demonstrated in this and previous studies [37–41]. Furthermore application of more than one NOS inhibitor had no greater effect than l-NAME alone indicating that the concentration of NOS inhibitor used in these studies was maximally effective. Classically the remnant relaxation to endothelium-dependent vasodilators in the presence of NOS and COX inhibitors has been attributed to EDHF. However, the NO scavengers, OxyHb and carboxy-PTIO, profoundly suppressed ACh-induced relaxation in both preparations. This finding is in contrast to that of Garland and McPherson [42] where it was shown that OxyHb had no effect on ACh-induced relaxations in rat mesenteric small arteries. This difference may relate to the concentration of OxyHb used; in the above study a single concentration of OxyHb (20 μM) was applied twice (10 μM given 15 min prior to pre-constriction and then a second 10 μM with a 10 min pre-treatment was used. Although low-dose OxyHb was shown to block the responses to exogenously applied NO, it is possible that endogenous NO (including NO in putative stores) may not have been scavenged. In addition in the current study similarly to OxyHb, a structurally unrelated NO scavenger,
Table 2

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Mean pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Maximum relaxation (%)</th>
<th>n value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.9±0.5</td>
<td>90.7±0.5</td>
<td>16</td>
</tr>
<tr>
<td>l-NAME (300 µM) + Indo (5 µM) + OxyHb (20 µM)</td>
<td>6.9±0.1</td>
<td>48.7±3.2*</td>
<td>6</td>
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<tr>
<td>l-NAME + Indo + Ba&lt;sup&gt;2+&lt;/sup&gt; (30 µM) + ouabain (1 nM)</td>
<td>6.3±0.5*</td>
<td>18.7±7.4***</td>
<td>8</td>
</tr>
<tr>
<td>l-NAME + Indo + OxyHb + Ba&lt;sup&gt;2+&lt;/sup&gt; + ouabain</td>
<td>6.9±0.7</td>
<td>24.9±10.4**</td>
<td>11</td>
</tr>
</tbody>
</table>

Data are shown as mean±S.E.M. l-NAME=1-Nitro-arginine methyl ester, Indo=indomethacin, OxyHb=oxy-haemoglobin, Ba<sup>2+</sup>=barium. Statistical analysis depicted as *P<0.05, **P<0.01, ***P<0.001 compared to the control vessels using one-way ANOVA followed by Bonferroni post-hoc tests.

carboxy-PTIO, also attenuated ACh-induced relaxation. Results from this study suggest that l-NAME does not optimally inhibit NO-dependent responses in these preparations; indeed studies measuring ACh-induced NO release, using an NO microsensor, from arteries including rat mesenteric artery [15,23] demonstrate residual NO measurement despite NOS inhibition.

From the present study it is clear that NO mediates a major component of ACh-induced relaxation in mesenteric and hepatic arteries, and a significant concentration-dependent relaxation to ACh still remains in the presence of optimal NO and prostacyclin blockade. This response is most likely due to EDHF activity since it is abolished by raising extracellular K<sup>+</sup> and by CTX+apamin treatment, as in other arteries [43–45] and confirms that these modulators of K<sup>+</sup> flux antagonise EDHF-mediated relaxations. Additionally following complete NO, prostacyclin and EDHF blockade ACh causes concentration-dependent contraction. This is in contrast to other studies [4,46], where blockade of EDHF responses resulted merely in an absence of any response to ACh (i.e., no relaxation and no contraction). The difference between these studies and the current may be explained by the fact that only in the absence of all endothelial derived relaxing factors, does ACh produce a contractile response. This is only observed in the presence of an NO scavenger and suggests that remnant NO may account for the absence of ACh-induced contraction in other vessels.

The l-NAME-insensitive but OxyHb-sensitive NO component of ACh-induced relaxation, in mesenteric arteries, did not involve activation of sGC, since the selective inhibitor, ODQ, had no effect on ACh responses in the presence of l-NAME+Indo. This dose of ODQ blocks ACh-induced relaxation of rabbit aorta, a response known to be entirely NO mediated, inhibits endothelium-dependent relaxation of rat aorta, and produces maximal inhibition of GC activity [25,47]. Indeed there is precedent for sGC-independent mechanisms of NO mediated relaxation, including direct hyperpolarization of vascular smooth muscle by authentic NO and NO donors [21,48–50]. It is probable that this is the mechanism for NO-mediated relaxation in the current study, since Ba<sup>2+</sup>+ouabain...
alter the exact mechanisms involved in ACh-induced relaxation [51]. However the data do show that OxyHb, which profoundly suppresses ACh-induced relaxation, also significantly suppresses ACh-induced hyperpolarization. Consistent with this, relaxations to the NO donor SNP are partially inhibited by Ba\(^{2+}\) ouabain in rat hepatic arteries suggesting NO-induced activation of K\(_{IR}\) and the Na/K ATPase: activity which is shared by a variety of different NO donors in several different artery types [52,53]. Our data support the hypothesis that endogenously generated NO similarly to NO donors also causes hyperpolarisation of vascular smooth muscle. Interestingly, unlike CTX+ apamin or raised K\(^+\), treatment with Ba\(^{2+}\) and ouabain, in the presence of OxyHb, whilst having a slightly greater effect than OxyHb alone did not convert ACh relaxation into contraction. These results suggest that a major component of EDHF responses previously shown to be sensitive to blockade of K\(_{IR}\) and the Na/K ATPase, reflects only a minimal EDHF blocking capacity and, in the large part is due to block of NO-mediated responses.

The source of this NOS inhibitor-insensitive, but Oxy-Hb-sensitive component of ACh-induced relaxation may be NO release from a pre-formed thiol store. Exposure to ultraviolet light releases NO from preformed stores in certain blood vessels [54]. Accordingly in the present study ultraviolet light caused relaxation of endothelium intact mesenteric arteries treated with L-NMMA + Indo. These responses where significantly inhibited by either OxyHb or Ba\(^{2+}\) ouabain, but insensitive to CTX+ apamin. Consistent with this, other studies also show NO release from such stores mediates OxyHb sensitive vasorelaxation by causing hyperpolarisation of vascular smooth muscle [55–58]. The location of this store is likely to be within the endothelial cell, since removal of the endothelium abolished ultraviolet light-induced relaxation. Therefore, it appears that the NO component of ACh-induced relaxation in these arteries is mediated by two distinct NO sources; one is susceptible to NOS inhibitors and likely to be produced by de novo synthesis by eNOS, and the other is NOS-inhibitor insensitive, sensitive to NO scavengers and might be attributed to a pre-formed store. In addition that NO from these stores cause relaxation by directly hyperpolarizing vascular smooth muscle. An alternative explanation for our findings is that NO-store derived NO might operate by stimulating the release of EDHF. This is unlikely since several lines of evidence point to an inhibitory effect of NO on EDHF release [16]. However further experiments investigating the hyperpolarizing nature of NO in endothelium-denuded and intact preparations might address this issue.

The clinical relevance of NO stores is unclear but it is possible that in diseases where NO production is impaired, e.g., endotoxaemia or hypertension, NO-stores may serve as a compensatory vascular NO supply. Indeed, Kubaszewski et al. [59], demonstrated that photorelaxation in hypertensive rats was enhanced compared to controls. It is

Fig. 6. Effect of oxy-haemoglobin (OxyHb, 20 \(\mu\)M) on ACh-induced hyperpolarization of rat mesenteric arteries. Intracellular microelectrode recordings of vascular smooth muscle cells. The effect of ACh (10 \(\mu\)M) on membrane potential in a vessel treated with L-NAME (300 \(\mu\)M) and indomethacin (Indo, 5 \(\mu\)M) or with L-NAME + Indo+OxyHb is shown in A and B, respectively. Mean ACh (10 \(\mu\)M) induced peak hyperpolarization in vessels treated with L-NAME+Indo (n=5) or L-NAME+Indo+ OxyHb (n=6) is shown in C. Values shown are mean±S.E.M. Statistical analysis using unpaired \(t\)-test shown as *\(P<0.05\).
possible that since there is often an induction of iNOS in situations where eNOS is dysfunctional, the blood vessel retains the capacity to store NO. Thereby potentially providing a source of NO that may be released in response to endothelium-dependent stimuli. Much evidence suggests that NO stores play an important role in the physiology of the endothelium, however the mechanisms by which NO is released from these stores is still unclear.

In summary a large component of the response to ACh that has previously been attributed to EDHF and mediated by Ba$^{2+}$ and ouabain sensitive mechanisms is likely secondary to NO released from a store located in the endothelial cell. Our findings highlight the importance of using NO scavengers in addition to NOS inhibitors to eliminate NO when attempting to study EDHF responses in the vasculature.

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