Endothelium-derived NO, but not cyclic GMP, is required for hypoxic augmentation in isolated porcine coronary arteries

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The exact mechanism underlying hypoxic endothelium-dependent augmentation of vasoconstriction is not fully understood. Bioassay studies (45) on canine coronary arteries demonstrated that a diffusible substance released by the endothelium contributes to the response. Further studies (22) in the same artery showed that a nitric oxide (NO) synthase inhibitor, a NO scavenger, and a soluble guanylyl cyclase inhibitor abolished the response, implying the involvement of NO.

NO is one of the known gaseous molecules, besides oxygen, carbon monoxide, and hydrogen sulfide (20, 66), that can regulate vascular tone. It is synthesized by different isoforms of NO synthase. In blood vessels, the isoform found in the endothelium [endothelial NO synthase (eNOS)] plays the predominant role (36). NO produced by eNOS diffuses to the underlying vascular smooth muscle cells where it binds to the heme group of soluble guanylyl cyclase, which catalyzes the conversion of GTP to cyclic GMP. The latter activates protein kinase G (PKG) and its downstream targets (35). NO can also directly activate sarco/endoplasmic reticular calcium ATPase (SERCA; Ref. 1) as well as cause hyperpolarization, allowing it to be considered as one of the endothelium-derived hyperpolarizing factors (18). These different actions of NO concur to make it a powerful endogenous vasodilator. Hence, its involvement in an endothelium-dependent vasoconstrictor response seems paradoxical (10, 22, 41). The possibility of contribution of other endothelium-derived vasoactive factors, whether constrictor or dilator (20), in the hypoxic augmentation has not been examined systematically, although earlier bioassay studies make a contribution of endothelin unlikely (3). The present study was designed to determine whether or not eNOS-derived NO is solely responsible for the endothelium dependency of hypoxic augmentation of the constrictor responses of isolated coronary arteries. When this appeared to be the case, the roles played in this paradoxical effect of NO by its major target in vascular smooth muscle, soluble guanylyl cyclase, and by the second messenger that it produces, cyclic GMP, were examined. When the results demonstrated that activity of the former, but not production of the latter, was required, the hypothesis was tested that the cyclic GMP-independent effects of NO involve movements of, or sensitivity to, the activator ion calcium.

MATERIALS AND METHODS

Tissue preparation. Porcine hearts were collected from the local abattoir and immersed in Krebs-Ringer bicarbonate buffer of the following composition: 120 mM NaCl, 25 mM NaHCO3, 5.5 mM glucose, 4.76 mM KCl, 1.18 mM MgSO4, 7H2O, 1.18 mM NaH2PO4·2H2O, and 2.5 mM CaCl2·2H2O (control solution). The coronary arteries were isolated, and the fat and connective tissues of
the adventitia were removed. They were cut into rings (~3 mm in length) for the recording of isometric tension and the measurement of cyclic GMP levels. In some preparations, the endothelium was removed mechanically (21, 52) by inserting the tip of a small forceps into the lumen and rolling the preparation on tissue paper soaked in control solution.

Isometric tension recording. Rings of artery were equilibrated for 1 h in organ chambers containing control solution aerated with 95% O2-5% CO2 and maintained at 37°C. They were connected to force transducers (AD Instruments, Sydney, Australia) for isometric tension recording (PowerLab; AD Instruments) and allowed to equilibrate at optimal basal tension [2.0 g; determined in preliminary experiments (data not shown)]. The rings were exposed to 60 mM potassium chloride twice before the actual experiment. The resulting increase in tension was used as reference tension. The rings were then incubated with different antagonists or inhibitors (Table 1) for 40 min (unless stated otherwise) to allow cell permeation and full inhibition of the target. This period of incubation also allowed basal tone to return to normal in the individual cases where the antagonist or inhibitor causes transient contractions. For calcium-depletion experiments, rings were submerged in calcium-free control solution, exposed three times to 60 mM potassium chloride, and washed in calcium-free control solution to remove extracellular calcium. The rings were exposed to 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α, (U46619, 3 × 10⁻⁸ M to 10⁻⁸ M) until a sustained contraction with a magnitude of ~30% of the reference contraction to 60 mM KCl was obtained. The organ chamber was then aerated with 95% N₂-5% CO₂ to induce hypoxia, reaching a Po₂ [measured with dissolved oxygen electrode (Lazar Research Laboratories, Los Angeles, CA)] of 30–40 mmHg in ~2 min.

Cyclic GMP immunoassay. Rings with or without endothelium were quickly frozen in liquid nitrogen and then stored in ~80°C at different time points of the hypoxic contraction until the rings were homogenized. Rings from two different hearts receiving the same treatment were weighted, pooled, and homogenized in 200 μL of tricarboxylic acid. They were then sonicated to extract the cyclic nucleotides. The cyclic GMP content was assayed using ELISA kits (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions.

### Table 1. Hypoxic augmentation in porcine coronary arteries

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Change of Tension During Hypoxia (560 mKCl contraction)</th>
<th>Concentration Used</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.6 ± 2.8</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>l-NAME</td>
<td>1.5 ± 1.1*</td>
<td>10⁻⁴ M</td>
<td>Nonselective inhibitor of ENOS</td>
<td>(18)</td>
</tr>
<tr>
<td>NS2028</td>
<td>5.2 ± 1.8*</td>
<td>10⁻⁵ M</td>
<td>Inhibitor of soluble guanylyl cyclase</td>
<td>(39)</td>
</tr>
<tr>
<td>YC-1 + l-NAME</td>
<td>32.5 ± 4.3</td>
<td>10⁻⁵ M (YC-1)</td>
<td>Stimulator of soluble guanylyl cyclase</td>
<td>(15, 40)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>32.1 ± 5.7</td>
<td>10⁻⁵ M</td>
<td>Nonselective inhibitor of iyclooxygenase</td>
<td>(11, 45)</td>
</tr>
<tr>
<td>Bosentan</td>
<td>27.4 ± 3.8</td>
<td>10⁻⁵ M</td>
<td>Nonselective Inhibitor of endothelin receptor</td>
<td>(4)</td>
</tr>
<tr>
<td>Tiron + DETCA</td>
<td>21.7 ± 0.9</td>
<td>10⁻² M (Tiron), 10⁻⁴ M (DETCA)</td>
<td>Superoxide anion scavenger (Tiron). Inhibitor of superoxide dismutase (DETCA)</td>
<td>(54)</td>
</tr>
<tr>
<td>Apocynin</td>
<td>21.6 ± 1.5</td>
<td>10⁻⁴ M</td>
<td>Inhibitor of NADPH oxidase</td>
<td>(2, 49, 61)</td>
</tr>
<tr>
<td>VAS2370</td>
<td>26.5 ± 8.9</td>
<td>3 × 10⁻⁵ M</td>
<td>Inhibitor of NOS4</td>
<td>(55)</td>
</tr>
<tr>
<td>Tram34 + UCL1684</td>
<td>28.5 ± 3.7</td>
<td>5 × 10⁻⁷ M</td>
<td>Inhibitor of IKCa and SKCa</td>
<td>(18)</td>
</tr>
<tr>
<td>Carbeneoxaline</td>
<td>28.6 ± 3.9</td>
<td>10⁻⁵ M</td>
<td>Blocker of gap junction</td>
<td>(18)</td>
</tr>
<tr>
<td>Iberiotoxin</td>
<td>17.4 ± 1.8</td>
<td>10⁻⁷ M</td>
<td>Inhibitor of BKCa</td>
<td>(18)</td>
</tr>
<tr>
<td>Ouabain</td>
<td>30.5 ± 3.4</td>
<td>10⁻⁵ M</td>
<td>Nonselective Inhibitor of Na⁺/K⁺-ATPase</td>
<td>(18)</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>20.4 ± 3.3</td>
<td>10⁻⁴ M</td>
<td>Inhibitor of KIR</td>
<td>(18)</td>
</tr>
<tr>
<td>α,Methyl-ATP</td>
<td>29.6 ± 5.8</td>
<td>10⁻⁵ M</td>
<td>Blocker of P2X receptor</td>
<td>(29, 44)</td>
</tr>
<tr>
<td>Ca²⁺-free buffer</td>
<td>21.6 ± 3.7</td>
<td>N/A</td>
<td>Inhibitor of cystathionine γ-Lyase</td>
<td>(5)</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>23.2 ± 2.9</td>
<td>10⁻⁶ M</td>
<td>Blocker of L-type calcium channel</td>
<td>(8)</td>
</tr>
<tr>
<td>EGTA</td>
<td>1.9 ± 0.6</td>
<td>5 × 10⁻⁷ M</td>
<td>Calcium chelator</td>
<td>(5)</td>
</tr>
<tr>
<td>β-cyano-l-alanine</td>
<td>22.8 ± 5.6</td>
<td>10⁻⁵ M</td>
<td>Inhibitor of cystathionine γ-Lyase</td>
<td>(66)</td>
</tr>
<tr>
<td>dl-proparglyglycine</td>
<td>29.2 ± 6.0</td>
<td>10⁻² M</td>
<td>Inhibitor of cystathionine γ-Lyase</td>
<td>(66)</td>
</tr>
<tr>
<td>KT5823</td>
<td>41.5 ± 4.8</td>
<td>10⁻⁶ M</td>
<td>Inhibitor of protein kinase G</td>
<td>(9, 30)</td>
</tr>
<tr>
<td>KT5720</td>
<td>35.0 ± 5.5</td>
<td>3 × 10⁻⁷ M</td>
<td>Inhibitor of protein kinase A</td>
<td>(9, 30)</td>
</tr>
<tr>
<td>KT5823 + KT5720</td>
<td>34.7 ± 6.1</td>
<td>As above</td>
<td>As above</td>
<td>(9, 30)</td>
</tr>
<tr>
<td>Rp-PET-8-Br-cGMP</td>
<td>32.3 ± 8.3</td>
<td>3 × 10⁻⁹ M</td>
<td>Inhibitor of protein kinase G</td>
<td>(30, 48)</td>
</tr>
<tr>
<td>Rp-8-Br-cAMP</td>
<td>29.8 ± 4.4</td>
<td>10⁻⁹ M</td>
<td>Inhibitor of protein kinase A</td>
<td>(30, 48)</td>
</tr>
<tr>
<td>Rp-PET-8-Br-cGMP + Rp-8-Br-cAMP</td>
<td>27.5 ± 9.6</td>
<td>As above</td>
<td>As above</td>
<td>(30, 48)</td>
</tr>
<tr>
<td>SQ22536</td>
<td>41.2 ± 3.8</td>
<td>10⁻⁸ M</td>
<td>Inhibitor of adenylyl cyclase</td>
<td>(30, 47)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Effects of various pharmacological inhibitors or desensitizers on the hypoxic augmentation in contracted (U46619, 3 × 10⁻⁸ M to 10⁻⁸ M) porcine coronary arteries with endothelium. l-NAME, Nα-nitro-l-arginine methyl ester; DETCA, diethyldithiocarbamic acid; 8-Br-CGMP, 8-bromo-cGMP. *P < 0.05 vs. control; n = 6–9.
signals then either went through a band pass filter to measure the emission at 510–525 nm by fluo-4 or a long pass filter to measure the emission at >590 nm by fura red. A region of 256 × 256 pixels was randomly chosen on the focused plane, and all of the emission in this region was recorded by a computer package LSM (Carl Zeiss) at a 20-s interval. The same settings were used throughout all experiments: zoom = 4, intensity = 40%, pinhole size = 1.2, and gain = 80%.

All solutions up to the actual experiments were aerated with 95% O2-5% CO2 and kept at 37°C, resembling the conditions for the measurement of isometric changes in tension in the organ chambers. The perfusion to the positive control group was switched to solution containing KCl (60 mM) to obtain a reference signal of maximal calcium influx. In the experimental group, the chamber was perfused for 15 min with U46619 (3 × 10⁻⁶ M) in control solution aerated with 95% O₂-5% CO₂. Then, in the control preparations, the same solution was applied for a further 15 min, whereas in the hypoxic group, a perfusion solution containing U46619, which had been equilibrated with a gas mixture containing 95% N₂-5% CO₂, was applied also for 15 min. The fluo-4-emitted signal was divided by that of fura red to cancel out the effects of natural fading of the signals. The maximal change of ratio of the two signals for KCl, U46619, and U46619 plus DETCA was considered to indicate statistically significant differences.

RESULTS

The contractile response to hypoxia was biphasic with a transient increase in tension that typically lasted ~5 min, followed by a secondary profound relaxation (Fig. 1). The present study focuses on the transient increases in tension. Such hypoxic augmentations were reduced significantly by the removal of the endothelium (Figs. 1 and 2). L-NAME (10⁻⁴ M) nearly abolished the augmentation by hypoxia (Fig. 2). Indomethacin did not significantly affect the hypoxic augmentation (Fig. 2).

In preparations contracted with U46619 the following inhibitors, alone or in combination, did not significantly affect the hypoxic augmentation (Table 1): bosentan (antagonist of endothelin receptor, 10⁻⁵ M), VAS2370 (inhibitor of Nox4, 3 × 10⁻⁴ M), apocynin (antioxidant, inhibitor of NADPH oxidase, 10⁻⁴ M), tiron (intracellular superoxide anion scavenger, 10⁻² M) plus DETCA (inhibitor of superoxide dismutase, 10⁻⁴ M), and gray bars represent the period of aeration with 95% N₂-5% CO₂ (N₂).

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TRAM34 (inhibitor of small conductance calcium-activated potassium channel, $5 \times 10^{-7}$ M) plus UCL1684 (inhibitor of intermediate conductance calcium-activated potassium channel, $5 \times 10^{-7}$ M), carbenoxolone (blocker of gap junctions, $10^{-4}$ M), iberiotoxin (inhibitor of large conductance calcium-activated potassium channel, $10^{-7}$ M), ouabain (inhibitor of sodium potassium ATPase, $10^{-7}$ M), BaCl2 (inhibitor of inward rectifier potassium channel, $10^{-4}$ M), α,β-methyl-ATP (desensitizer of receptors responsible for U46619 contraction, $10^{-5}$ M), nifedipine (inhibitor of voltage operated calcium channel, $10^{-6}$ M), β-cyano-L-alanine and L-propargyglycine (inhibitors of cystathionine γ-lyase, $10^{-5}$ M and $10^{-2}$ M, respectively), and SQ22536 (inhibitor of adenylyl cyclase, $10^{-4}$ M).

The NO donor DETA NONOate, restored the hypoxic augmentation (during contraction to U46619) in a concentration-dependent manner in rings with endothelium incubated with L-NAME (Fig. 3A) or in preparations without endothelium (data not shown). Similar results were obtained with sodium nitroprusside (data not shown). However, such restoration of the hypoxic augmentation was not observed with isoproterenol (data not shown).

The soluble guanylyl cyclase inhibitors ODQ ($10^{-5}$ M, Fig. 3B) and NS2028 ($10^{-5}$ M; Table 1) abolished the hypoxic augmentation during contractions to U46619 in rings with endothelium or in preparations without endothelium incubated with DETA NONOate in the presence of l-NAME (Fig. 3B). The soluble guanylyl cyclase stimulators Bay 412272 ($5 \times 10^{-6}$ M; Fig. 4) and YC-1 ($10^{-5}$ M; Table 1) (15) significantly restored the hypoxic contractions in preparations without endothelium or in preparations with endothelium incubated with l-NAME.

In rings with endothelium, contracted with U46619 and incubated with ODQ ($10^{-5}$ M), the cyclic GMP analog 8-BrcGMP did not restore the hypoxic contraction (Fig. 5A). A similar absence of restoration of the hypoxic contraction was observed with dibutyryl cyclic GMP (data not shown). Incubation with atrial natriuretic peptide to increase cyclic GMP did not restore the hypoxic contraction (Fig. 5A). A similar absence of restoration of the hypoxic contraction was observed with isoproterenol (data not shown). The inhibitors of protein kinase G (KT5823, $10^{-6}$ M; and Rp-PET-8-cGMP, $3 \times 10^{-5}$ M) and protein kinase A (KT5720, $3 \times 10^{-7}$ M; and Rp-8-Br-cAMP, $10^{-5}$ M) did not significantly affect the response to hypoxia (Table 1).

In rings with endothelium, the cyclic GMP level decreased significantly when the contraction to U46619 reached its plateau under control condition. Hypoxia did not cause further significant changes in cyclic GMP content (Fig. 6). Moreover, in rings with endothelium, the relaxations to DETA NONOate and 8-Br-cGMP were not reduced compared with control conditions under hypoxic conditions. On the contrary, the relaxation to 8-Br-cGMP was enhanced significantly under hypoxic conditions (Fig. 7).
The hypoxic contraction persisted in calcium free buffer (Table 1) but was abolished only when the calcium chelator EGTA (5 mM) was added to the calcium-free solution (Table 1). It was not significantly affected by the L-calcium channel inhibitor nifedipine (10^{-5} M, Table 1). The SERCA inhibitor thapsigargin (10^{-6} M) augmented the hypoxic contraction significantly (Fig. 8), and this potentiation was not affected when the compound was administered in combination with nifedipine (data not shown). In the presence of the Rho kinase inhibitors Y27632 and HA1077 (10^{-5} M), the contraction to hypoxia was reduced by almost two-thirds (Fig. 8). Fluorescence confocal microscopy revealed no significant changes in the intracellular level of calcium in the arterial smooth muscle exposed to hypoxia (Fig. 9).

**DISCUSSION**

The present experiments demonstrate that hypoxia causes endothelium-dependent contractions in isolated porcine coronary arteries as it does in those of dogs and humans and confirms the involvement of NO (22, 41, 45). Endothelium-dependent hypoxic augmentation could be due to the aug-

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**Fig. 5.** Hypoxic responses in contracted (U46619, ED_{50}) porcine coronary arteries with endothelium incubated with ODQ (10^{-5} M) in the absence and presence of increasing concentrations of 8-Br-cGMP (10^{-6} to 10^{-3} M; A; *P < 0.05 vs. control; n = 6–7) or L-NAME (10^{-4} M) in the absence and presence of increasing concentrations of atrial natriuretic peptide (10^{-9} to 10^{-7} M; B; *P < 0.05 vs. control; n = 6).

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**Fig. 6.** A: concentration of cyclic GMP in porcine coronary arteries at different time points before and during hypoxia in contracted preparations (U46619, ED_{50}) with (+EC, black bars) or without endothelium (−EC, white bars). Time points represent the time in the tracings (B) as indicated by the arrows: 1) basal concentration before U46619 was added, 2) concentration after contraction by U46619, and 3) peak of the hypoxic augmentation. *P < 0.05, +EC vs. −EC; #P < 0.05 vs. basal; n = 14.
mented release of endothelium-derived contracting factors. The present experiments do not support this interpretation. Indeed, although endothelial cells can generate the potent vasoconstrictor peptide endothelin-1 (62), the nonselective antagonist of endothelin-receptor bosentan (4) did not affect the hypoxic augmentation in preparations contracted with U46619, confirming that the peptide cannot be held responsible for the response (59). In rings with endothelium contracted with U46619, the absence of effect of the nonselective inhibitor of cyclooxygenase indomethacin (11, 45) on the hypoxic augmentation implies that endothelium-derived vasoconstrictor prostanoids do not contribute to the phenomenon. In addition to endothelin-1 and vasoconstrictor prostanoids, endothelial cells can generate uridine adenosine tetraphosphate (UPyA), which has been regarded as an endothelium-derived contracting factor (29). However, a known desensitizer of the receptor responsible for UPyA contraction, α,β-methyl-ATP, did not affect the hypoxic augmentation. Reactive oxygen species (ROS) mediate or amplify endothelium-dependent contractions (26, 46), and the production of ROS increases during hypoxia (50, 58, 61). However, the combination of tiron, an intracellular scavenger of superoxide anion, and DETCA (46), or inhibitors of NADPH oxidase [VAS2370 (55) or apocynin (2, 49, 61)] did not affect the hypoxic augmentation, demonstrating that the response cannot be attributed to endothelium-derived ROS. Although hypoxia may favor the combination of NO and superoxide anions in the vascular smooth muscle cells to form peroxynitrite (38, 60), the absence of effect of the antioxidants (apocynin and the combination of tiron plus DETCA) makes it an unlikely that peroxynitrite plays a role in the hypoxic augmentation in the porcine coronary artery.

An alternative explanation would be that the hypoxic augmentation is due to inhibition of the tonic release of endothelium-derived relaxing factor(s) (21). The lack of effects of indomethacin and of inhibitors of adenylyl cyclase (SQ22536) and protein kinase A (KT5720 and Rp-8-Br-cAMP) (30, 31) on the hypoxic augmentation rules out the contribution of prostacyclin (37) and the cyclic AMP pathway. Likewise, the combination of TRAM34 and UCL1394 (inhibitors of endothelial...

Fig. 7. Relaxation to DETANONOate (A) or 8-bromo (Br)-cyclic GMP (B) under hypoxic (95% N2) or control conditions (95% O2) in porcine coronary arteries with endothelium. *P < 0.05; n = 6.

Fig. 8. Hypoxic augmentation in porcine coronary arteries. Contracted preparations (U46619, 3 × 10⁻⁹ M to 10⁻⁸ M) with endothelium (+EC) incubated with thapsigargin (10⁻⁶ M) or Rho kinase inhibitors (Y27632, 3 × 10⁻⁶ M or HA1077, 3 × 10⁻⁶ M) and controls without endothelium (−EC). *P < 0.05 vs. +EC; n = 6.

Fig. 9. Changes of calcium level in the vascular smooth muscle layer of porcine coronary arteries were measured by florescent signals using confocal microscopy. Rings with endothelium were incubated in a Perspex chamber with constant perfusion with high potassium solution (KCl, 60 mM) or solution containing U46619 (3 × 10⁻⁹ M) and aerated with either 95% N₂-5% CO₂ (hypoxia) or 95% O₂-5% CO₂ (control). Data are expressed changes in the ratio between the fluo-4- and fura-red-emitted signals. *P < 0.05 vs. KCl; n = 6.
K⁺ channels) and carbenoxolone (inhibitor of gap junctions) did not affect the hypoxic augmentation, making a contribution of endothelium-dependent hyperpolarization most unlikely (17). Another endothelium-derived gaseous modulator of vascular tone, hydrogen sulfide is also unimportant in the phenomenon, as β-cyano-L-alanine and β-propargylglycine, which inhibit its synthesis sulfide in the endothelium (66) do not affect the hypoxic augmentation. However, the present experiment demonstrates that in the porcine coronary artery the endothelium-dependent part of the response to hypoxia is inhibited by agents that target the NO pathway, in particular inhibitors of eNOS and soluble guanylyl cyclase. The present findings thus corroborate the interpretation reached in canine arteries that NO is required in order for the endothelium-dependent hypoxic contraction to occur (22) and thus that endothelium-derived NO is likely to be the only diffusible substance (28, 45) solely responsible for the phenomenon. This conclusion is strengthened by the present findings demonstrating that exogenous NO donors (DETA NONOate or sodium nitroprusside) restore the contractile response to hypoxia in preparations with endothelium treated with l-NAME. Since restoration of contraction to hypoxia is not observed with isoproterenol, which acts on the cyclic AMP pathway, this phenomenon is likely to be NO specific.

ODQ (63) and NS2028 (39) inhibited the hypoxic augmentation, whether it was caused by the endothelium or restored by DETA NONOate in the presence of l-NAME. These compounds act by oxidizing heme moieties (39, 65) of heme-containing proteins, such as soluble guanylyl cyclase, a major target for endothelium-derived NO. Thus the present findings support the earlier postulation (22) that soluble guanylyl cyclase plays a key role in the hypoxic augmentation. This role is demonstrated further by the restoration of the hypoxic response in the presence of l-NAME when incubated with the soluble guanylyl cyclase stimulators Bay 412272 (15) and YC-1, which enhances the activation of the enzyme (15). These observations thus suggest that the activation of soluble guanylyl cyclase is essential for the hypoxic augmentation to occur.

However, a surprising conclusion of the present study is that the signaling cascade downstream of soluble guanylyl cyclase is unlikely to involve cyclic GMP. Indeed, the addition of cell permeable analogs of cyclic GMP [8-Br-cGMP (6) and dibutyryl cyclic GMP (22)] or stimulation of the production of cyclic GMP by particulate guanylyl cyclase with atrial natriuretic peptide (33, 35) did not restore the hypoxic facilitation in the porcine coronary artery. When the cyclic GMP levels were measured at different time points of the contraction, no changes due to hypoxia were observed. Since soluble guanylyl cyclase is the major target of NO in vascular smooth muscle (34), the fact that the levels of cyclic GMP do not change implies that the amount of NO released by the endothelium is not altered during the hypoxic exposure under the present experimental conditions. Hence, it seems logical to conclude that hypoxia reduces either the inhibitory effect of unchanged levels of cyclic GMP on the contractile process or a cyclic GMP-independent vasodilator effect of NO. The former is not likely because the present results show that, under hypoxic conditions, the relaxations to both DETA NONOate and 8-Br-cGMP are not reduced and that in fact the relaxation to the cyclic GMP analog is enhanced.

Cyclic GMP-independent effects of soluble guanylyl cyclase have been reported (14, 19, 64). The present results indicate another cyclic GMP-independent effect of soluble guanylyl cyclase activation during hypoxic augmentation. This interpretation is strengthened by the results obtained with inhibitors of protein kinase G and protein kinase A (24) at concentrations believed to be effective in vascular smooth muscle (30, 31). When soluble guanylyl cyclase is activated and produces cyclic GMP, the cyclic nucleotide predominately activates protein kinase G (34, 42, 43). Hence the lack of significant effect of these inhibitors on hypoxic augmentation implies that the response does not depend on cyclic GMP. A potential cyclic GMP-independent effect of NO is hyperpolarization (13), the inhibition of which could explain the hypoxic augmentation. This possibility is made unlikely by the lack of effect of TRAM34, UCL1684, iberiotoxin (17), ouabain (inhibitor of Na⁺/K⁺-ATPase; Ref. 27) and BaCl₂ (17). Alternatively, the activation of SERCA by NO is also cyclic GMP independent (1). However, in the present study the SERCA antagonist thapsigargin enhanced rather than reduced the hypoxic augmentation. Thus the latter is not likely explained by inactivation of SERCA.

U46619 causes an increased calcium entry in vascular smooth muscle (12). As both nifedipine (8) and extracellular calcium depletion do not prevent the hypoxic augmentation, an increased calcium influx cannot not explain the current phenomenon. The lack of significant changes in intracellular calcium levels shown by confocal fluorescence microscopy with hypoxia confirms that it does not augment the influx of the activator ion. However, since the hypoxic augmentation is abolished by the calcium chelator (EGTA), calcium must be present for it to occur. This logically leads to the postulation of the involvement of calcium sensitization causing further vasoconstriction during hypoxia. This conclusion is prompted by the reduction in hypoxic augmentation caused by the two chemically distinct tested inhibitors of Rho kinase [Y27632 (23) and HA1077 (16)], which is important in mediating calcium sensitization (25) as well as endothelium-dependent contractions at the level of vascular smooth muscle (7). The enhancement in hypoxic augmentation caused by thapsigargin must be due to the reduced removal of calcium from the cytosol, thus causing an elevated level of calcium allowing more binding to the sensitized contractile apparatus (51).

In summary, the present study confirms that NO is required for the occurrence of endothelium-dependent hypoxic augmentation. The results demonstrate, that despite the obligatory involvement of soluble guanylyl cyclase, cyclic GMP and its known downstream targets do not contribute. The endothelium-dependent contraction is likely to be caused by calcium sensitization involving Rho kinase.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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