Hypoxia sensing in the fetal chicken femoral artery is mediated by the mitochondrial electron transport chain

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1Department of Pediatrics, Maastricht University Medical Centre, GROW School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands; 2Department of Pharmacology, School of Medicine, Universidad Complutense Madrid and Centro de Investigaciones Biomedicas en Red de Enfermedades Respiratorias, Madrid, Spain; and 3Department of Pharmacology and Toxicology, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands

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Zoer B, Cogolludo AL, Perez-Vizcaino F, De Mey JG, Blanco CE, Villamor E. Hypoxia sensing in the fetal chicken femoral artery is mediated by the mitochondrial electron transport chain. Am J Physiol Regul Integr Comp Physiol 298: R1026–R1034, 2010. First published January 20, 2010; doi:10.1152/ajpregu.00500.2009.—Vascular hypoxia sensing is transduced into vasoconstriction in the pulmonary circulation, whereas systemic arteries dilate. Mitochondrial electron transport chain (mETC), reactive O2 species (ROS), and K+ channels have been implicated in the sensing/signaling mechanisms of hypoxic relaxation in mammalian systemic arteries. We aimed to investigate their putative roles in hypoxia-induced relaxation in fetal chicken (19 days of incubation) femoral arteries mounted in a wire myograph. Acute hypoxia (Po2 ~ 2.5 kPa) relaxed the contraction induced by norepinephrine (1 μM). Hypoxia-induced relaxation was abolished or significantly reduced by the mETC inhibitors rotenone (complex I), myxothiazol and antimycin A (complex III), and NaN3 (complex IV). The complex II inhibitor 3-nitropropionic acid enhanced the hypoxic relaxation. In contrast, the relaxations mediated by acetylcholine, sodium nitroprusside, or forskolin were not affected by the mETC blockers. Hypoxia induced a slight increase in ROS production (as measured by 2,7-dichlorofluorescein-fluorescence), but the mETC blockers. Hypoxia induced a slight increase in ROS production (as measured by 2,7-dichlorofluorescein-fluorescence), but the mETC blockers. Hypoxia-induced relaxation was endothelium independent, but the sensing/signaling mechanisms involved were not further characterized. Also, in previous studies, our laboratory has shown that incubation of chicken embryos under chronic, moderate hypoxia induced sustained structural and functional changes in systemic and pulmonary vasculature (40, 41, 49, 50). However, whether vascular acute hypoxia sensing is affected by a previous exposure to chronic hypoxia is controversial (34, 49, 61, 63), and the chicken model might provide new perspectives to the question. Therefore, in the present study, we tested two hypotheses: 1) the mETC/ROS/K+ channels axis is involved in the hypoxic relaxation of fetal chicken femoral arteries; and 2) in ovov exposure to chronic hypoxia induces changes in the responsiveness of fetal chicken femoral arteries to acute hypoxia.

METHODS

Egg incubation. All experimental procedures were carried out according to the regulation of the Dutch Law on Animal Experimentation and the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/ 609/EU) and approved by the Committee on Animal Experimentation of the University of Maastricht. Fertilized eggs from White Leghorn chickens (‘t Anker, Ochten, The Netherlands) were incubated at 37.8°C, 45% humidity, and rotated once per hour over an angle of 90° (incubator model 25HS, Masalles Commercial, Spain). The majority

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VASCULAR SMOOTH MUSCLE CELLS belong to a specialized homeostatic O2-sensing system, which responds rapidly to moderate changes in O2 tension (3, 58). Vascular hypoxia sensing is transduced into vasoconstriction in the pulmonary circulation [hypoxic pulmonary vasoconstriction (HPV)], whereas systemic arteries dilate. It is considered that O2-sensing systems consist of a sensor that alters the production of a mediator in response to changes in Po2. The mediator, in turn, alters the function of one or more effectors, which ultimately mediate the physiological response of the system (3, 58). Numerous experimental evidences point to the mitochondrial electron transport chain (mETC) as the sensor, reactive O2 species (ROS) as mediators, and K+ channels as effectors of the vascular response to acute hypoxia (3, 10, 26, 35, 44, 52, 53, 55, 56, 58, 59), and it has been suggested that differences between pulmonary and systemic arteries in this sensing/signaling system may account for the opposite responses of these vessels to the hypoxic stimulus (52). The present information about the response to hypoxia in blood vessels from nonmammalian species suggests largely similar mechanisms as in mammals (32, 42, 43). In the last few years, the chicken embryo/fetus has emerged as a suitable model for the study of numerous aspects of developmental vascular biology, including the response to acute and chronic changes in oxygenation (1, 9, 38, 39, 47, 50, 51, 63). In a previous work from our group, it was shown that acute in vitro hypoxia reduced the tone induced by norepinephrine (NE) (38). This hypoxia-induced relaxation was endothelium independent, but the sensing/signaling mechanisms involved were not further characterized. Also, in previous studies, our laboratory has shown that incubation of chicken embryos under chronic, moderate hypoxia induced sustained structural and functional changes in systemic and pulmonary vasculature (40, 41, 49, 50). However, whether vascular acute hypoxia sensing is affected by a previous exposure to chronic hypoxia is controversial (34, 49, 61, 63), and the chicken model might offer new perspectives to the question. Therefore, in the present study, we tested two hypotheses: 1) the mETC/ROS/K+ channels axis is involved in the hypoxic relaxation of fetal chicken femoral arteries; and 2) in ovov exposure to chronic hypoxia induces changes in the responsiveness of fetal chicken femoral arteries to acute hypoxia.
of the eggs were incubated under normoxic conditions (21% O₂, 0.03% CO₂). However, to assess the effects of chronic prenatal hypoxia on the responsiveness to acute hypoxia, a group of eggs was incubated under chronic hypoxic conditions (15.0 ± 0.3% atmospheric O₂, 0.03% CO₂) from day 6. Chronic hypoxia was maintained by providing a constant flow of N₂ and compressed air (3,000 and 4,500 ml/min, respectively) with a flow meter (AGA Gas BV), as previously described (63). The O₂ and CO₂ concentrations in the incubator were monitored with a DrDAQ O₂ sensor (Pico Technology) and an infrared CO₂ analyzer (Beckman Instruments, Fullerton, CA).

Vascular reactivity studies. At day 19 of the 21-day incubation period, noninternally pipped chicken fetuses were decapitated, and femoral arteries were isolated and stored in cold Krebs-Ringer bicarbonate (KRB) buffer. Rings of 1.7- to 2-mm length were mounted between an isotropic force transducer (Kistler Morphoscope 656, Seattle, WA) and a displacement device in a myograph (model 610M, Danish Myotechnology, Aarhus, Denmark) using two stainless steel wires (diameter 40 μm). The KRB buffer in the organ chamber was maintained at 39°C and aerated with 21% O₂/74% N₂/5% CO₂ (Po2 19.16 kPa, SD 1.15, n = 12, measured with an ARL 510 blood-gas analyzer, Radiometer, Copenhagen, Denmark). After an equilibration period of 30 min, the vessels were distended to a resting tension corresponding to a transmural pressure of 20 mmHg. This pressure corresponds to the mean arterial blood pressure reported in 19-day chicken fetuses (2) and elicited the highest contractile response to KCl and NE, as determined in pilot experiments. After 30 min of incubation under resting tone, a control contraction was elicited by raising the K⁺ concentration of the buffer (62.5 mM) in exchange for Na⁺.

To study the effects of acute hypoxia, the organ chambers were wrapped in cling film, and femoral artery rings were contracted with NE (1 μM), as previously described (38). Acute hypoxia was induced when NE-induced contraction was stable (after ~10 min) by switching the gas mixture aerating the organ bath from 21% O₂/74% N₂/5% CO₂ to 95% N₂/5% CO₂ (Po2 2.48 kPa, SD 0.34, n = 12). After 15–20 min of hypoxic exposure, the gas mixture was switched back to 21% O₂/74% N₂/5% CO₂.

The effects of acute hypoxia were studied under control conditions and the presence of one of the following pharmacological tools: the mETC inhibitors rotenone (complex I, 30 μM), 3-nitropropionic acid (3-NPA, complex II, 5 mM), myxothiazol (complex III, 10 μM), antimycin A (complex III, 5.7 μM), and sodium azide (complex IV, 1 mM); the NADPH-oxidase inhibitor apocynin (0.3 mM); the inhibitor of glycolysis 2-deoxy glucose (10 mM); the ROS scavengers polyethylene glycol-superoxide dismutase (PEG-SOD, superoxide scavenger, 100 U/ml), PEG-catalase (H₂O₂ scavenger, 100 U/ml); the nonselective K⁺ channel inhibitor tetraethylammonium (TEA, 1 mM); and the voltage-gated K⁺ channel inhibitor 4-aminoptyridine (4-AP, 5 mM). The K⁺ channel blocker diphenyl phosphate oxide-1 (DPO-1, 0.3 μM); the ATP-sensitive K⁺ channel (KATP) inhibitor glibenclamide (10 μM); the long-conductance Ca⁺²-activated K⁺ channel inhibitoriberiotoxin (0.1 μM); the inward-rectifying K⁺ channel (Kir) inhibitor BaCl₂ (30 μM); and the Na⁺-/K⁺-ATPase inhibitor ouabain (0.1 mM). These drugs were added 15 min before NE and maintained during the whole experiment. Due to the scarce information on the effects of the above-mentioned pharmacological tools in chicken tissues, doses of probes were selected based on their effects in mammalian tissues (10, 22–24, 33, 35, 44, 54, 56, 57, 60).

In another set of experiments, and to analyze which critical level of oxygenation was necessary to elicit relaxation, NE-contracted femoral artery rings were consecutively exposed to gas mixtures containing 15% (Po2 15.93 kPa, SD 1.11, n = 12), 10% (Po2 10.42 kPa, SD 0.85, n = 12), 5% (Po2 6.96 kPa, SD 0.52, n = 12), and 0% O₂ (5% CO₂-balance N₂ in all of the mixtures). Finally, in another group of experiments, the effects of cumulative concentrations of authentic H₂O₂ (1 μM to 0.1 mM) were tested in vessels contracted with NE and bubbled with 21% O₂.

**Determination of ROS.** ROS generation in isolated femoral artery rings was assessed using 2,7-dichlorofluorescein (DCF), which mainly detects H₂O₂ (9). Rings were incubated with the membrane-permeable diacetate form of DCF (DCF-DA, 10 μM) for 60 min at room temperature, then placed in the stage of a fluorescent inverted microscope (Leica DM IRB, Wetzlar, Germany) and superfused at 2 ml/min with a physiological salt solution (PSS) of composition (in mmol/l): 130 NaCl, 5 KCl, 1.2 MgCl₂, 1.8 CaCl₂, 10 glucose, 10 HEPES (pH 7.3 with NaOH, temperature 39°C). Pilot myograph experiments showed that, using this PSS, NE-contracted femoral arteries responded to hypoxia with relaxation. During the first 30 min, the tissues were maintained under normoxia induced by bubbling the PSS solution with air. Once fluorescence values were stable, NE (1 μM) was added, and, after 10 min, the preparations were challenged with hypoxic solution (PSS bubbled with 100% N₂). After 10 min of hypoxia, vessels were exposed to the membrane-permeable H₂O₂ analog t-butyl-hydroperoxide (0.1 mM). Vessels were illuminated continuously through the luminal surface using a 450- to 490-nm band-pass filter. The emitted fluorescence was filtered using a 515-nm long-pass emission filters. Images were taken at 1-min intervals with a Leica DC300F color digital camera. Fluorescence, after subtracting background, was quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih.gov/ij). Intensity values are reported as percentage of the values before the hypoxic challenge.

**Drugs and solutions.** KRB contained the following (in mmol/l): 118.5 NaCl, 4.75 KCl, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, 25.0 NaHCO₃, 2.5 CaCl₂, 5.5 glucose. Solutions containing different concentrations of K⁺ were prepared by replacing part of the NaCl by an equimolar amount of KCl. In experiments in which 2-deoxy glucose was used, the KRB solution was identical to that described above, except that glucose was omitted and replaced with 2-deoxy glucose (10 mM). Apocynin, 3-NPA, and myxothiazol were obtained from Fluka (Buchs, Switzerland). NaN₃ was obtained from Merck (Darmstadt, Germany). TEA and glibenclamide were obtained from Alexis Biochemicals (San Diego, CA). All other drugs were obtained from Sigma (St. Louis, MO). All drugs were dissolved initially in distilled, deionized water (except rotenone, antimycin A, DPO-1, and glibenclamide, which were dissolved in ethanol) to prepare adequate stock solutions, and further dilutions were made in deionized water. Total DMSO or ethanol added to the organ bath was, at the most, 0.1% vol/vol and did not affect arterial tone or the reactivity to NE and hypoxia.

**Statistical analysis.** Results are described as means (SD) of measurements in n animals. For clarity, results in Figs. 1 and 3–6 are shown as means ± SE. Contractions are expressed in terms of active wall tension (N/m), calculated as the force divided by twice the length of the segment, while the relaxant responses are expressed as the percent reduction of the contraction induced by NE. Differences between mean values were assessed by one-way ANOVA, followed by Dunnett post hoc t-test comparing control with the other groups. Nonpaired t-tests were used when only two groups were compared. Differences were considered statistically significant at a P < 0.05. All analyses were performed using a commercially available statistics package (GraphPad InStat version 3.00, GraphPad Software, San Diego, CA).

**RESULTS**

**Hypoxia-induced relaxation.** In fetal chicken femoral artery rings contracted with NE (1 μM), hypoxia (Po2 2.5 kPa) induced a sustained relaxation (Fig. 1A), as previously described. This relaxation was preceded by a transient contraction in ~50% of the vessels. Reoxygenation with 21% O₂ caused a transient additional relaxation before the recovery of normoxic levels of tension (Fig. 1A). We observed that vaso-
incubated under chronic hypoxia (15% O₂ from femoral arteries from control chicken fetuses (19 day, SE) responses to acute hypoxia as percentage of NE-induced constriction in (H11006)

7.0 –2.5 kPa) in a NE-contracted 19-day fetal chicken femoral artery. Changing back the PO₂ from 2.5 to 7.0 different from the one observed by directly switching the PO₂

1.2, step (10.4 to 7.0 kPa) elicited a minimal relaxation (1.21%, SD/H11011

19.2–15.9 –10.4 –7.0 –2.5 kPa), it was observed that the third motion (cyclical variations in vasomotor tone) was induced in ~20% of the NE-contracted vessels, independent of the presence or absence of hypoxia or the different drugs (see Figs. 2B and 6A and for examples). When NE-contracted vessels were exposed to a stepwise decrease of O₂ (21–15–10–5-0% O₂; PO₂ ~19.2–15.9–10.4–7.0–2.5 kPa), it was observed that the third step (10.4 to 7.0 kPa) elicited a minimal relaxation (1.21%, SD 1.2, n = 8), whereas the fourth step (7.0 to 2.5 kPa) induced a 33.14% (SD 22.9) relaxation, which was not significantly different from the one observed by directly switching the PO₂ from 19.2 to 2.5 kPa. Switching back the PO₂ from 2.5 to 7.0 kPa completely reversed hypoxia-induced relaxation (see Fig. 1B for a representative example).

**Table 1. Mean (SD) wall tension induced by NE under the different experimental conditions**

<table>
<thead>
<tr>
<th>Drug Effect</th>
<th>NE Contraction</th>
<th>NE + Hypoxia</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.79 (0.41)</td>
<td>1.01 (0.33)</td>
</tr>
<tr>
<td>Chronic hypoxia</td>
<td>1.98 (0.35)</td>
<td>1.34 (0.16)</td>
</tr>
<tr>
<td>Rotene</td>
<td>1.73 (0.46)</td>
<td>1.90 (0.33)</td>
</tr>
<tr>
<td>Rotene + succinate</td>
<td>1.42 (0.32)</td>
<td>1.47 (0.24)</td>
</tr>
<tr>
<td>3-NPA</td>
<td>1.44 (0.30)</td>
<td>0.36 (0.38)</td>
</tr>
<tr>
<td>Myxothiazol</td>
<td>1.12 (0.28)*</td>
<td>0.93 (0.13)</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>0.97 (0.44)*</td>
<td>1.03 (0.18)</td>
</tr>
<tr>
<td>NaN₃</td>
<td>1.07 (0.32)*</td>
<td>1.23 (0.57)</td>
</tr>
<tr>
<td>2-Deoxy glucose</td>
<td>1.10 (0.27)*</td>
<td>0.70 (0.17)</td>
</tr>
<tr>
<td>Apocynin</td>
<td>1.56 (0.34)</td>
<td>0.91 (0.25)</td>
</tr>
<tr>
<td>PEG-catalase</td>
<td>2.08 (0.23)</td>
<td>1.16 (0.29)</td>
</tr>
<tr>
<td>PEG-SOD</td>
<td>1.52 (0.32)</td>
<td>0.68 (0.27)</td>
</tr>
<tr>
<td>KCl</td>
<td>1.81 (0.49)</td>
<td>1.17 (0.15)</td>
</tr>
<tr>
<td>4-AP</td>
<td>2.29 (0.40)</td>
<td>1.32 (0.58)</td>
</tr>
<tr>
<td>DPO-1</td>
<td>1.71 (0.40)</td>
<td>1.83 (0.25)</td>
</tr>
<tr>
<td>Gibenclamide</td>
<td>1.78 (0.36)</td>
<td>1.20 (0.45)</td>
</tr>
<tr>
<td>Ibotoxin</td>
<td>1.62 (0.35)</td>
<td>0.96 (0.26)</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>1.69 (0.38)</td>
<td>1.19 (0.31)</td>
</tr>
<tr>
<td>Ouabain</td>
<td>1.76 (0.36)</td>
<td>0.98 (0.18)</td>
</tr>
</tbody>
</table>

Values are means (SD) in N/m. NE, norepinephrine; 3-NPA, 3-nitropropionic acid; PEG, polyethylene glycol; SOD, superoxide dismutase; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; DPO-1, diphenyl phosphate oxide-1. *P < 0.05 vs. control.
we studied hypoxia-induced relaxation in the presence of 2-deoxy glucose, an inhibitor of glycolysis. As shown in Table 1, 2-deoxy glucose (together with omission of glucose in the KRB solution) impaired NE-induced contraction, but did not significantly affect hypoxia-induced relaxation (36.42%, SD 12.9, n/H11005 6).

**Hypoxia-induced relaxation and ROS.** To analyze the production of ROS, we tested the effects of hypoxia in femoral artery rings incubated with DCF-DA. NE was present during the fluorescence experiments to reproduce the conditions of the myograph experiments. As shown in Fig. 4A, hypoxia induced only a weak elevation in DCF fluorescence. In contrast, when the H2O2 analog t-butyl-hydroperoxide (0.1 mM) was added, a marked increase in DCF fluorescence was observed.

In another set of experiments, we analyzed whether exogenous H2O2 reproduced the vascular effects of hypoxia. Authentic H2O2 was used in these experiments. Figure 4B shows that only the highest concentration of H2O2 tested (0.1 mM) relaxed NE-contracted femoral arteries. The relaxant effects of H2O2 were abolished by the presence of the H2O2 scavenger PEG-catalase (not shown). In contrast, PEG-catalase, the superoxide scavenger PEG-SOD, or the NADPH-oxidase inhibitor apocynin did not significantly change hypoxia-induced relaxation in NE-contracted fetal chicken femoral arteries (Fig. 5).

**Hypoxia-induced relaxation and K+ channels.** As shown in Fig. 6, hypoxia-induced relaxation was not significantly affected by the presence of 40 mM K+-depolarizing solution, TEA (nonselective K+ channel blocker), DPO-1 (Kv1.5 blocker), glibenclamide (KATP blocker), iberiotoxin (large-conductance Ca2+-activated K+ channel blocker), BaCl2 (KiR blocker), or ouabain (Na+-K+-ATPase blocker). In contrast, in the presence of the Kv channel blocker 4-AP, hypoxia-induced relaxation was enhanced ~1.8-fold (P < 0.01 vs. control, Fig. 5).

**DISCUSSION**

In contrast to pulmonary arteries, hypoxia induces relaxation of systemic arteries. In the present study, we aimed to characterize the putative role of the mETC, ROS, and K+ channels in the relaxation induced by hypoxia in NE-contracted fetal chicken femoral arteries (38). Our results show the following.

1) Inhibition of the mETC complexes I, III, and IV impaired hypoxia-induced relaxation. 2) Hypoxia induced a slight increase in ROS production (as measured by DCF-fluorescence), but hypoxia-induced relaxation was not affected by scavenging of superoxide or H2O2. 3) Hypoxia-induced relaxation was not affected by the blockade of KATP, KCa, or Kir channels and was enhanced by Kv blockade.

Although our experimental setup was based on switching the O2 tension from normoxia (PO2 ~19 kPa) to ~2.5 kPa, we observed that a decrease in PO2 from 7 to 2.5 kPa elicited a similar relaxant effect, and that changes between normoxia and a PO2 of 7 kPa did not affect vascular tension. In contrast, Fredricks et al. (12) reported concentration-dependent relaxant effects of reducing the O2 percentage (15, 10, 5, 0% O2) in isolated resistance arteries from the gracilis muscle of adult rats. The arterial PO2 of 19-day chicken fetuses is ~7 kPa (48), and our study demonstrates that vascular response to O2 changes occurs only below that level.

mETC. The mitochondrion’s role as the primary site for O2 consumption, energy generation, and determinant of cellular redox state makes it an ideal candidate for O2 sensing. (52).
This idea is supported by a significant number of publications demonstrating that pharmacological inhibition of the mETC strongly affects vascular O₂ sensing (9, 15, 22, 54, 60). However, significant controversy exists concerning the mechanisms that link mitochondrial function to the effectors of O₂ sensing in mammalian blood vessels. The main hypotheses of mitochondrial vascular O₂ sensing involve cytosolic redox state, ROS, and energy state (3, 52, 53, 55, 56, 58, 59). As mentioned above, we observed that hypoxic relaxation of fetal chicken femoral arteries was almost abolished by the presence of inhibitors of mETC complexes I, III, and IV and enhanced by complex II inhibition. The effects of the mETC blocker appear to be specific for hypoxia-induced relaxation, since the relaxant effects of ACh (which induces in chicken femoral arteries endothelium-dependent, NO-mediated relaxation) (38), SNP (cGMP-mediated relaxation), or forskolin (cAMP-mediated relaxation) were unaffected by the different mETC inhibitors.

During mitochondrial respiration, most of the O₂ consumed is reduced to H₂O at complex IV (cytochrome oxidase). However, superoxide can be generated at sites upstream from complex IV when single electrons escape from the various transport proteins (45, 46, 56). Complexes I and III are the most widely studied sites for the generation of ROS within the mETC. ROS formation within complex III can theoretically be dissected by using myxothiazol vs. antimycin A, which targets the more distal oxidation of the ubisemiquinone radical to ubiquinone (22, 54). Inhibition of a complex proximal to the ROS production site would decrease ROS production, whereas inhibition distal to this site would disrupt the flow of electrons down the chain, diverting them to react with O₂ and generate ROS (26, 45, 46). In contrast to our results, inhibition of the mETC downstream from ubisemiquinone did not alter, or even enhanced, the hypoxic response of mammalian vascular smooth muscle cells (22, 54). These effects are thus, in principle, in accordance with the hypothesis of increased superoxide release from complex III as the underlying mechanism of vascular hypoxic signaling. However, other groups demonstrated that also distal inhibition of the mETC abolished the vascular response to hypoxia (60).

Inhibition of the mETC not only alters ROS production, but ultimately prevents the ATP production due to a lack of protons to flow through and activate the ATP synthase. During profound hypoxia, mitochondria can change from being ATP producers to potentially powerful ATP consumers (7). From our data, it could be speculated that hypoxic relaxation of fetal chicken femoral arteries depends more on reduced mitochondrial ATP production than on ROS generation during electron transport. However, the available evidence in mammals does not support an energy limitation mechanism for the O₂ sensitivity of vascular smooth muscle (35, 44). Moreover, hypoxia-induced relaxation of fetal chicken femoral arteries occurred abruptly, whereas the decay in intracellular ATP levels is described, even under anoxia, as slow and progressive (7). To gain further insights into the role of oxidative metabolism in hypoxia-induced relaxation, we performed some experiments in which extracellular glucose was omitted and glycolysis was inhibited by the presence of 2-deoxy glucose (57). Under these conditions, the contraction elicited by NE was impaired, but hypoxia-induced relaxation did not change. Although we have not determined ATP levels, and, therefore, we can only provide

\[ pD_2 (-\log EC_{50}) \]

for the relaxations induced by acetylcholine (ACh; 1 nM–0.1 mM), the NO donor sodium nitroprusside (SNP; 1 nM–0.1 mM), and the adenylate cyclase stimulator forskolin (10 nM–10 μM) in 1 μM NE-contracted fetal chicken femoral arteries in the absence or the presence of rotenone (30 μM, n = 6), 3-NPA (5 mM, n = 6), myxothiazol (10 μM, n = 6), antimycin A (5.7 μM, n = 6), or NaN₃ (1 mM, n = 6). Values are means ± SE.

A: mean data showing the relaxation induced by acute hypoxia in the absence (control, n = 38) or presence of rotenone (n = 8), rotenone + the complex II substrate succinate (n = 4), 3-NPA (n = 7), myxothiazol (n = 11), antimycin A (n = 7), and the complex IV inhibitor NaN₃ (n = 8), *P < 0.05 vs. control. B: pD₂ (-log EC₅₀) of the relaxations induced by acetylcholine (ACh; 1 nM–0.1 mM), the NO donor sodium nitroprusside (SNP; 1 nM–0.1 mM), and the adenylate cyclase stimulator forskolin (10 nM–10 μM) in 1 μM NE-contracted fetal chicken femoral arteries in the absence (control, n = 6) or the presence of rotenone (30 μM, n = 6), 3-NPA (5 mM, n = 6), myxothiazol (10 μM, n = 6), antimycin A (5.7 μM, n = 6), or NaN₃ (1 mM, n = 6). Values are means ± SE.

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Fig. 3. A: mean data showing the relaxation induced by acute hypoxia in the absence (control, n = 38) or presence of rotenone (n = 8), rotenone + the complex II substrate succinate (n = 4), 3-NPA (n = 7), myxothiazol (n = 11), antimycin A (n = 7), and the complex IV inhibitor NaN₃ (n = 8), *P < 0.05 vs. control. B: pD₂ (-log EC₅₀) of the relaxations induced by acetylcholine (ACh; 1 nM–0.1 mM), the NO donor sodium nitroprusside (SNP; 1 nM–0.1 mM), and the adenylate cyclase stimulator forskolin (10 nM–10 μM) in 1 μM NE-contracted fetal chicken femoral arteries in the absence (control, n = 6) or the presence of rotenone (30 μM, n = 6), 3-NPA (5 mM, n = 6), myxothiazol (10 μM, n = 6), antimycin A (5.7 μM, n = 6), or NaN₃ (1 mM, n = 6). Values are means ± SE.
functional evidences, our data suggest that O₂ sensing in the chicken fetus femoral artery requires mitochondrial electron flow, but not oxidative phosphorylation.

We observed that the mETC complex IV inhibitor NaN₃ not only abolished hypoxia-induced relaxation, but also relaxed femoral arteries and impaired the contraction evoked by NE. These effects could be related to an increase in NO production, since NaN₃ has been shown to be metabolized to NO by catalases (30). We also observed that the inhibition of mETC complex II with 3-NPA enhanced hypoxia-induced relaxation. In contrast, complex II inhibition selectively suppressed hypoxia-induced ROS production in murine pulmonary artery smooth muscle cells (33) and impaired HPV in isolated, perfused rabbit lungs (60). The presence of 3-NPA blocks electron entry and electron backflow into complex II but increases ROS, which are produced using electrons derived from complex I (5). However, complex II and complex I display inverse regulatory properties, whereby the heightened activity of one complex represses the other, a phenomenon likely due to competition between the two complexes for available ubiquinone (29). The relative importance of complexes I and II in providing electrons to the ubiquinone pool and producing ROS appears to vary among tissues and species (5, 6). On the other hand, previous observations provide evidence that, under hypoxic conditions, complex II may switch function from succinate dehydrogenase to fumarate reductase (5, 33). This might be the reason why succinate was unable to reverse the inhibitory effect of rotenone in our experiments. Elucidation of this and other unclear points would require further research, analyzing mitochondrial respiration and mitochondrial ROS production during hypoxia.

**ROS.** Mechanisms involving changes in ROS have been key components of current hypotheses for explaining O₂-induced changes in vascular tone (16–18, 28, 62). A growing body of data implicates superoxide and its degradation product H₂O₂ as participants in signal transduction of HPV, hypoxic relaxation of systemic arteries, and normoxic constriction of the ductus arteriosus (9, 21, 27, 36, 37, 62). The role of H₂O₂ as a vascular mediator of hypoxia is supported by the fact that low concentrations of exogenous H₂O₂ induce pulmonary vasoconstriction and systemic relaxation, i.e., the effect of hypoxia is mimicked. In addition, the presence of catalase (which breaks down H₂O₂) suppresses the vascular effects of hypoxia (23, 24, 55, 56). In the present work, we observed that exogenous H₂O₂ relaxed NE-contracted femoral arteries, as did hypoxia. However, this effect was only observed at high concentrations (100 μM) of...
H2O2. The presence of PEG-SOD (which would increase the breakdown of superoxide to H2O2) or PEG-catalase did not significantly affect hypoxia-induced relaxation. We also tested the effects of the NADPH oxidase inhibitor apocynin, because a number of theories regarding vascular O2-sensing mechanisms include NADPH oxidases that increase or decrease ROS production in a O2-dependent manner (20). We did not observe a significant effect of NADPH oxidase inhibition on hypoxia-induced relaxation of fetal chicken femoral arteries. Finally, we tested the effects of hypoxia in femoral artery rings incubated with DCF, a dye widely used for detecting H2O2, and we found that only a slight elevation of DCF fluorescence occurred during the hypoxic challenge. Therefore, although detection of ROS is fraught with problems and all methods have come under close scrutiny (52, 53, 59), our results do not suggest a relevant role for ROS in the hypoxic signal transduction in fetal chicken femoral arteries. Accordingly, the group of Wolin has shown that ROS are not primary mediators of the hypoxic relaxation of bovine coronary arteries and sustains the presence of an O2 sensing/signaling model based on mitochondrial control of piruvate metabolism associated with cytosolic NADPH redox regulation (13, 14, 17, 18, 62).

**Potassium channels.** K+ channels are known targets of vascular hypoxia. Numerous research groups have demonstrated that hypoxia inhibits Kv currents in pulmonary artery vascular smooth muscle cells (3, 4, 8, 58). Also, normoxic contraction of the DA has been related to Kv current inhibition (9, 25). It has been suggested that hypoxic relaxation of systemic arteries could be a consequence of the decrease of intracellular ATP and the subsequent hyperpolarization caused by the opening of KATP channels (10). However, Quayle et al. (35) demonstrated that the activation of KATP current required, not hypoxia, but anoxia. Other authors reported that hypoxia produced relaxation of systemic arteries by opening Kv channels (44). Our laboratory’s previous finding that hypoxia-induced relaxation of fetal chicken femoral arteries was decreased when the vessels were contracted with high KCl (which would prevent K+ outflow) suggested a role for K+ channel opening in this response (38). However, we observed that hypoxia-induced relaxation was unaffected by the blockade of KATP, KCa, or Kir channels. Interestingly, in the presence of the Kv channel blocker 4-AP, hypoxia-induced relaxation was enhanced. As shown in Table 1, we found that fetal chicken femoral arteries constrict in response to 4-AP, whereas the inhibition of the other K+ channels caused no contractile effect. This suggests that, in this vessel, Kv channels are the predominant channel subtype controlling K+ outflow. It has been reported that the effects of hypoxia are more pronounced in partially depolarized systemic vascular smooth muscle cells (11). Therefore, the effects of 4-AP on membrane potential could be responsible for the enhanced hypoxic relaxation observed in its presence. In contrast to this, we did not observe changes in the hypoxic relaxation when the femoral arteries were simultaneously exposed to 40 mM K+-depolarizing solution and NE.

**Chronic fetal hypoxia did not affect the response to acute hypoxia.** Pre- and postnatal exposure to chronic hypoxia induces sustained structural and functional changes in both pulmonary and systemic vascular beds (19, 40, 41, 49, 50). However, whether vascular acute hypoxia sensing is affected by chronic...
Hypoxia is controversial. Previous studies in mammals have reported conflicting results with respect to the vasoconstrictor response of pulmonary arteries to an acute hypoxic challenge after chronic hypoxia, and reduced, enhanced, or unchanged HPV has been reported after exposure to chronic alveolar hypoxia (61). Exposure of adult rats to short-term chronic intermittent hypoxia abolished hypoxia-induced relaxation in systemic arteries (34). In the chicken, exposure to chronic in vivo hypoxia did not affect HPV at the age of 2 wk (63). Moreover, chronic hypoxia during incubation did not alter normoxic ductus arteriosus constriction in 19-day chicken fetuses (49). As reported elsewhere, chronic moderate hypoxia in the chicken fetus led, in the femoral artery, to impairment of endothelium-dependent relaxation (40, 50) and increased periartrial sympathetic innervation (41). However, the femoral relaxation induced by acute hypoxia was not affected by exposure to chronic hypoxia. Taken together, our previous and the present data suggest that chronic moderate prenatal hypoxia does not affect the development of vascular O2-sensing systems.

Perspectives and significance. Our experiments indicate that the mETC plays a critical role in O2 sensing in fetal chicken femoral arteries. In contrast, hypoxia-induced relaxation appears not to be mediated by ROS, and K+ channels are unlikely effectors. Other proposed mechanisms for hypoxic relaxation of systemic arteries (62), such as increased sarcoplasmic reticulum uptake of Ca2+, decreased sarcoplasmic reticulum Ca2+ release, decreased Ca2+ influx across the plasma membrane, or Ca2+ desensitization remain to be investigated. The mechanisms of vascular O2 sensing are still the subject of intense scrutiny and debate. Aside from the role of mETC, NADPH oxidases, ROS, or K+ channels in hypoxic vascular responses, new sensors/transducers, such as hydrogen sulfide (31) or ceramide (8), have been recently proposed. HPV (42, 63), normoxic contraction of the ductus arteriosus (1, 9, 15), and hypoxic relaxation of systemic arteries (Ref. 38 and present work) are present in the chicken, and the responses appear to implicate several of the mediators described in the mammalian vessels, offering an interesting model for the analysis of old and new mediators involved in the biology of vascular O2-sensing systems.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


