Maturation of O₂ sensing and signaling in the chicken ductus arteriosus

Angel L. Cogolludo,1,2 Javier Moral-Sanz,1 Saskia van der Sterren,2 Giovanna Frazziano,1 Anne N. H. van Cleef,2 Carmen Menéndez,1 Zoer B, Moreno E, Roman A, Pérez-Vizcaíno F, Villamor E. Maturation of O₂ sensing and signaling in the chicken ductus arteriosus. Am J Physiol Lung Cell Mol Physiol 297; L619–L630, 2009. First published July 17, 2009; doi:10.1152/ajplung.00092.2009.—The increase in O₂ tension after birth is a major factor stimulating ductus arteriosus (DA) constriction and closure. Here we studied the role of the mitochondrial electron transport chain (ETC) as sensor, H₂O₂ as mediator, and voltage-gated potassium (Kᵥ) channels and Rho kinase as effectors of O₂-induced contraction in the chicken DA during fetal development. Switching from 0% to 21% O₂ contracted the pulmonary side of the mature DA (mature pDA) but had no effect in immature pDA and relaxed the aortic side of the mature DA (mature aDA). This contraction of the pDA was attenuated by inhibitors of the mitochondrial ETC and by the H₂O₂ scavenger polyethylene glycol (PEG)-catalase. Moreover, O₂ increased reactive oxygen species (ROS) production, measured with the fluorescent probes dihydroethidium and 2′,7′-dichlorofluorescein, only in mature pDA. The H₂O₂ analog t-butyl-hydroperoxide mimicked the responses to O₂ in the three vessels. In contrast to immature pDA cells, mature pDA cells exhibited high-amplitude O₂-sensitive potassium currents. The Kᵥ channel blocker 4-aminopyridine prevented the current inhibition elicited by O₂. The L-type Ca²⁺ (CaL) channel blocker nifedipine and the Rho kinase inhibitors Y-27632 and hydroxysafsidil induced a similar relaxation when mature pDA were stimulated with O₂ or H₂O₂. Moreover, the sensitivity to these drugs increased with maturation. Our results indicate the presence of a common mechanism for O₂ sensing/signaling in mammalian and nonmammalian DA and favor the idea that, rather than a single mechanism, a parallel maturation of the sensor and effectors is critical for O₂ sensitivity appearance during development.

THE DUCTUS ARTERIOSUS (DA) is an artery that connects the main pulmonary artery with the aorta during fetal life. DA smooth muscle cells (DASMC) belong to those specialized cell types that sense local O₂ tension, which include, among others, glomus cells of the carotid body, neuroepithelial cells in the lungs, and smooth muscle cells of the resistance pulmonary arteries and of the feto-placental arteries (4, 38). In term infants, the increase in O₂ tension at birth is a key factor stimulating DA constriction, which precedes the anatomic and permanent closure of the vessel (9, 29). In contrast, the DA from preterm babies is frequently unresponsive to O₂ and less likely to constrict after birth. Failure of DA closure in very preterm infants is associated with several comorbidities, such as necro-

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METHODS

Experiments were performed in accordance with the Spanish and Dutch laws for animal experimentation, and the procedures were approved by our institutional review boards.

Egg incubation and vessel isolation. Fertilized eggs of White Leghorn chickens were incubated at 37.8°C and 45% humidity and rotated once per hour (incubator model 25HS, Masalles Comercial). Embryos were incubated for 15 or 19–20 days of the 21-day incubation period. For clarity, we refer to immature DA (15 days) or mature DA (19–20 days) based on their ability to respond to acute changes in O2. On the experimental day, the fetuses were killed by decapitation and both the right and the left DA were carefully dissected and severed distal to the takeoff of the pulmonary arteries and proximal to the insertion into the aorta. Each DA was divided in two segments referred to as pulmonary and aortic DA (pDA and aDA, respectively). The junction between the two segments was discarded for experiments.

Recording of arterial reactivity. Two stainless steel wires (diameter 40 μm) were inserted into the lumen of the DA, which was mounted as a ring segment between an isometric force transducer and a displacement device in a myograph (Danish Myo Technology model 610M, Aarhus, Denmark). The myograph organ bath (5-ml vol) was filled with Krebs-Ringer bicarbonate buffer (in mmol/l: 118.5 NaCl, 4.75 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 2.5 CaCl2, and 5.5 glucose) maintained at 39°C and continuously aerated with 0% O2-95% N2-5% CO2 (P02 = 2.6–3.3 kPa). Each DA was stretched to its individual optimal lumen diameter, i.e., the diameter at which it developed the strongest contractile response to KCl (62.5 × 10–3 M), with a diameter-tension protocol as previously described (1, 36). The response of the DA rings to oxygen was assessed by bubbling the organ chamber with 21% O2-74% N2-5% CO2 (P02 = 17–19 kPa).

Determination of reactive oxygen species. Reactive oxygen species (ROS) generation in isolated DA was assessed with 2′,7′-dichlorofluorescein (DCF) or dihydroethidium (DHE), which detect mainly H2O2 and superoxide, respectively. Endothelium-denuded DA were incubated with the membrane-permeant diacetate form of DCF (DCF-DA, 10–3 M) or with DHE (10–3 M) for 60 min. DA were 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 the following composition (in mmol/l): 130 NaCl, 5 KCl, 1.2 MgCl2, 1.8 CaCl2, 10 glucose, and 10 HEPES (pH 7.3 with NaOH). During the first 30 min the tissues were maintained under hypoxia, induced by bubbling the PSS solution with 100% N2 to achieve a P02 of ~3–4 kPa in the chamber. Once fluorescence values were stable, preparations were challenged with normoxic solution for 5 min. DA were illuminated through the luminal surface with a 450–490 nm (for DCF) or 530-nm (for DHE) hand-pass filter. The emitted fluorescence was filtered with 515-nm (for DCF) or 610-nm (for DHE) long-pass emission filters. Images were taken at 1-min intervals with a Leica DC300F color digital camera. Fluorescence, after subtraction of background, was quantified with ImageJ (version 1.32j, National Institutes of Health; http://rsb.info.nih/gij/). Intensity values are reported as a percentage of the values before the normoxic challenge.

Electrophysiological studies. DASMC were isolated by enzymatic digestion. Briefly, DA rings were opened along their longitudinal axis and placed into a nominally calcium-free PSS (Ca2+-free PSS). To establish a hypoxic environment during the DASMC isolation procedure, the O2 scavenger sodium dithionite (0.8 × 10–3 M; pH adjusted to 7.4 with NaOH) was included in the Ca2+-free PSS as reported previously (26). Tissues were initially incubated at 4°C in this solution containing elastase I (0.28 mg/ml) for 5 min. Thereafter, preparations were incubated at 37°C in a low-Ca2+ (10–3 M) PSS containing collagenase I (1 mg/ml), collagenase XI (1 mg/ml), papain (0.15 mg/ml), and dithiothreitol (1.5 mg/ml) for an additional 5 min. Afterwards, tissues were washed in Ca2+-free PSS and disaggregated with a wide-bore, smooth-tipped pipette. Cells were then placed in a perfusion chamber on the stage of an inverted microscope. After a brief period of time (~10 min) to allow cells to adhere to the bottom of the chamber, cells were superfused with hypoxic Ca2+-free PSS (in the absence of sodium dithionite) at a rate of 2 ml/min. Hypoxia was induced as described in Determination of reactive oxygen species. Cells were kept in hypoxia for at least 30 min before initiation of the experiment. Normoxia was established by bubbling the solution with room air. Membrane currents were recorded with the whole cell configuration of the patch-clamp technique, normalized for cell capacitance and expressed in picoamperes per picofarads as previously described (12, 13). Currents were recorded under essentially Ca2+-free conditions with an external Ca2+-free PSS (see above) and a Ca2+-free pipette (internal) solution containing (in mmol/l) 110 KCl, 1.2 MgCl2, 5 Na2ATP, 10 HEPES, 10 EGTA, pH adjusted to 7.3 with KOH. EGTA and ATP were included in the pipette solution to minimize the component of ATP-dependent and Ca2+-activated K+ currents. Under these conditions, currents were evoked after the application of 200-ms depolarizing pulses from ~60 mV to test potentials from ~60 mV to +60 mV in 10-mV increments.

Western blot analysis. After dissection, DA were immediately frozen in liquid nitrogen and homogenized as previously described (13). The protein content was determined with the Bradford assay (reagents from Bio-Rad). Western blotting was performed with 20 μg of protein per lane. SDS-PAGE (7.5% acrylamide) electrophoresis was performed with the method of Laemmli in a minigel system (Bio-Rad). Samples from mature pDA, immature pDA, and mature

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Fig. 1. Heterogeneity of oxygen-induced response in ductus arteriosus (DA) preparations. Representative traces (A) and mean data (B) showing the responses induced by 21% O2 in mature pulmonary DA (pDA), immature pDA, and mature aortic DA (aDA). Results are means ± SE; n = 6–9.
aDA were run in parallel. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes overnight at 4°C and incubated with rabbit anti-KV1.5 primary antibody and then with horseradish peroxidase-conjugated anti-rabbit secondary antibody. The bands were visualized by chemiluminescence (ECL, Amersham). Results are expressed relative to α-actin expression in each sample.

**RT-PCR analysis.** Total RNA was isolated and purified from DA homogenates with the RNeasy Fibrous Tissue Mini kit (Qiagen). Total RNA was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer’s instructions. Real-time PCR was performed with a Taqman system (Roche-Applied Biosystems, Mannheim, Germany) in the Unidad de Genomica, Universidad Complutense de Madrid. Specific primers were designed for chicken superoxide dismutase (SOD)1 (right 5'-GGTCCGGTAAGAGAAATGACAG-3' and left 5'-GACCTCGGCAATGTGACTG-3'), SOD2 (right 5'-ATATGACCCCCATTGAA-3' and left 5'-GCTGCAAAAGGTGATGTTAC-3'), catalase (right 5'-TGGATCCTTCAAATGAGTCTGA-3' and left 5'-GATGCAATGTGTTTCCATCC-3'), and GAPDH (right 5'-ACCATGTAGTTCAGATCGGAAG-3' and left 5'-GTGCCCTCTGCGCAAGTCCA-3').

Data analysis. Values are expressed 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. Relaxations are expressed as a percentage of decrease of the initial tone previously induced by O₂, r-buty1-hydroperoxide, or KCl. Sensitivity [expressed as apparent affinity (pD₂) = −log EC₅₀] to agonists was determined for each artery by fitting individual concentration-response data to a nonlinear sigmoidal regression curve (GraphPad Prism version 2.01; GraphPad Software, San Diego, CA). Differences between mean values were assessed by one-way ANOVA followed by Bonferroni post hoc t-test. Nonpaired t-tests were used if only two groups were compared. Differences were considered significant at P < 0.05.

![Fig. 2. Inhibition of mitochondrial electron transport chain (ETC) blunts O₂-induced constriction in mature pDA. Representative traces (A–C) and mean data (D) show the responses induced by 21% O₂ in the absence (control) or in the presence of complex I (rotenone) or complex III (myxothiazol and antimycin A) inhibitors. Results are means ± SE; n = 5–9. **P < 0.01 vs. control (ANOVA followed by Bonferroni’s test).](http://ajplung.physiology.org/ by 10.220.33.5 on October 13, 2017)
RESULTS

O₂-induced contractions and effects of ETC inhibitors. After an equilibration period of 45 min in hypoxia (0% O₂), vessels were exposed to normoxia (21% O₂) for 10 min. O₂ induced a contractile response in mature pDA but not in immature pDA as we had previously reported (1), whereas in mature aDA it even caused a relaxant response (Fig. 1). O₂-induced contraction in mature pDA was easily reversible when returning to hypoxia and highly reproducible in two consecutive challenges (Fig. 2A). Thus each vessel was exposed twice to normoxia. To test the role of the mitochondrial ETC, the second challenge to normoxia was elicited in the absence or presence of inhibitors. The contraction induced by O₂ was abolished by rotenone (10⁻⁶ M), an inhibitor of complex I of the mitochondrial ETC. However, at this concentration rotenone had no effect on the contraction induced by the thromboxane A₂ mimetic U-46619 (10⁻² M and 96 ± 3% of a previous stimulation with U-46619, in the absence and the presence of rotenone, respectively; P > 0.05, n = 4–5). Inhibition of the ETC complex III with antimycin A (10⁻⁶ M) or myxothiazol (10⁻⁷ M) also blunted O₂-induced constriction (Fig. 2). At the concentrations used, antimycin A induced a slight decrease (Fig. 2B) and myxothiazol a slight increase (Fig. 2C) of the basal tone.

Role of ROS as mediators. Mature pDA exhibited an increase in DHE fluorescence following 5-min exposure to 21% O₂, and this effect was prevented by the SOD mimetic tiron (10⁻⁵ M), indicating increased superoxide generation (Fig. 3A). Thereafter, we tested the effects of normoxia in DA incubated with DCF, a dye widely used for detecting H₂O₂. A marked elevation in DCF fluorescence was found in mature pDA after normoxic challenge (Fig. 3, B and C). This effect was evident within the first minute of normoxic challenge. In contrast, normoxia did not increase DCF fluorescence in either immature pDA or mature aDA.

Fig. 3. Normoxia selectively increases reactive oxygen species (ROS) production in mature pDA. A: changes in dihydroethidium (DHE) fluorescence induced by normoxia in mature pDA incubated with vehicle (control) or the superoxide dismutase mimetic tiron (10⁻⁵ M). B and C: time course (B) and representative pictures (C) of the changes in 2’,7’-dichlorofluorescein (DCF) fluorescence following exposure to 21% O₂ in mature pDA, immature pDA, and mature aDA. Pictures were taken from the same vessel before and after 5-min exposure to normoxia. Results are means ± SE; n = 3–6. P < 0.05 vs. *immature pDA and #mature aDA (ANOVA followed by Bonferroni’s test).
We also examined the mRNA expression of SOD1, SOD2, and catalase (Fig. 4). Compared with immature pDA, mature pDA had lower expression of SOD1 but similar expression of SOD2 and catalase. SOD1 and SOD2 were similarly expressed in mature pDA and aDA, whereas catalase was twofold higher in mature aDA than in pDA. In addition, the contraction of mature pDA evoked by normoxia in the presence of the membrane-permeant analog of the H₂O₂ scavenger catalase [polyethylene glycol (PEG)-catalase 100 U/ml] only reached 51 ± 12% of that obtained under control conditions.

In the next set of experiments, the effect of the membrane-permeant H₂O₂ analog tert-butyldihydroperoxide (10⁻⁵–10⁻⁴ M) was tested. The H₂O₂ analog had no contractile effect on immature pDA but caused a concentration-dependent contraction of mature pDA (Fig. 5, A and C). In contrast, tert-butyldihydroperoxide relaxed mature aDA (Fig. 5, B and C) in a concentration-dependent manner. Therefore, application of the H₂O₂ analog mimicked the effects of normoxia on the three vessels.

Role of voltage-gated potassium channels as effectors. Addition of the Kv channel blocker 4-aminopyridine (4-AP, 10⁻² M) contracted immature pDA and mature pDA and aDA. The contractile responses induced by the Kv channel blocker were significantly smaller in immature pDA (0.14 ± 0.01 N/m, n = 5) compared with mature pDA (0.43 ± 0.04 N/m, n = 12) and aDA (0.48 ± 0.04 N/m, n = 8). However, no significant differences were found between vessels when values were expressed as a percentage of a previous response to KCl (79 ± 11%, 67 ± 6%, 82 ± 10% for immature pDA, mature pDA, and mature aDA, respectively).

The possible modulation of Kv channels by normoxia was tested in isolated DASMC with the patch-clamp technique. Potassium current amplitudes were about threefold higher in cells isolated from mature pDA than in those from immature pDA (Fig. 6A). Currents present in cells from mature pDA were markedly inhibited by normoxia, whereas no effects were observed in DASMC isolated from immature pDA (Fig. 6, B and C). Addition of the Kv channel blocker 4-AP (10⁻³ M) inhibited the current to a similar extent in mature and immature DASMC (Fig. 6, D and E), whereas at 10⁻² M the potassium current in mature pDA cells was nearly abolished (not shown). Moreover, in the presence of 4-AP (10⁻³ M), the effects of normoxia in mature pDA cells were prevented. To test whether the difference in the effect of normoxia on potassium currents was caused by different Kv1.5 protein content, we analyzed its expression in immature pDA, mature pDA, and mature aDA. Surprisingly, the expression of Kv1.5 channels was lower in

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**Fig. 4.** Expression of superoxide dismutase (SOD)1 (A), SOD2 (B), and catalase (C) analyzed by RT-PCR. Results are normalized to GAPDH and represent means ± SE; n = 6. *P < 0.05 vs. mature pDA (ANOVA followed by Bonferroni’s test).

**Fig. 5.** H₂O₂ analog tert-butyldihydroperoxide mimics the effects of normoxia in chicken DA. Representative pictures (A and B) and mean data (C) show responses induced by tert-butyldihydroperoxide (10⁻⁵–10⁻⁴ M) in mature pDA, immature pDA, and mature aDA. Results are means ± SE; n = 4–6. P < 0.01 vs. **immature pDA and ##mature aDA (ANOVA followed by Bonferroni’s test).
mature pDA than in immature pDA or mature aDA (Fig. 6, F and G).

Role of Ca$^{2+}$/H11001 channels as effectors. The inhibitory effect of normoxia on $K_v$ currents is expected to activate L-type Ca$^{2+}$/H11001 (CaL) channels, contributing to O$_2$-induced constriction. Consistent with this, incubation with the CaL channel blocker nifedipine inhibited the contractile response to O$_2$ in mature pDA (Fig. 7). Additionally, O$_2$-induced constriction was nearly blunted in the absence of extracellular calcium. Nifedipine also relaxed pDA previously contracted with O$_2$. Interestingly, nifedipine caused a similar relaxant response in mature pDA contracted with 21% O$_2$ or with $\tau$-butyl-hydroperoxide (10$^{-4}$ M) (Fig. 7D).

In another set of experiments, DA were initially contracted with KCl (62.5 × 10$^{-3}$ M), and thereafter nifedipine was added in a cumulative fashion. A concentration of 10$^{-5}$ M nifedipine was needed to achieve a near-full relaxant effect in KCl-contracted mature pDA (Fig. 8A). Moreover, immature pDA were less sensitive to nifedipine than mature pDA. To test whether this difference was due to a maturation of CaL channels we tested the effects of the CaL opener BAY K8644. This drug had no effects in immature pDA but contracted mature pDA in a concentration-dependent manner (Fig. 8B). This contractile response was suppressed in the presence of nifedipine (not shown). Finally, pretreatment with BAY K8644 did not modify the contractile response to O$_2$ in mature (98 ± 11%
of the response to oxygen in the absence of the drug, $P > 0.05$; Fig. 8C) or immature (Fig. 8D) pDA.

**Role of Rho kinase as effector.** In mature pDA stimulated with 21% O$_2$, the addition of Rho kinase inhibitors hydroxyfasudil or Y-27632 induced a concentration-dependent relaxation (Fig. 9). Interestingly, contractions induced by O$_2$ and t-butyl-hydroperoxide showed a similar sensitivity to hydroxyfasudil ($pD_2 = 5.82 \pm 0.16$ and $5.68 \pm 0.176$, respectively, $P > 0.05$; Fig. 9C) and to Y-27632 ($pD_2 = 5.92 \pm 0.16$ and $5.79 \pm 0.2$, respectively, $P > 0.05$; Fig. 9D). In the last series of experiments, we examined the relaxation induced by the two Rho kinase inhibitors in mature pDA, mature aDA, and immature pDA stimulated with KCl. The potency of hydroxyfasudil (Fig. 9E) and Y-27632 (Fig. 9F) was higher in mature pDA ($pD_2 = 5.75 \pm 0.13$ and $6.37 \pm 0.1$, respectively) than in immature pDA ($pD_2 = 5.22 \pm 0.11$ and $5.51 \pm 0.13$, respectively; $P < 0.05$ vs. mature pDA for both drugs). Moreover, both drugs showed a similar potency to relax mature aDA ($pD_2 = 5.71 \pm 0.14$ and $6.11 \pm 0.13$ for hydroxyfasudil and Y-27632, respectively) compared with pDA (data above).

**DISCUSSION**

The preparation of the DA for its specific task of postnatal closure is reflected in critical maturational changes in reactivity (1–3, 9, 29). The chicken lacks the limitations of most available models and has arisen as an excellent model for studying the developmental biology of the DA (31). In the present study, we have examined the developmental changes in the cellular mechanisms (sensor, mediator, and effectors) involved in the chicken DA response to O$_2$. Our results can be summarized as follows. 1) Contraction induced by O$_2$ is restricted to the mature pulmonary side of the DA. 2) This response is nearly abolished by mitochondrial ETC inhibitors. 3) Normoxia increases ROS production in the pulmonary side of mature DA but not in the immature DA or in the aortic side. 4) Exogenous addition of the H$_2$O$_2$ analog t-butyl-hydroperoxide mimics the effects of normoxia in all vessels tested. 5) KV currents change during development from low-amplitude O$_2$-insensitive currents in immature pDA to high-amplitude O$_2$-sensitive currents in mature pDA.
The inhibition of Ca\textsubscript{L} channels and Rho kinase attenuates the normoxic constriction. Mitochondria have been proposed as O\textsubscript{2} sensors in several specialized O\textsubscript{2}-sensing tissues such as the resistance pulmonary arteries, glomus cells of the carotid body, fetal adrenomedullary chromaffin cells, and the DA (4, 14, 23, 37, 38, 40). Michelakis et al. (23) reported that complex I and complex III ETC inhibitors fully relaxed O\textsubscript{2} constriction in human DA. In the chicken DA, the inhibition of complex I or III also abolishes the contractile response to O\textsubscript{2} (Ref. 16 and present study). The effect of the complex I inhibitor rotenone was selective since the contraction induced by U-46619 was not affected.

**H\textsubscript{2}O\textsubscript{2} as mediator.** An increased production of ROS (specifically H\textsubscript{2}O\textsubscript{2}) has been proposed to underlie the O\textsubscript{2} response in mammalian DA (18, 23, 27). Here, we found that normoxia augmented fluorescence to DHE, which mainly detects changes in cytosolic concentrations of superoxide. The increase in DHE fluorescence was prevented by the superoxide mimetic tiron, confirming that it was due to the elevation in superoxide levels. Superoxide is rapidly dismuted by SOD to H\textsubscript{2}O\textsubscript{2}, a stable and mobile molecule that is more likely to act as the signaling mediator. Thus we measured the changes in H\textsubscript{2}O\textsubscript{2} levels by using the fluorescent dye DCF. O\textsubscript{2} increased the fluorescence to DCF in mature pDA, but not in either mature aDA or immature pDA. Therefore, the three preparations exhibit a profound parallelism in terms of tension generation and H\textsubscript{2}O\textsubscript{2} production after normoxia. Moreover, O\textsubscript{2} induces contraction and increases H\textsubscript{2}O\textsubscript{2} production in the DA with a similar time course. Another argument in favor of H\textsubscript{2}O\textsubscript{2}...
as the O2 mediator in the chicken DA was the marked attenuation of the O2 constriction following the application of the H2O2 scavenger PEG-catalase. In accordance with this, Greyner and Dzialowski (16) observed that the H2O2 scavenger N-mercaptopropionylglycine relaxed O2-induced contraction in the mature pDA. Furthermore, in striking similarity with O2, the H2O2 analog t-butyl-hydroperoxide contracted mature pDA, had no effect on immature pDA, and relaxed mature aDA. In mammalian DA, H2O2 has been shown to induce constriction because of its ability to inhibit Kv channels (23, 27) and to activate Rho kinase (18), which are considered effectors of the O2 response (see below). In the chicken DA, nifedipine (which reverses depolarization-induced contraction) and Rho kinase inhibitors relaxed O2- and H2O2-induced contraction with a similar potency and efficacy, which is also consistent with the involvement of H2O2 as the mediator molecule of the O2 response.

*Kv* channels as effectors. In rabbit (27, 39) and human (23) DA it has been reported that O2 inhibits Kv channels, depolarizes membrane potential, and causes vasoconstriction. In our previous study (1), we found that the chicken DA, nifedipine (which reverses depolarization-induced contraction) and Rho kinase inhibitors relaxed O2- and H2O2-induced contraction with a similar potency and efficacy, which is also consistent with the involvement of H2O2 as the mediator molecule of the O2 response.
central portion of the DA, which contains a similar proportion of tissue from pulmonary and aortic sides. Here we observed that the inhibition of Kᵥ channels causes a comparable vasconstriction in the pulmonary and aortic sides of mature DA. Similar results have been recently reported (16). In addition, no differences were found in the response to 4-AP between mature and immature pDA when values were normalized to a previous response to KCl. Altogether these data indicate that under basal conditions Kᵥ channels make a similar contribution in controlling tone in the chicken DA regardless of gestational age or the portion of the vessel studied.

This is the first study examining potassium currents in the chicken DA. Under our experimental conditions, Kᵥ channels were the main contributors to the total outward current in both mature and immature pDASMC as evidenced by the sensitivity to 4-AP. Perfusion with normoxic solution dramatically decreased Kᵥ current amplitude in mature pDA. Therefore, in agreement with studies performed in mammals, our present work identifies the presence of O₂-sensitive Kᵥ channels in the chicken DA. These data support the idea that the Kᵥ channel inhibition in response to O₂ is preserved during evolution, as recently suggested (31).

Inhibition of Kᵥ channels leads to membrane depolarization activation of Ca₂⁺ channels, increase in intracellular Ca²⁺, and vasoconstriction (12, 22, 24, 32, 38). Thus Kᵥ channel inhibition could be a key mechanism mediating O₂-induced contraction in the chicken DA, as previously reported in mammals (23, 25, 27, 28). In agreement with this, the contractile response to O₂ was partly inhibited by the Ca₉ channel blocker nifedipine and nearly abolished in the absence of extracellular calcium (present work and Ref. 16). It is very likely that, apart from Ca₉ channel opening, Ca²⁺ entry through store-operated channels (11, 16, 17) or its release from intracellular stores (19) may also contribute to the normoxic contraction. Interestingly, compared with other chicken vascular beds (like the pulmonary arteries; unpublished observations) the DA appears to be less sensitive to dihydropyridines, as evidenced by the low potency of BAY K₈₆₄₄ to induce contraction and of nifedipine to relax KCl constriction. This may explain the relatively high concentration of nifedipine (≥10⁻⁶ M) needed to significantly reduce O₂-induced contraction.

Rho kinase as effector. Activation of Rho kinase can phosphorylate the 130-kDa myosin binding subunit of myosin light chain phosphatase, causing its inhibition. This prevents myosin light chain dephosphorylation and hence increases Ca²⁺ sensitivity and smooth muscle contractility. Thus Rho kinase-mediated Ca²⁺ sensitization is an important mechanism controlling vascular smooth muscle tone (20, 30). In the present study, we found that O₂ constriction is diminished by ~75% when extracellular Ca²⁺ is removed. Similar results have been reported by Greyner and Dzialowski (16). The remaining Ca²⁺-independent contraction is likely to be due to Rho kinase-induced Ca²⁺ sensitization, as reported in human and rabbit DA (17, 18). Consistent with this view, O₂-induced constriction in mature pDA was relaxed by the Rho kinase inhibitors Y-27632 and hydroxyfasudil.

Maturation of O₂-induced contraction. The mechanisms underlying the failure of the preterm DA to constrict and close after birth are not completely elucidated. In the chicken DA at 15 days of incubation, we have found (Ref. 1 and present study) that the response to O₂ was very weak or absent. On the other hand, 19–20 days of incubation all pDA were markedly constricted in response to O₂. Our results are in agreement with previous studies in other species showing that the constriction of DA to O₂ is developmentally regulated (10, 21, 32). The reduced response to O₂ in preterm DA may reflect a deficiency in the sensor, the mediator, the effector, or a combination of these. In this regard, we observed that O₂ increased ROS content in mature but not in immature pDA. These differences are not related to changes in mRNA expression of SOD1 (which is even higher in the immature DA), SOD2, or catalase, and rather may reflect an impairment of the mitochondrial sensing mechanism in the immature pDA. In accordance with this, Kajimoto et al. (18) found that in response to O₂, superoxide levels increased to higher levels in term than in preterm rabbit DASMC. Interestingly, we also observed that when the putative mediator (i.e., H₂O₂) was exogenously provided it induced contraction of the mature but not the immature pDA, suggesting that the unresponsiveness to O₂ also involves immaturity of the final effectors.

Focusing on the putative effectors, we show that Kᵥ currents are markedly reduced in immature versus mature pDA smooth muscle cells. In accordance with this, Thebaud et al. (32) observed that Kᵥ current amplitudes in preterm rabbit DA are reduced compared with term DA. Among the different Kᵥ channels, Kᵥ1.5 have been shown to be O₂ sensitive and proposed as candidates to underlie O₂-sensitive Kᵥ currents in DA (23, 32) and pulmonary arteries (5). However, the developemental sensing mechanism in the immature pDA. In accordance with this, Michelakis et al. (23) for normoxic constriction of the mammalian DA (mitochondrial ETC as sensor, H₂O₂ as mediator, and Kᵥ channels and Rho kinase as effectors) is also responsible for normoxic constriction of chicken DA. Scheme also shows the inhibitors used targeting different steps of the pathway.

Fig. 10. Mechanism proposed by Michelakis et al. (23) for normoxic constriction of the mammalian DA (mitochondrial ETC as sensor, H₂O₂ as mediator, and Kᵥ channels and Rho kinase as effectors) is also responsible for normoxic constriction of chicken DA. Scheme also shows the inhibitors used targeting different steps of the pathway.
opmental increase in Kv current found in the chicken DA seems to be unrelated to changes in Kv1.5 channel expression, which even decreases with gestational age. In fact, contradictory data regarding the changes in Kv1.5 channels expression during the last days of gestation have been reported in different species (32, 41). One possibility is that other O2-sensitive Kv channels apart from Kv1.5 (such as Kv2.1) make a major contribution to the total Kv current in chicken DA.

Very recently, Thebaud et al. (33) suggested that CaL channels are O2 sensitive in the rabbit DA and their functional immaturity contributes to the impaired response to O2 in the preterm animal. We found that nifedipine, a CaL channel blocker, was more potent to inhibit KCl-induced contractions in the mature than the immature pDA. In addition, the CaL channel activator BAY K8644 was also much more effective in inducing a contraction in the mature than in the immature pDA. Nifedipine and BAY K8644 belong to the same chemical class of dihydropyridines and bind to the same site in the channel (15). Thus our data indicate a maturational change in the response to dihydropyridines. In the study of Thebaud et al. (33), the presence of BAY K8644 enhanced the O2 constriction in preterm (to levels observed at term) but not in term DA. On the contrary, we found that in the preterm chicken DA the immaturity cannot be reversed by BAY K8644.

Finally, the reduced response to O2 in preterm compared with term DA has also been associated with a diminished expression/activity of components of the Rho–Rho kinase pathway (11, 18). Consistent with this, we have observed that the sensitivity to the Rho kinase inhibitors hydroxyfasudil and Y-27632 increased with development.

Changes in response to O2 along the DA. One of the most striking features of the chicken DA is the radically opposite response to changes in O2 (relaxation vs. contraction) seen in the two portions (aortic vs. pulmonary) of the vessel. This functional difference correlates with morphological changes along the chicken DA between the pulmonary artery and the aorta (1, 7, 8), which may reflect the proposed different embryological origin of the cells composing the aortic and the pulmonary portions of the vessel (8). Here we have examined the mechanisms involved in the different responses to O2 in both sides of the vessel. The major finding was that O2 did not increase ROS content in mature aDA. This may be explained, at least partly, by a higher expression of the H2O2 scavenging enzyme catalase. Nevertheless, this does not seem to be the only difference along the vessel. Thus, unlike the pulmonary side, the aortic side of the DA relaxed in response to the H2O2 analog (the proposed mediator). This opposite behavior cannot be explained by a different expression/activity of the effector proteins (i.e., Kv channels and Rho kinase) in both sides, since the inhibition of Kv1 channels caused a similar contraction in both sides of the vessel and, conversely, the Rho kinase inhibitors relaxed both the aortic and the pulmonary ductal segments.

Conclusions. In summary, our results offer supportive evidence implicating the mitochondrial ETC as sensor, H2O2 as mediator, and Kv channels and Rho kinase as effectors of O2-induced contraction in the chicken DA (Fig. 10). Therefore, there is a conserved mechanism for O2 sensing/signaling in mammalian and nonmammalian DA. Our results also indicate that the developmental response to O2 involves a parallel maturation of the three components: sensor, mediator, and effectors.

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