Mechanisms mediating the oxygen-induced vasoreactivity of the ductus arteriosus in the chicken embryo

Henry Greyner and Edward M. Dzialowski
Department of Biological Science, University of North Texas, Denton, Texas

Submitted 1 January 2008; accepted in final form 17 September 2008

Greyner H, Dzialowski EM. Mechanisms mediating the oxygen-induced vasoreactivity of the ductus arteriosus in the chicken embryo. Am J Physiol Regul Integr Comp Physiol 295: R1647–R1659, 2008. First published September 17, 2008; doi:10.1152/ajpregu.00001.2008.—The avian embryo provides a novel model for studying the ductus arteriosus (DA) during the transition from in ovo to ex ovo life. Here we examined the mechanisms regulating the vasoreactivity of the two morphologically distinct portions of the chicken DA (proximal and distal) in response to O2. Oxygen-induced contraction is redox sensitive and reversed by the reducing agent diethiothreitol and the H2O2 scavenger N-mercaptopyrrolyglycine. As in the mammalian DA, inhibiting mitochondrion-derived reactive oxygen species production with rotenone and antimycin A relaxed the O2-constricted DA. The contractile response to O2 matures during hatching and is mimicked by the K+ channel inhibitor 4-amino-pyridine (4-AP) on day 19 and externally pipped (EP) embryos. Together, O2 and 4-AP significantly increase DA tone above that observed with either alone. The O2-induced contraction is mediated by influx of extracellular Ca2+ through L-type Ca2+ and store-operated channels. Inositol 1,4,5-trisphosphate-sensitive Ca2+ stores play a minor role in the O2-induced contraction. The O2-induced contraction is mediated by the Rho kinase pathway, as fasudil and Y-27632 significantly relax the O2 contracted DA. Prostaglandins E2, F2α, and D2 produce significant contraction of the proximal DA. The O2-induced relaxation of the distal portion of the DA is mediated by an endothelial-derived nitric oxide/cGMP pathway. Both 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and endothelial cell removal inhibit O2-induced relaxation in the distal segment. Mechanisms regulating O2-induced contraction in chicken proximal DA are similar to those found in mammalian DA, making the chicken a useful model for studying development of this O2-sensitive vessel.

Oxygen; calcium L-channels; store-operated channels; Rho kinase; reactive oxygen species

THE DUCTUS ARTERIOSUS (DA) are embryonic blood vessels that connect the pulmonary arteries to the aorta allowing for a right-to-left shunt of the blood away from the fluid-filled lungs. The DA of terrestrial vertebrates originates from the sixth aortic arch (5). However, differently from mammals, birds have bilaterally developed DA that originate from the right and left pulmonary arteries and connect to the descending aorta. The morphology of the right and left DA are not uniform along their length in the chicken (4). The portion of the DA closer to the pulmonary arteries, proximal DA, contains a well-developed smooth muscle layer. Distal to this and connecting with the descending aorta, the distal DA contain more elastin, less smooth muscle, and are thicker than the proximal DA (4, 5). It is the proximal portion of the DA that is most morphologically similar to the mammalian DA.

Mammalian DA patency and closure during in utero development depends on several factors with prostaglandins and O2 playing crucial roles (38). In the lamb DA, PGE2 acts as a potent vasoconstrictor of the DA maintaining patency (11, 14). In contrast, PGF2α has little effect or, in some cases, contracts the precontracted lamb DA (11, 16), while PGD2 produces relaxation in the lamb DA (16). In mammals, PGE2 is the major contributor to ductal patency prior to birth (7, 8, 11, 14, 15, 23, 46). The role of prostaglandins in maintaining DA patency in the chicken DA is unknown. However, the nonselective cyclooxygenase (COX) inhibitor indomethacin (1, 17) and the COX-1 selective inhibitor valeryl salicylic acid (1) do not produce responses in the chicken or emu DA, suggesting a diminished role for prostaglandins in the avian DA when compared with mammals.

Closure of the chicken DA begins when the embryo breaks the egg shell and breathes normoxic air with its lungs (4). The pathways regulating this closure in the chicken DA are unknown. In mammals, it is the rise in blood PO2 associated with the onset of lung ventilation that stimulates DA closure. The mechanism for the initial O2-induced closure of the mammalian DA involves changes in membrane potential of the smooth muscle cells and initiation of an initial Ca2+-dependent pathway. Inhibition of K+ channels in the DA smooth muscle cells are thought to lead to membrane depolarization (44). This opens voltage-gated L-type Ca2+ channels and results in the initial vasoconstriction due to influx of Ca2+. Additionally, store-operated Ca2+ channels (SOC) and inositol 1,4,5-trisphosphate (IP3)-sensitive sarcoplasmic reticulum (SR) Ca2+ stores have been implicated in the O2-induced contraction (13, 19). This O2-induced constriction of the mammalian DA is mediated by a rise in mitochondrial-derived reactive O2 species (ROS; 3). Additionally, The sensitivity of the mammalian DA to Ca2+ increases through the upregulation of the Rho kinase pathway (22). During contraction, myosin light-chain (MLC) is phosphorylated by MLC kinase (MLCK)-producing contraction. This is countered by the action of MLC phosphatase (MLCP) that cleaves the phosphate from the MLC to produce relaxation. Rho kinase inhibits MLCP activity producing a sustained contraction requiring less Ca2+ (13, 22). A number of properties of developing chicken embryos make them an excellent model to study DA physiology (1, 39). The major advantage of the chicken embryo for studies of the DA is the ease with which the incubation environment, such as O2 levels, can be manipulated and directly measured. However, very little is known about the pathways involved in regulating tone and closure of the avian DA, especially in

http://www.ajpregu.org
0363-6119/08 $8.00 Copyright © 2008 the American Physiological Society R1647

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
regard to the two different morphological tissue types that compose this vessel. The goal of this study was to characterize the regulators of ductal patency and O2-induced closure of the chicken ductus during hatching. We hypothesized that the mechanisms outlined above that maintain ductal patency and regulate closure of the mammalian ductus would be similar to those found in the proximal portion of the chicken DA. An understanding of the closure of the chicken DA will allow its use as a novel system to further study the development of the mechanisms involved in the O2-induced closure of this unique vessel and how they respond to changes in the developmental environment.

MATERIALS AND METHODS

All experiments were approved by the University of North Texas IACUC.

Incubation and stages of hatching. Eggs from white leghorn chickens were obtained from Texas A&M University and incubated at 37.5°C with a relative humidity of 70%. Eggs were turned automatically every 4 h. The DA was examined from day 19 prepped, day 19–20 internally pipped (IP), and day 20 externally pipped (EP) embryos. Hatching in chicken embryos begins on day 19 of incubation and involves two phases, internal pipping and external pipping. Embryos internally pip the inner membrane of the air cell with their beak late on day 19 and early on day 20 and begin to respire hypoxic gas in the air cell (45). Internal pipping was determined by candling the egg and noting the presence of the beak and head in the air cell and associated breathing movements. On day 20, the embryos externally pip the eggshell with their beak and begin to breathe normoxic air. Hatching occurs 8 and 12 h after external pipping.

Vessel preparation and isometric tension in vitro. The right and left DA from day 19, IP, and EP chicken embryos were excised and placed in a physiologically saline solution (PSS composed of (in mM) 120.5 NaCl, 4.8 KCl, 1.2 MgSO4, 1.6 CaCl2, 1.2 NaH2PO4, 20.4 NaHCO3, and 10 glucose) equilibrated with 95% N2-5% CO2. The proximal and distal portions of the DA were separated based on visual inspection of vessel morphology and diameter (4).

Isometric tension generated by the DA was measured in vitro using a four-chamber 610M Danish Myo Technologies myograph. Vessel rings ranging in length from 0.8 to 2 mm were mounted in the organ chamber by threading two 40-μm diameter stainless steel wires through the vessel and then attaching one wire to a force transducer and the other to a micromanipulator. The vessels were suspended in PSS and bubbled with a 95% N2-5% CO2 gas mixture resulting in a PO2 of 4 kPa (4% O2) and a PCO2 of 5 kPa (5% CO2). During exposure to increased O2 levels, the organ bath was bubbled with a 67% N2-28% O2-5% CO2 gas mixture resulting in a PO2 of 25 kPa (25% O2) and a PCO2 of 5 kPa (5% CO2). Bath PO2 and PCO2 were monitored with a Radiometer ABL5 blood gas meter. Isometric force was recorded as mN/mm (1) with Chart 5.2 data acquisition software and a Powerlab 8SP (ADInstruments) connected to the 610M DMT myograph.

The vessels were stretched to the length that produced the largest contraction in response to 120 mM KCl as determined in preliminary experiments, and the vessels were allowed to equilibrate at 4 kPa O2 for 30 min prior to conducting any experiments. The baseline tensions for the distal and proximal portions were similar. All experiments were conducted under low-light conditions.

Oxygen-mediated contraction. The role of K+ channels in the O2-induced contraction was examined in rings of proximal sections from day 19, IP, and EP embryos and the distal section of IP animals. Vessels were initially contracted by increasing O2 from 4 kPa to 25 kPa, and this was followed by a decrease in O2 back to 4 kPa. After the vessel had relaxed back to the baseline tension, 10 mM 4-aminopyridine (4-AP), a K+ channel inhibitor, was added to the organ bath, and the vessel was allowed to contract. In another set of EP vessels, the O2 was increased from 4 kPa to 25 kPa after the addition of 4-AP to determine whether there was an additive effect with both 4-AP and O2.

The role of Ca2+ in the O2-induced contraction of the proximal DA was examined using a combination of a number of pharmacological agents to examine the importance of extracellular Ca2+ entry through SOCs, voltage-gated L-type Ca2+ channels, and T-type Ca2+ channels and intracellular release of Ca2+ from IP3-sensitive SR stores. This was done by using various combinations of the following: 4 μM mibebradil (a T-type Ca2+ channel antagonist), 10 μM nifedipine (voltage-gated L-type Ca2+ channel antagonist), 30 μM 2-aminoethoxydiphenylborane [2-APB; IP3 receptor and transient receptor potential (TRP) channel and SOC antagonists], 0.1 μM thapsigargin (a potent sarcoplastic reticulum Ca2+-ATPase inhibitor), and 1 μM Bay K 8644 (a L-type Ca2+ channel agonist). Nifedipine, thapsigargin, and 2-APB were tested in both the standard PSS and Ca2+-free PSS with 5 mM EGTA to determine the role of internal Ca2+ stores.

To test for the role of reactive oxygen species (ROS) in stimulating the O2-induced contraction, the proximal and distal vessels from EP embryos were exposed to rotenone (10 μM; 28), an inhibitor of electron transport chain complex I, or antimycin A (10 μM; 27), an inhibitor of electron transport chain complex III. The redox sensitivity of the DA was examined with the reducing agent DTT (3 mM) (34) using proximal rings from day 19, IP, and EP embryos and the distal section of IP embryos. To further test the role of redox sensitivity and ROS in the O2-induced contraction, O2-contracted proximal DA from EP embryos was exposed to the cell-permeable ROS scavenger N-mercaptopropionylglycine (NMPG; 10, 20, and 30 mM) (34). During these four sets of experiments, the vessels were precontracted with 25 kPa O2, and the drug was added to the organ bath after vessel tension had stabilized. Each vessel was tested with only one drug.

Because the PO2 used in the experiments was higher than that found in vivo, we examined the response of the EP proximal DA to increases in O2 to a more physiological level (12 kPa) (40) and compared this with the contraction produced by an increase in O2 to 25 kPa. After the 30-min equilibration period at 4 kPa, the O2 level was raised to 25 kPa, and the vessel was allowed to contract until a plateau was reached. The O2 was then decreased to 4 kPa, and the vessel was allowed to relax. This was followed by an increase in O2 to 12 kPa.

The role of the Rho kinase pathway and calcium sensitization in the O2-induced contraction was examined in proximal DA segments from EP embryos. This was done by exposing the vessels to either Y-27632 (10-8 to 10-5 M), a selective inhibitor of the Rho-associated protein kinase p160ROCK, or fasudil (10-8 to 10-4 M), a cyclic nucleotide-dependent protein kinase inhibitor and Rho-associated kinase inhibitor. Vessels were precontracted at a PO2 of 25 kPa and, following stabilization, were exposed to stepwise increases in Y-27632 or fasudil.

Vasoreactivity to prostaglandins. Cumulative dose-response curves were constructed for PGE2 (10-8 to 10-5 M), PGF2α (10-8 to 10-5 M), and PGD2 (10-8 to 10-5 M), using the proximal and distal DA from day 19 and IP embryos. After the addition of each concentration, the vessel was allowed to stabilize before the next concentration was added. The effects of prostaglandins were tested at both 4 kPa O2 and 25 kPa O2.

Nitric oxide-mediated relaxation. The role of the endothelial-derived nitric oxide (NO)/cGMP pathway in the O2 response of the proximal and distal DA was examined from day 19 embryos. Endothelium-dependent relaxation was examined using cumulative addition of Ach (10-8 M to 10-5 M) on vessels precontracted with 10-5 M phenylephrine. To examine the influence of O2 on the Ach-stimulated responses, measurements were made under both 4 kPa and 25 kPa O2 conditions. Ductus rings were exposed in vitro to 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10-5 M), a selective inhibitor of NO-sensitive guanylyl cyclase, either before or after O2 was increased from 4 kPa to 25 kPa. To examine the role of
the endothelium in the NO-mediated relaxation, endothelial cells were removed from both proximal and distal sections of the DA by threading a piece of silk suture (size range 4-0 to 6-0) several times through the lumen of the rings after placing the vessels in the myograph. The response of the vessels was then measured to increasing O2 from 4 kPa to 25 kPa.

Drugs. PGE2, PGF2α, and PGD2 were purchased from Cayman Chemical and dissolved in ethanol. Preliminary experiments showed that the vehicle (ethanol at <0.1%) had no effect on vessel tension when provided by itself. Antimycin A, rotenone, DTT, NMPG, ACh, and nifedipine were purchased from Sigma-Aldrich. Fasudil, Y-27632, 4-AP, mibebradil dihydrochloride, Bay K 8644, and ODQ were purchased from Tocris Bioscience.

Statistical analysis. Significance of the results was examined using ANOVA followed by Tukey’s post hoc tests. Data are presented as the means ± SE of either the tension generated above the baseline tension, here noted as active tension (1), the change in tension after addition of drugs, or the % of the initial O2-induced contraction. In the experiments examining the role of Ca2+ in the O2-induced contraction, the tension of the initial O2-induced contraction was calculated using the minimum tension in the absence of external Ca2+ at 4 kPa O2 as the baseline. The responses to Y-27632 and fasudil are presented as the % change from the initial contraction above the initial baseline produced by O2. The level of significance for all tests was P < 0.05. All statistics were carried out with Sigmastat 3.5.

RESULTS

Mechanisms involved in the O2-induced DA contraction. The initial O2-induced contraction of the proximal DA significantly increased in strength with the progression of hatching (Fig. 1A). The weakest contractions in response to increased O2 significantly increased in strength with the progression of hatching (Fig. 1A). During IP, the O2-induced contraction was significantly greater than the contraction observed on day 19 in EP embryos (P < 0.05). In contrast, the distal DA from EP embryos relaxed significantly in response to increased O2. The level of O2 exposure (12 vs. 25 kPa) did not have a significant effect on the strength of the O2-induced contraction in the proximal DA from EP embryos (P = 0.155; n = 5; Fig. 2).

The Kv channel inhibitor 4-AP produced significant contraction of the proximal DA and the distal DA. In day 19 embryos, 4-AP (10 mM) produced a significant contraction of the proximal section of the DA that was not different from the O2-induced contraction (Fig. 1A). During IP, the increase in proximal DA tension produced by 10 mM 4-AP was significantly greater than the O2-induced contraction. By EP, the O2-induced contraction and the 4-AP contraction in the proximal DA were similar in magnitude. The distal DA from EP animals relaxed when exposed to 25 kPa O2, but contracted in response to 10 mM 4-AP. The 4-AP-induced contraction was of similar magnitude in the proximal and distal segments in the IP embryos. In another set of embryos, O2 alone (0.30 ± 0.04 mN/mm; n = 6) and 4-AP alone (0.28 ± 0.06 mN/mm; n = 6) produced similar significant contractile responses in the proximal DA from EP embryos. Increasing O2 after administration of 4-AP produced a further significant contraction of the EP proximal DA to 0.93 ± 0.13 mN/mm (Fig. 1B; P < 0.025).

The source of Ca2+ during the O2-induced contraction of the proximal DA from EP embryos includes L-type Ca2+ channels, SOCs, and internal IP3-sensitive Ca2+ stores. Inhibiting L-type Ca2+ channels with nifedipine (10 μM) revealed a role for L-type Ca2+ channels in the O2-induced contraction (Fig. 3A). Nifedipine significantly reversed the O2-induced contraction of the EP proximal DA in the presence of external Ca2+ (Fig. 3B; P < 0.001). However, the DA were still contracted to ~50% of the O2-induced contraction, suggesting a role for other Ca2+ channels, such as SOCs. In the absence of external Ca2+, nifedipine-treated vessels did not produce a significant contraction in response to an increase in O2 (Fig. 3B). Additionally, the L-type Ca2+ channel agonist Bay K 8644 enhanced the O2-induced contraction of the EP to a level significantly above the O2-induced contraction alone (Fig. 1B). Increasing O2 after depletion of the SR Ca2+ stores with thapsigargin in the presence of nifedipine and absence of external Ca2+ resulted in no change in tension (Fig. 3C). A significant contraction was produced under low O2 conditions when Ca2+ was added back to the solution, presumably due to Ca2+ entry through SOCs. Under these conditions, the DA

Fig. 1. Oxygen contracts the proximal ductus arteriosus (DA) and is mimicked by 4-aminopyridine (4-AP). A: response of the chicken DA to an increase in tissue bath O2 from 4 kPa to 25 kPa and to the addition of 10 mM 4-AP in day 19 proximal DA (19-Prox; n = 6), internally pipped (IP) proximal DA (IP-Prox; n = 13), IP distal DA (IP-Dist; n = 8), and externally pipped (EP) proximal DA (EP-Prox; n = 8). *Bars with different letters are significantly different from each other at P < 0.05. B: response of the EP proximal DA to 4-AP + 4 kPa O2 (n = 6), 4-AP + 25 kPa O2 (n = 6), and Bay K 8644 + 25 kPa O2 (n = 7) presented as a percentage of the initial O2-induced contraction. *Significant contraction at P < 0.05. +Significantly greater contraction than the O2-induced contraction alone.
tension was further increased after the O$_2$ was increased to 25 kPa, further supporting the role of SOCs in the O$_2$-induced contraction.

A small portion of the O$_2$-induced contraction of the proximal DA was due to release of Ca$^{2+}$/H$^{10}$ from IP$_3$-sensitive internal Ca$^{2+}$/H$^{10}$ stores (Fig. 4A). In the absence of external Ca$^{2+}$, O$_2$ produced a significant contraction of the DA (Fig. 4, A and B; \(P < 0.05\)). Addition of the IP$_3$ receptor antagonist 2-APB significantly relaxed the vessel below the baseline tension. In the next experiment, an increase in O$_2$ in Ca$^{2+}$/H$^{10}$-free conditions produced a significant contraction of the DA, presumably through the release of Ca$^{2+}$/H$^{10}$ from IP$_3$-sensitive Ca$^{2+}$/H$^{10}$ stores (Fig. 4C). Addition of Ca$^{2+}$ to the organ bath produced a significant contraction similar to the initial O$_2$-induced contraction. The IP$_3$ receptor and SOCs antagonist 2-APB reduced the O$_2$-induced contraction by approximately one-half (Fig. 4C), suggesting a role for Ca$^{2+}$ influx through SOCs and IP$_3$-sensitive Ca$^{2+}$/H$^{10}$ stores in response to an increase in O$_2$.

T-type Ca$^{2+}$/H$^{10}$ channels do not play a role in the O$_2$-induced contraction, as mibefradil did not alter the tension generated by the proximal DA in response to O$_2$ (data not shown; \(P = 0.44\); \(n = 4\)).

Nifedipine had no effect on the O$_2$-induced relaxation of the EP distal DA \((P = 0.78)\). The relaxation in response to O$_2$ was \(-0.11 \pm 0.03\) mN/mm and the relaxation in response to O$_2$ plus nifedipine was \(-0.11 \pm 0.06\) mN/mm (paired \(t\)-test \(P = 0.75\); \(n = 4\)).

The initial contractile response of the DA to increased O$_2$ is mediated in part by the redox status of the DA smooth muscle cells. The reducing agent DTT (3 mM) produced significant relaxation of the proximal DA, which had been precontracted with 25 kPa O$_2$ in day 19 \((P = 0.013)\), IP \((P = 0.006)\), and EP \((P < 0.001)\) embryos (Fig. 5, A and B). This response increased with age and was associated with the increase in initial contraction of each stage to O$_2$ seen in Fig. 5. The distal portion from IP embryos was insensitive to 3 mM DTT.

---

**Fig. 3.** L-type Ca$^{2+}$/H$^{10}$ channels play a role in the O$_2$-induced DA contraction from EP embryos. A: representative tension tracing showing the proximal DA contractile response to 25 kPa O$_2$ in the absence and presence of Ca$^{2+}$/H$^{10}$ and 10 \(\mu\)M nifedipine. The level of the bar indicating the Ca$^{2+}$/H$^{10}$ conditions in relation to the tracing is the tension of the initial O$_2$-induced contraction for this vessel. B: relative ductus contractions generated in response to 25 kPa O$_2$ and nifedipine in the absence and presence of external Ca$^{2+}\ (n = 6)\). C: relative DA contractions in response to changes in P$_O_2$ after depletion of Ca$^{2+}$/H$^{10}$ stores by thapsigargin and blocking of L-type Ca$^{2+}$/H$^{10}$ channels with nifedipine in the absence and presence of external Ca$^{2+}\ (n = 7)\). The contractile response of the DA is presented as the percentage of the initial O$_2$-induced contraction calculated from the Ca$^{2+}$/H$^{10}$-free plus 4 kPa O$_2$ baseline. *Significant contraction at \(P < 0.05\). + Significant reduction in tension compared with the initial O$_2$-induced contraction at \(P < 0.05\).
Rotenone and antimycin A, inhibitors of mitochondrial ROS production, produced significant relaxation of the O2-constricted proximal DA. Blocking in vitro mitochondrial production of ROS by inhibiting complex I and III of the electron transport chain with rotenone (10 μM) and antimycin A (10 μM), respectively, produced significant relaxation of the O2-constricted proximal DA (Fig. 5, C and D; P < 0.001). Rotenone (10 μM) completely abolished the O2-induced contraction of the proximal section from EP embryos. Antimycin A (10 μM) also produced significant relaxation of the EP proximal DA; however, the tension did not return to baseline levels. The distal portion from EP embryos was insensitive to both 10 μM rotenone and 10 μM antimycin A, remaining relaxed in the presence of 25 kPa O2. In addition, the reducing agent and H2O2 scavenger NMPG (10, 20, and 30 mM) produced a significant relaxation of the EP proximal DA (P < 0.001, Fig. 5E).

There was a significant Rho kinase-mediated Ca2+-independent component to the O2-induced contraction of the DA from EP embryos. Fasudil and Y-27632, inhibitors of the Rho kinase pathway, produced significant relaxation of the O2-constricted chicken EP proximal DA. Inhibiting Rho-associated protein kinase, which diminishes MLCP activity, with Y-27632 produced a significant relaxation of the O2-induced contraction (Fig. 6). The O2-induced contraction in the proximal DA from EP embryos was completely abolished by Y-27632 with the tension stabilizing below the initial steady state. Fasudil, a Rho-associated kinase inhibitor, produced a similar significant relaxation of the O2-contracted proximal DA from EP animals (Fig. 6). As with Y-27632, fasudil produced a relaxation that stabilized below the initial steady-state tension.

Ductus vasoreactivity to prostaglandins. PGE2, PGF2α, and PGD2 produced contraction of the O2 precontracted chicken DA. Adding PGE2 to the acutely O2-constricted chicken DA from day 19 and IP animals did not produce relaxation. After the acute contractile response to O2 in the proximal DA, addition of PGE2 did not alter the DA tone at low concentrations (10^-8, 10^-7 M; Fig. 7, A and B). At higher concentrations, the proximal DA section of both day 19 (threshold = 10^-5 M) and IP (threshold = 10^-6 M) embryos contracted significantly in response to PGE2. The distal DA of both day 19 and IP animals showed no significant response to PGE2 (Fig. 7, A and B). The other prostaglandins tested, PGF2α (Fig. 7C) and PGD2 (Fig. 7D), produced a similar response with the only difference being that the PGD2-induced contraction began at a concentration of 10^-6 M in both the day 19 and IP proximal DA.

The proximal DA contraction in response to the highest concentration of PGE2 (10^-5 M) was unaffected by the level of O2 on day 19 of incubation (Fig. 8). In the proximal DA from IP animals, 10^-5 M PGE2 produced a significantly stronger contraction in the presence of 25 kPa O2 than in the presence of 4 kPa O2. PGF2α produced a significant contraction that was independent of both age and concentration of O2 (Fig. 8). The addition of 10^-5 M PGD2 produced a contraction that was age dependent only at 4 kPa O2 (Fig. 8).

Role of NO in the O2-induced response. The proximal and distal DA exhibited an endothelium-dependent relaxing re-

Fig. 4. Store-operated Ca2+ (SOC) channels and Inositol 1,4,5-trisphosphate (IP3)-sensitive internal Ca2+ stores play a role in the O2-induced DA contraction from EP embryos. A: representative tension tracing showing the response of the DA to 25 kPa O2 and 2-aminoethoxydiphenylborane (2-APB) in the absence of external Ca2+. B: response of the proximal DA to 25 kPa O2 and 25 kPa O2 + 2-APB both in the absence of external Ca2+. C: O2-induced contraction in the absence of external Ca2+; after the addition of external Ca2+, and after inhibition of Ca2+ entry through SOCs and the IP3-sensitive Ca2+ stores with 2-APB. The response of the DA is presented as a percentage of the initial O2-induced contraction calculated from the Ca2+-free plus 4 kPa O2 baseline. *Significant change from the baseline tension at P < 0.05. n = 6 to 8 vessels. ‡Significant reduction in tension compared with the initial O2-induced contraction at P < 0.05.
Fig. 5. The O$_2$-induced contraction of the proximal DA is redox sensitive and mediated by reactive oxygen species (ROS). A: representative recording of proximal DA tone after exposure to a Po$_2$ of 25 kPa followed by the reducing agent DTT (3 mM). B: change in tension in the O$_2$-contracted DA after the addition of DTT (3 mM).

C: representative recording of EP proximal DA tone after exposure to 25 kPa O$_2$ and followed by inhibition of the mitochondrial electron transport complex I by rotenone (10 mM). D: change in tension of EP vessels precontracted with O$_2$ following administration of rotenone (10 mM) or antimycin A (10 mM). n = 6 vessels. E: ductus tension after exposure to 25 kPa O$_2$ followed by 10, 20, and 30 mM N-mercaptopropionylglycine (NMPG; n = 5 vessels). *Significant difference between the O$_2$-induced contraction and the response to the specific drug at P < 0.05.
DISCUSSION

The chicken embryo is a valuable new model for studying the changes occurring in the DA during the transition to a neonate. During hatching, the two DA in the chicken must close to ensure proper blood flow to the lungs. For the first time, we show that the pathways regulating the O2-induced contraction previously described in the mammalian DA are also involved in regulating the O2-induced contraction of the chicken DA (Figs. 1–6, and 11). The O2-induced contraction is mediated by Ca2+ influx through L-type Ca2+ channels and SOCs and to a smaller extent by Ca2+ from IP3-sensitive Ca2+ stores (Fig. 11). Additionally, we show that exposure to the major regulator of mammalian DA patency, PGE2, produces a contraction of the ductus (Figs. 7 and 8). Our results indicate that the O2-induced relaxation of the distal DA is mediated by an endothelium–dependent NO/cGMP pathway.

Mechanisms involved in the initial phase of O2-induced contraction. The O2-induced contractile response of the proximal portion of the chicken DA matures with in ovo development (Fig. 1). On day 19 and during IP, the proximal DA responded to O2 with a weaker contraction than the DA from EP embryos. Upon EP, the animals began to breathe normoxic air and the contractile response of the proximal DA to O2 increased when compared with the day 19 and IP response. This is in contrast to the developmental pattern observed by Ågren et al. (1). In response to an increase in O2, the DA from day 19 embryos produced contractions of similar magnitude to those found on day 19 in our study. They failed to observe a further increase in contraction strength upon EP, which is most likely due to the fact that their day 21 (EP) vessels had been precontracted with norepinephrine (1). As in the chicken DA, a similar developmental maturation of the O2 response was observed in the emu DA (17), the guinea pig DA (30), and the rabbit DA (41).

The mechanisms mediating the initial O2-induced closure of the DA in mammals, following the initiation of lung ventilation, have been divided into three stages. The first stage is thought to involve changes in membrane potential and ion flux and to be mediated by the production of ROS by the mitochondria and inhibition of K+ channels (3). The second stage is a subacute response involving modulators, such as upregulation of the Rho-kinase pathway or endothelin-1 (3). The third stage involves tissue remodeling and the anatomical closure of the DA. Here we have focused on the mechanisms regulating the initial electrical stage of the O2-induced contraction in the chicken embryo DA. Our data suggests that the same mechanisms are involved in this initial contractile phase of the DA and are conserved in the mammalian and chicken DA (Fig. 11).

Our results suggest that the initial O2-induced contractile response of the proximal chicken DA is mediated at least in part by the production of mitochondrial ROS. Rotenone and antimycin A inhibit ROS production by electron transport chain complexes I and III. In response to both, the O2 contracted proximal DA relaxed (Fig. 5). A similar rotenone-induced relaxation was observed in the O2-constricted emu DA on days 48–49 of incubation (50-day incubation; 17). In both the rabbit and human DA, rotenone and antimycin A mimicked hypoxia and relaxed the O2 constricted DA (28). A number of studies have shown an increase in mitochondrial-derived ROS, specifically H2O2, in the mammalian DA in response to increased O2 (28, 34). Administration of catalase reversed the H2O2-induced contraction in the rabbit DA (34). Additionally, the reducing agent DTT and the reducing agent and ROS scavenger NMPG both relaxed the O2 contracted proximal DA (Fig. 5). These results are in agreement with the results found in fetal rabbit pups (34) where DTT and NMPG relaxed the normoxia-constricted DA. Thus, as with mammals, it appears that mitochondrial-derived ROS are important modulators of the O2-induced contraction in the avian DA through a redox-sensitive pathway.
In mammals, the ROS-induced contraction is thought to be mediated by redox-sensitive K_v channels (3, 27, 28). The inhibitor of K_v channels 4-AP produced contraction of both the proximal and distal section of day 19, IP, and EP DA from chicken embryos. Inhibition of the K_v channels with 4-AP (10 mM) produced a DA contraction from day 19 and EP embryos that was similar in magnitude to the O_2-induced contraction (Fig. 1). This is in agreement with previous experiments done in rabbits (44), humans (28), and emus (17). In the proximal section of the chicken DA from IP animals, however, we found that the contraction produced by 10 mM 4-AP was significantly greater than the O_2-induced contraction. These findings suggest that there is an age-dependent maturation of the chicken proximal DA with the potential upregulation of K_v channels during IP. During EP the response of the proximal DA to O_2 was once again similar to the response to 10 mM 4-AP. However, in the presence of both 4-AP and O_2 the proximal DA contraction is significantly greater than the contraction with either 4-AP or O_2 alone. Additionally, the distal segment of the DA contracted in response to 4-AP, suggesting that O_2-insensitive K_v channels are present in the avian DA. Other K^+ channels that could potentially influence vessel tone are the K_ATP and K_Ca channel. However, Ågren et al. (1) showed that the chicken DA did not respond to the K_ATP inhibitor glibenclamide or the K_Ca channel inhibitor tetraethylammonium, suggesting that neither of these K^+ channel types are involved in the chicken DA contraction. Similar findings have been shown in the human DA (27). Therefore, it appears that K_v channels might have a role in the O_2-induced contraction of the chicken DA (Fig. 11); however, their identities need to be further characterized.

In mammals, the ROS-induced contraction is thought to be mediated by redox-sensitive K_v channels (3, 27, 28). The inhibitor of K_v channels 4-AP produced contraction of both the proximal and distal section of day 19, IP, and EP DA from chicken embryos. Inhibition of the K_v channels with 4-AP (10 mM) produced a DA contraction from day 19 and EP embryos that was similar in magnitude to the O_2-induced contraction (Fig. 1). This is in agreement with previous experiments done in rabbits (44), humans (28), and emus (17). In the proximal section of the chicken DA from IP animals, however, we found that the contraction produced by 10 mM 4-AP was significantly greater than the O_2-induced contraction. These findings suggest that there is an age-dependent maturation of the chicken proximal DA with the potential upregulation of K_v channels during IP. During EP the response of the proximal DA to O_2 was once again similar to the response to 10 mM 4-AP. However, in the presence of both 4-AP and O_2 the proximal DA contraction is significantly greater than the contraction with either 4-AP or O_2 alone. Additionally, the distal segment of the DA contracted in response to 4-AP, suggesting that O_2-insensitive K_v channels are present in the avian DA. Other K^+ channels that could potentially influence vessel tone are the K_ATP and K_Ca channel. However, Ågren et al. (1) showed that the chicken DA did not respond to the K_ATP inhibitor glibenclamide or the K_Ca channel inhibitor tetraethylammonium, suggesting that neither of these K^+ channel types are involved in the chicken DA contraction. Similar findings have been shown in the human DA (27). Therefore, it appears that K_v channels might have a role in the O_2-induced contraction of the chicken DA (Fig. 11); however, their identities need to be further characterized.

As with the mammalian DA, the O_2-induced contraction of the chicken’s proximal DA is mediated in part by the influx of external Ca^{2+} through voltage-gated L-type Ca^{2+} channels. The L-type Ca^{2+} channel inhibitor nifedipine (10 μM) partially reversed the O_2-induced contraction in the proximal
The O$_2$-induced contraction of the chicken DA relies on the Rho pathway in a similar fashion. The inhibitors of Rho kinase pathway, Y-27632 and fasudil, exerted strong relaxation of the O$_2$-contracted DA from EP embryos. Kajimoto et al. (22) reported that Ca$^{2+}$ sensitization is important in human DA and rabbit DA, because after the O$_2$-induced contraction is initiated, the phosphorylation and subsequent deactivation of myosin phosphatase by Rho kinase maintains smooth muscle contraction, even if Ca$^{2+}$ availability decreases. In the rabbit DA, both Y-27632 and fasudil completely eliminated the O$_2$-induced contraction (19). When given prior to an increase in O$_2$, both Y-27632 and fasudil blocked the O$_2$-induced contraction. As with the human and rabbit DA (19, 22), it appears that the Rho pathway is important for DA contraction in the chicken DA (Fig. 11).

Vasoactivity of prostaglandins. It is well documented that a major contributor to maintaining patency of the mammalian DA is PGE$_2$ (38). In the lamb DA, PGE$_2$ added in vitro to precontracted rings of the vessel act as a potent vasodilator (10, 11, 14). Unlike the mammalian DA, it appears that PGE$_2$ does not play a similar role in maintaining chicken DA patency. Rather than relaxing the vessel, PGE$_2$ constricts it in an O$_2$-sensitive manner during IP. This contractile response to PGE$_2$ is opposite the response of the only other avian examined, the emu. The O$_2$-contracted emu DA relaxed upon exposure to exogenous PGE$_2$ with a threshold concentration of 10$^{-6}$ M (17). Agren et al. (1) found that the chicken DA do not respond to the COX inhibitor indomethacin, the COX-1 selective inhibitor valerylsaliclyc acid, and the COX-2 inhibitor nimesulide, suggesting that the chicken DA is not regulated by locally produced prostaglandins. Additionally, the proximal

![Graph](image-url)
DA from day 19 (threshold 10^{-5} M) and IP (threshold 10^{-6} M) chicken contracted when exposed to exogenous PGE_{2} (Fig. 7, A and B). These threshold levels are well above the mammalian levels of circulating PGE_{2} (9).

The mechanisms by which the chicken DA constricts in response to PGE_{2} remains to be clarified. The effects of PGE_{2} are dependent on the receptor subtypes present in the tissue. In adult porcine large cerebral arteries activation of EP1 and EP3 receptors by PGE_{2} caused constriction (20). This study also found that the prostanoid receptors EP1 and EP3, which activate the phosphatidyl-inositol pathway, are located in the smooth muscle layers of the arteries. Therefore, it may be that the chicken DA contains PGE_{2} receptors of the EP1 and EP3 subtype.

The other prostaglandins studied, F_{2\alpha} and D_{2}, also produced constriction of the proximal portion of the chicken DA (Fig. 7, C and D). PGF_{2\alpha} acts on a FP receptor that increases intracellular Ca^{2+} levels in the smooth muscle cell by stimulating a phospholipase C pathway, resulting in smooth muscle contraction in lamb and calf DA (32). However, in the mammalian DA, the physiological role of PGF_{2\alpha} in controlling DA tone is considered small, since contractions are only produced at high doses. Similar high PGF_{2\alpha} thresholds were observed in the proximal DA on day 19 (threshold 10^{-5} M) and IP (threshold 10^{-5} M). PGD_{2} acts on the DP receptor, which causes relaxation in smooth muscle cells (25). In the proximal DA, PGD_{2} produced contraction with a threshold concentration of 10^{-6} M (Fig. 7D). Similar affinities of PGD_{2} for the DP and FP receptors were found in the mouse ovary cell (23). Additionally, the bronchoconstriction produced by PGD_{2} in the anesthetized dog is mediated by the FP receptor (29). Therefore, it is possible that PGD_{2} is acting through the FP receptors present in the chicken DA.

These findings taken together suggest that prostaglandins play no discernible role in maintaining chicken DA patency when compared with the mammalian DA. A similar diminished role of PGE_{2} in maintaining DA patency has been suggested for the emu DA (17) and in one mammalian model,
the guinea pig DA (6). The major source of prostaglandins in the developing mammalian fetus is the placenta (43). The levels of circulating prostaglandins in the avian embryo are unknown, but are expected to be low. There is little tonic contraction of the vessel prior to days 16 or 17 of incubation (1), and NO does not appear to be important in maintaining patency (1). Therefore, patency may be maintained in the chicken DA by the internal blood pressure in conjunction with the weak ability of the vessel to contract (1).

**Proximal vs. distal sections.** The DA of the developing chicken embryo are composed of two different morphological phenotypes along their lengths (1, 4). The proximal DA descends from the pulmonary arteries and are composed of a well-defined muscular layer. In contrast, the distal DA continues from the proximal DA to connect with the aorta and contains more elastin and lacks a defined muscular layer (4). Normal morphological closure of the chicken DA during hatching occurs at the proximal end, while the distal portion remains patent after hatching (4, 18).

We show that the proximal and distal section of the chicken DA react differently to changes in O2. Oxygen induces contraction of the proximal DA through the same mechanisms found in the mammalian DA (Fig. 11). In contrast, the distal DA did not react to nifedipine, DTT, rotenone, antimycin A, Y-27632 (data not shown), or fasudil (data not shown), suggesting a significantly different cellular pathway involved in the response to O2, such as an endothelial-derived NO/cGMP pathway.

We found that the endothelium-dependent relaxing response of the chicken DA to ACh was O2-dependent. ACh had little effect on either the distal DA or proximal DA tension at 4 kPa O2 but produced significant relaxation in both when O2 was increased to 25 kPa. A similar O2-dependent relaxation in response to ACh was observed in the chicken DA using vessel segments that contained both the proximal and distal portions (2). Oxygen-dependent relaxation responses to ACh have been observed in the day 19 femoral artery (24), carotid artery (24), and pulmonary arteries of chicken (31, 35) and rabbit (21). As with the chicken DA, the fetal lamb DA was less sensitive to NO under hypoxic conditions (12). Under our higher O2 level (25 kPa), ACh produced a relaxation response that was similar to the relaxation of the pulmonary arteries (26). A similar interaction between NO and O2 has been observed in the fetal lamb DA (12).

While both the proximal and distal segments of the chicken DA exhibit endothelium-derived NO relaxation in response to ACh, the O2-induced relaxation of the distal segment is endothelium-derived. In response to increased O2, the distal portion responded with an endothelium-dependent relaxation that was blocked by the soluble guanylate cyclase inhibitor ODQ and the removal of the endothelium. In contrast, neither ODQ nor removal of the endothelium resulted in any change in the O2-induced contraction of the proximal DA. This suggests that endothelium-derived NO is actively regulating distal DA tone in response to an increase in O2. This O2-induced relaxation response of the distal portion of the ductus may be important for the changing hemodynamics occurring during the closure of the proximal portion of the DA.

ACh-mediated relaxation acts through the NO/cGMP pathway to increase intracellular levels of cGMP. Under the hypoxic conditions used in our study, it is possible that the endothelium was unable to produce NO, as O2 is a necessary substrate for NO production. Both the chicken and mammalian pulmonary arteries show a decrease in the pulmonary endothelium-dependent NO pathway under hypoxic conditions (21, 31, 36, 37, 47). This hypothesis is supported by the fact that sodium nitroprusside, which produces an NO endothelium-independent relaxation, was able to relax the chicken DA under hypoxic conditions (2). In the mouse DA in vivo, there is a decrease in the DA response to blocking NO production with L-NAME with gestation (33). This decreased response of the mouse DA in vivo to L-NAME correlates with decreases in blood Po2 levels. In the developing chicken embryo at this stage, the Po2 of oxygenated blood returning from the choioallantoic membrane has been measured between 4 and 6.5 kPa (33, 40). Our findings suggest that these blood gas levels are low enough in vivo to influence the production of NO by the endothelium prior to hatching. Upon initiation of lung ventilation during EP, blood gas tensions rise (40), which would stimulate a NO-mediated relaxation of the distal but not proximal DA.

**Perspectives and Significance**

Tone of the mammalian DA is regulated by a balance between the relaxing forces of prostaglandins and the constrictive effects of O2 (38). Based on our findings, we provide the proposed mechanism for the closure of the chicken DA in Fig. 11. An increase in O2 (Fig. 11, a) produces an acute response involving the mitochondria of smooth muscle cells acting as O2 sensors and producing ROS (Fig. 11, b). This increase in ROS inhibits K+ channels (Fig. 11, c) depolarizing the cell membrane (Fig. 11, d) and causing the opening of L-type Ca2+ channels, followed by opening of SOCs and IP3-sensitive Ca2+ channels in the SR (Fig. 11, e). Additionally, ROS may be directly influencing the L-type Ca2+ channels. Once the Ca2+ channels open, there is an increase in the intracellular concentration of Ca2+, which leads to contraction of the smooth muscle cells (44). As with the mammalian DA, the Ca2+- insensitive Rho kinase pathway increases the sensitivity of the chicken DA to Ca2+ (Fig. 11, i). The pathways regulating the O2-induced contraction in the mammalian DA and the chicken DA during birth/hatching appear to be highly conserved.

In contrast, we have shown that prostaglandins are unable to relax the chicken DA, and we suggest they play no discernible role in maintaining DA patency during in ovo development. It may be that the role of prostaglandins evolved with the appearance of the mammalian placenta, the major source of prostaglandins in the developing mammalian fetus (43). The avian embryo provides a model system in which the pathways governing the O2-induced contraction can be studied without the confounding influence of prostaglandins. Additionally, the chicken has the advantage of developing in ovo without direct inputs from the mother, allowing for greater manipulation of the developmental environment and providing a novel system for studying the development of the O2-sensitive DA (39).

**ACKNOWLEDGMENTS**

Three anonymous reviewers provided valuable suggestions during the revision of this manuscript.
REFERENCES


GRANTS

This study was supported by National Science Foundation Grant IOS0417205 and an American Heart Association Award (both to E. M. Dzialowski).


