Title: In vitro oxygen exposure promotes maturation of the oxygen sensitive contraction in pre-term chicken ductus arteriosus

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Running Head: Oxygen maturation of pre-term chicken ductus arteriosus

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Abstract

The ductus arteriosus (DA) are O₂-sensitive, embryonic blood vessels that serve as a right-to-left shunt in the developing avian embryo (Dzialowski et al., 2011). Prior to internal pipping, the chicken DA produces a weak O₂-induced contraction. During hatching, the O₂-sensitivity of the avian DA vessels increases significantly. To see if we could accelerate the maturation of chicken DA O₂-sensitivity, we exposed the vessel *in vitro* to elevated O₂ (25 kPa) for 3-hours prior to internal pipping on day 19 of incubation. The DA initially responded to increasing O₂ with a weak contraction (0.15 ± 0.04 N/m) that significantly increased in strength (0.63 ± 0.06 N/m) during 3-hour 25 kPa O₂ exposure. A tonic influence of nitric oxide, not present at low O₂, appeared during the 3-hour 25 kPa O₂ exposure. The long-term O₂-induced contraction was mediated by both L-type Ca²⁺ channels and internal Ca²⁺ stores. The Rho-kinase pathway inhibitors Y-27632 and fasudil produced significant relaxation, suggesting a role for Ca²⁺ sensitization in the contractile response to the three hours of elevated O₂. While the day 19 DA initially exhibited an immature contractile response to O₂, maturation of the pathways regulating O₂-induced contraction was accelerated by exposure to 25 kPa O₂, producing contractions similar in magnitude to those found during the final stage of hatching. This suggests that maturation of O₂-sensitivity may be accelerated *in vivo* by increasing arterial O₂ levels.

Key Words: ductus arteriosus, chicken, oxygen, rho kinase

1. Introduction

The ductus arteriosus (DA) is a cardiovascular shunt present during embryonic development in birds and mammals (Dzialowski et al., 2011). In birds, a pair of DA vessels divert right ventricular outflow through the pulmonary arteries away from fluid filled lungs and into the descending aorta. This shunt ensures adequate blood flow to the descending aorta and the embryonic gas exchanger, the chorioallanotic membrane (CAM). At hatching, the DA must close to ensure proper blood flow to the now ventilated lungs.

The mammalian and avian DA are O₂ sensitive tissues much like the pulmonary arteries and carotid body cells. Initially, avian embryonic DA are insensitive to changes in O₂ (Ågren et al., 2007). Beginning around day 18 or 19 of incubation in the chicken, O₂-sensitive pathways in the DA begin to mature and the vessel constricts weakly in response to increasing O₂ (Ågren et al., 2007; Belanger et al., 2008; Cogollundo et al., 2009). Hatching in the chicken begins on day 20 of incubation when the embryo internally pips (IP) the air cell and begins to breathe hypoxic gas with its lungs while still relying heavily on the CAM for gas exchange. This stage of hatching is followed by external pipping (EP) where the embryo breaks the eggshell with its beak and becomes more reliant on lungs for O₂. As hatching progresses to the EP stage, the DA contractile response to O₂ increases significantly (Belanger et al., 2008; Copeland and Dzialowski, 2008) and the vessel begins to constrict (Belanger et al., 2008). This developmental responsive pattern is similar to mammalian species with increases in DA O₂-sensitivity occurring at the end of gestation and at birth in mammals (Coceani et al., 1978, 1979; Kajino et al., 2001).

The increase in O₂-sensitivity of the chicken DA correlates with increases in arterial Po₂ as the bird transitions from the embryonic gas exchanger, the CAM, to the lungs during hatching (Belanger et al., 2008). During later stages of incubation, including the IP stage, the embryo is in a hypoxic state with low arterial and venous Po₂ due to O₂ diffusion limitations of the porous eggshell. Tazawa et al. (1983) found that both arterial and venous Po₂ rise significantly during hatching. The greatest rise in arterial Po₂ occurs during the EP stage of hatching when the embryo begins to respire normoxic air. This increase in blood Po₂ correlates with maturation of O₂-sensitivity of the DA during EP (Belanger et al., 2008). Additionally, we showed that chronic hypoxic (15% O₂) or hyperoxic incubation (30% O₂) of chicken embryos influenced the developmental trajectory of DA O₂-sensitivity (Copeland and Dzialowski, 2009). In contrast, van

der Sterren et al. (2014) found that hyperoxic incubation (60% O₂) during the last few days of incubation had no effect on the O₂ induced contraction of the embryonic day 19 DA.

Between embryonic day 19 (D19) and hatching the DA becomes more sensitive to O₂, producing a greater contraction in response to elevated O₂. The mechanism driving this increase in O₂-sensitivity of the chicken DA during hatching is not well understood. Oxygen levels during incubation may influence the maturation of the O₂-sensitive constriction pathway in the avian DA. Oxygen-sensitivity maturation could be stimulated by increased blood gas Po₂ associated with hatching. Hypoxic incubation delayed the appearance of O₂-sensitivity of the DA, while hyperoxic incubation resulted in earlier maturation of O₂-sensitivity during IP (Copeland and Dzialowski, 2009). We hypothesized that exposing D19 chicken DA to elevated O₂ would accelerate maturation of the O₂-sensitive contractile pathways, leading to increased O₂-stimulated contraction of the DA. To test this hypothesis, we characterized the *in vitro* contractile response of the D19 chicken DA to a three-hour exposure of elevated O₂.

2. Methods

2.1 Incubation

White leghorn chicken eggs were purchased from the Texas A&M University Poultry Science Center and incubated at 37.5 °C with a relative humidity of 70%. Eggs were automatically turned every 4 hours.

2.2 Isometric tension in vitro

The proximal and distal portion of the left DA from day 19 chicken embryos was excised and placed in a physiological saline solution (PSS composed of 120.5 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.6 mM CaCl₂, 1.2 mM NaH₂PO₄, 20.4 mM NaHCO₃, and 10 mM glucose) equilibrated with 95% N₂ and 5% CO₂. The proximal and distal portions of the DA were separated based on visual inspection of vessel morphology and diameter. Isometric tension generated by the DA was measured *in vitro* using a 4 chamber 610M Danish Myo Technologies myograph. Vessel rings 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 and 5% CO₂ gas mixture resulting in a Po₂ of 4 kPa (4% O₂) and a Pco₂ of 5.3 kPa (5% CO₂).

Bath P_{O_2} and P_{CO_2} were monitored with a Radiometer ABL5 blood gas meter. Isometric force was recorded by Chart data acquisition software and a Powerlab 8SP (ADInstruments) connected to the 610M DMT myograph.

2.3 Oxygen-mediated contraction.

The vessel tension was set to approximately 0.5 N/m (Greyner and Dzialowski, 2008) and allowed to equilibrate at 4% O_2 for 30 minutes prior to conducting any experiments. After the equilibration period, vessels were exposed to 25% O_2 , 5% CO_2 , and balance N_2 for 5 to 10 minutes to test for the initial acute response to increases in O_2 . This was followed by a 3 hour exposure to 25% O_2 , during which vessels were washed with fresh PSS every 15 minutes during the first hour and then every 30 minutes. After exposure for 3 hours, O_2 was decreased to 4% for 5 minutes and returned to 25% to see if baseline tension had changed and determine how the vessel would respond to O_2 . All protocols listed below were run on vessels after a continual 3-hour exposure O_2 (see Fig 1A for representative trace). In certain cases, to determine the influence of prostaglandins and nitric oxide during the 3-hour exposure to 25% O_2 the prostaglandin inhibitor indomethacin (5.6 μM) and/or the nitric oxide inhibitor L-NAME (0.1 mM) were added to the PSS.

To test for the role of voltage gated K_v channels, the proximal DA from day 19 embryos were exposed to the K^+ -channel (K_v) blocker, 4-aminopyridine (10 mM, 4-AP). In these experiments, 4-AP was added after the vessels were contract during the 3-hour O_2 exposure and then allowed to relax in response to the return to 4% oxygen. Three hour O_2 pre-contracted proximal DA were also exposed to the L-type Ca^{2+} channel blocker nifedipine (10 μM). After the addition of nifedipine, the PSS was substituted by a modified 0 mM Ca^{2+} PSS to study effects of external Ca^{2+} on O_2 induced constriction DA.

To explore the role of reactive oxygen species in stimulating contraction, the proximal DA was exposed to rotenone (10 μM), an inhibitor of electron transport complex I. The role of calcium sensitization on the chicken DA was examined by exposing 3-hour O_2 conditioned vessels to step-wise increases in Y-27632 (10^{-8} to 10^{-5} M), a selective inhibitor of the Rho-associated protein kinase p160ROCK, and fasudil (10^{-8} to 10^{-4} M), a cyclic nucleotide-dependent protein kinase inhibitor and Rho-associated kinase inhibitor.

2.4 Vasoreactivity to prostaglandin

To examine the responsiveness of the DA to prostaglandin E₂, cumulative dose-response curves were constructed for prostaglandin E₂ (10^{-10} to 10^{-5} M; PGE₂) after 3-hour exposure. After the addition of each concentration, the vessel was allowed to stabilize before the next concentration was added.

2.5 Drugs

Prostaglandin E₂ was purchased from Cayman Chemical and dissolved in ethanol. Preliminary experiments showed that the vehicle (ethanol) had no effect on vessel tension when used by itself. Indomethacin, L-NAME, rotenone, and nifedipine were purchased from Sigma-Aldrich. Fasudil, 4-aminopyridine, and Y-27632 were purchased from Tocris Bioscience.

2.6 Statistical analysis

Comparisons were made using repeated measures ANOVA followed by Tukey's post hoc tests. All data are presented as the mean ($\pm$ SE) net active tension generated above the baseline tension and the level of significance for all tests was $P < 0.05$. All statistics were carried out with SigmaPlot 10.5.

3. Results

3.1 Contraction in response to 3 hour O₂ exposure

Three hour exposure to 25% O₂ produced a significant increase in tension of the proximal section of D19 DA (see Fig. 1A for representative trace). Initially, O₂ produced a weak proximal DA contraction and distal DA relaxation (Fig. 1A). Although the O₂ induced contraction in the proximal portion of the DA was weak, it was typically significantly greater than baseline tension (Fig 1B; $p < 0.05$). The subsequent 3-hour exposure to 25% O₂ resulted in a gradual increase in proximal DA tension which was 4 times greater than the O₂ stimulated contraction that occurred within the first 10 min of increasing O₂. After 3-hour 25% O₂ exposure, returning O₂ to 4% produced a relaxation of the proximal DA to a new, higher baseline tension. At this point, increasing O₂ concentration to 25% produced a rapid increase in tension. There was little change in tension of the distal portion of the DA in response to 3-hour 25% O₂ exposure (Fig. 1A), therefore the rest of the study focuses only on proximal DA response.

3.2 Role of prostaglandin E_2 and nitric oxide

Endogenous nitric oxide influenced the tension of the DA during 3-hour 25% O_2 exposure; however blocking prostaglandins had no effect (Fig. 1B). In response to the initial increase in O_2 , neither the prostaglandin antagonist indomethacin, nor the NO antagonist L-NAME, influenced vessel tension. During 3-hour exposure, indomethacin did not alter DA tension when compared to control. In the presence of L-NAME and 3-hour 25% O_2 exposure, DA tension was significantly greater than control tension ($p < 0.05$).

Prostaglandin E_2 produced a weak relaxation in the DA after 3-hour O_2 exposure (Fig. 2). Relaxation was observed with the addition of 10^{-8} M PGE_2 ; however, only the 10^{-6} M dose produced a significant relaxation during the 3-hour O_2 -induced contraction ($p < 0.05$). The DA produced a significant contraction in response to 10^{-5} M PGE_2 .

3.3 Oxygen Mechanisms

The 3-hour O_2 -induced contraction of the DA was not mimicked by the K_v channel antagonist 4-AP (Fig. 3). After 3-hour exposure, the 4-AP produced a weak contraction at 4% O_2 that was not significantly different from the initial O_2 induced contraction or the new baseline tension. Returning to 25% O_2 after addition of 4-AP resulted in a significant increase in tension above the 3-hour O_2 -induced contraction.

L-type Ca^{2+} channel blocker nifedipine produced relaxation of the D19 O_2 contracted proximal DA (Fig. 4). After the 3-hour 25% O_2 exposure, addition of nifedipine resulted in a significant DA relaxation ($p < 0.05$). Removal of Ca^{2+} from the PSS resulted in greater vessel relaxation than simply blocking L-type Ca^{2+} channels. At this point, additional nifedipine produced no change in vessel tension; however, returning O_2 to the original 4% resulted in a decrease in tension to a point indistinguishable from the new baseline (Fig. 4).

Rotenone, an inhibitor of electron transport complex I and ROS production produced a significant relaxation of the 3-hour O_2 -contracted DA (Fig. 5).

There was a significant Rho kinase-mediated, Ca^{2+} independent component to the 3-hour 25% O_2 exposure response. Rho kinase inhibitors Y-27632 and fasudil produced significant relaxation of the 3-hour O_2 -induced contracted DA (Fig. 6A and B, respectively). Both inhibitors produced relaxation beginning with a dose of 10^{-6} M. With addition of 10^{-4} M, the tension decreased to a level significantly below the new baseline.

4. Discussion

On D19, when the DA is relatively insensitive to O₂, a 3-hour exposure to elevated O₂ levels enhanced the O₂-induced contraction of the DA to levels similar to those previously observed during the EP stage of hatching (Belanger et al., 2008, Fig 1B). These findings suggest that increased blood Po₂, which rises during hatching in birds and birth in mammals, stimulates the maturation of cellular pathways involved in the O₂-induced contractile response of the DA.

4.1 Contraction in response to long-term exposure

The proximal portion of day 19 chicken embryo DA responded to increased O₂ in a manner dependent upon the length of exposure (Fig. 1A). Immediately after increasing O₂, a weak but significant contraction occurred. This contraction was similar in strength to the contractions found by others at this time of development and is lower than the O₂ induced contractions of the DA from EP embryos (Belanger et al., 2008; Greyner and Dzialowski, 2008; van der Sterren et al., 2014). This weak contraction was followed by a steady increase in DA tension that began within one hour of exposure to 25% O₂ and continued for approximately two hours. After 3-hour exposure, O₂-induced tension of the D19 DA was similar to that seen in the DA from an EP stage embryo (Belanger et al., 2008).

The data suggest oxygen exposure can influence maturation of the O₂-sensitivity of the chicken DA. We were able to accelerate sensitivity by exposing the immature DA to elevated O₂ for 3 hours. Longer manipulation of O₂ levels during incubation have been shown to alter the timing of O₂-sensitivity (Copeland and Dzialowski, 2009). Incubation in hyperoxia (30% O₂) resulted in a strong DA response to O₂ earlier in the hatching process (IP stage) than control. Hypoxic incubation (15% O₂) had the opposite effect on the O₂-sensitivity of the chicken DA during hatching. In contrast, exposure to 60% O₂ from embryonic days 15 to 19 had no effect on the DA response to O₂ (van der Sterren et al., 2014).

During normal development and hatching, O₂-sensitivity of the chicken DA increases from day 19 through EP. During this period, arterial Po₂ levels increase (Tazawa et al., 1983) as the animal begins to rely more on its lungs as it transitions away from CAM respiration. A strong correlation exists between the increase in arterial Po₂ and the O₂-induced tension of the DA (Belanger et al., 2008). Given this natural correlation and our ability to accelerate the O₂-

sensitivity by exposure to increased O₂, our data suggest that O₂ exposure may play a strong role in stimulating the O₂-sensitivity maturation.

4.2 Role of prostaglandin E₂ and nitric oxide

Prostaglandin E₂ (PGE₂) had no *in vitro* contribution in maintaining tone of the proximal DA during either short or long term O₂ exposure. Exposing the proximal DA from D19 embryos to the COX inhibitor indomethacin for a three-hour period resulted in no change in vessel tension compared with control, regardless of O₂ concentration (Fig. 1B). Ågren et al. (2007) found no response in the chicken DA when exposed to the COX-1 and COX-2 inhibitor indomethacin, the COX-1 inhibitor valeryl salicylate, or the COX-2 inhibitor nimesulide. The emu DA was also insensitive to indomethacin (Dzialowski and Greyner, 2008). In contrast, the DA of many mammals constricts in the presence of indomethacin (Smith, 1998; Takahashi et al., 2000; Kajino et al., 2001).

In the 3-hour O₂ pre-contracted D19 DA, PGE₂ produced a weak relaxation followed by contraction at the highest concentration (Fig. 2). PGE₂ relaxed the 3-hour O₂ contracted DA at the 10⁻⁷ and 10⁻⁶ M PGE₂ concentrations (Fig. 2). Increasing concentration to 10⁻⁵ M PGE₂ resulted in a contraction of the DA. This is counter to what has been observed with the D19 DA pre-contracted with short term O₂ or high KCl (Ågren et al., 2008; Greyner and Dzialowski, 2008). In these studies, the D19 DA was found to only contract at the highest doses of PGE₂ tested. The emu DA and the DA from less mature day 15 chicken embryos both relaxed in response to PGE₂ (Dzialowski and Greyner, 2008; Ågren et al., 2009). The mammalian DA has a much stronger relaxation response to PGE₂ when compared with the chicken DA (Clyman et al., 1978, 1980a, 1980b, 1999; Bouayad et al., 2001, 2002; Kajino et al., 2001; Waleh et al., 2004). These results suggest that regardless of O₂ availability, the role of prostaglandins in maintaining ductal patency is less important in the chicken embryo than in the mammalian DA.

On the other hand, nitric oxide (NO) may have a larger role in keeping the DA from closing in an oxygen dependent manner during the 3-hour exposure (Fig. 1B). Adding L-NAME during the 3-hour exposure to 25% O₂ resulted in a contraction significantly higher than control (p<0.05). A similar O₂ dependent response to L-NAME was also found in the fetal sheep DA (Clyman et al., 1998). These findings suggest that in the chicken DA, increases in O₂ are capable of signaling the release of NO, most probably from the endothelial cells surrounding the lumen

of the DA (Ågren et al., 2008). NO may play a role in countering the O₂-induced contraction which occurs after the initiation of lung ventilation during the IP stage. Because of the timing of hatching, birds take longer to transition from embryonic CAM respiration to sole reliance on the lungs than mammals. Thus, NO mediated relaxation during 3-hour O₂ exposure may ensure the DA does not close too rapidly. Alternatively, the NO mediated relaxation may be an oxidative stress response to elevated *in vitro* Po₂.

4.3 Constricting pathways

Changes in reactive oxygen species (ROS) produced by the mitochondria inhibiting K_v channels resulting in membrane depolarization have been proposed as one of the mediators of O₂ induced contraction of the mammalian and avian DA (Reeve et al., 2001; Michelakis et al., 2002; Archer et al., 2004; Greyner and Dzialowski, 2008). The electron transport chain complex I inhibitor rotenone relaxed the 3-hour O₂ contracted day 19 proximal DA (Fig. 5), presumably inhibiting the production of ROS through complex I. These results support the conclusions of Michelakis et al. (2002) who reported the addition of rotenone or antimycin A, blockers of the electron transport chain complex I and III respectively, caused relaxation of the O₂ constricted human DA. A similar response was seen in DA from late stage emu embryos (Dzialowski and Greyner, 2008).

However, when looking directly at the role of K_v channels in the 3-hour O₂-induced contraction its role is not as clear. In mammals such as rabbits (Tristani-Firouzi et al., 1996) and birds such as emus (Dzialowski and Greyner, 2008), the K_v channel inhibitor 4-AP mimics the O₂ acute contraction. 4-AP produces a contraction of similar magnitude to short-term exposure to 25% O₂ in both the chicken and emu DA (Dzialowski and Greyner, 2008). This has caused some to suggest that inhibition of K_v channels may be important for the acute O₂ induced contraction. In contrast, when the D19 DA was exposed to 4-AP under low O₂ there was little contraction followed by significant contraction after being exposed to 3-hour 25% O₂ (Fig. 3). While the vessel relaxes in response to blocking ROS production, the connection with K_v channels may be diminished as the DA becomes more sensitized to O₂.

Calcium for the 3-hour O₂-induced contraction comes from both external and internal sources. Blocking of the L-type Ca²⁺ channels with nifedipine relaxed the 3-hour O₂-induced contracted chicken DA (Fig. 4). Nifedipine exerted less influence during 3-hour exposure

compared with the response of the EP DA to short term O₂ exposure (Greyner and Dzialowski, 2008). L-type Ca²⁺ channels have been shown to play a role in the human DA constriction (Michelakis et al., 2000). There also appears to be a large contribution of internal Ca²⁺ stores on the 3-hour O₂-induced contraction (Fig. 4). Upon removal of external Ca²⁺, DA tension was still close to 50% of the O₂-induced tension. In the DA from EP animals, we previously reported that blocking with nifedipine and removal of external Ca²⁺ abolished most of the O₂-induced contraction (Greyner and Dzialowski, 2008). This finding suggests that during 3-hour O₂ induced contraction another mechanism or mechanisms may be facilitating availability of internal Ca²⁺ or increasing the Ca²⁺ sensitivity for sustained DA contraction. This response seen in the D19 chicken embryo is in agreement with Clyman, et al. (2007), where 50% of the mature lamb DA maximal contraction is due to cellular entry of Ca²⁺ through L-type Ca²⁺ and store operated Ca²⁺ channels.

The increased contraction in response to 3-hour O₂ exposure may be due to increased sensitization of the smooth muscle cells to Ca²⁺. The Rho kinase pathway inhibits the action of myosin light chain phosphatase (MLCP), maintaining smooth muscle contraction. The Rho kinase inhibitors Y-27632 and fasudil completely abolished the 3-hour O₂ induced contraction (Fig. 6). The relaxations produced by Y-27632 and fasudil in this experiment are similar to those obtained from chicken DA (Greyner and Dzialowski, 2008). Rho kinase mediated calcium sensitization and inhibition of MLCP could in part explain why even after removal of the Ca²⁺ from the bath solution there was some persistent contraction. Kajimoto et al. (2007) explained that inhibition of MLCP could reduce the requirement of Ca²⁺ in the smooth muscle cells to produce and sustain contraction. The Rho kinase pathway, however, has a larger role than simply reducing the amount of Ca²⁺ needed in the smooth muscle cell. The balance between MLCP and myosin light chain kinase (MLCK) is an integral part of DA tone and decreasing MLCP activity may tip the balance towards MLCK. This relationship is evident as seen by the addition of Y-27632 and fasudil relaxing the DA.

4.3 Conclusions

The embryonic chicken embryo DA is an O₂-sensitive tissue that must constrict and close upon hatching. With the progression of hatching, the O₂ contractile sensitivity matures from a weak O₂ stimulated contraction on day 19 to a stronger O₂ stimulated contraction during the EP

stage (Belanger et al., 2008). We were able to accelerate maturation of this O₂ contractile sensitivity by exposing the DA to elevated levels of O₂ on day 19 of incubation. While not fully mature at day 19, the pathways responsible for O₂ induced contraction are able to be upregulated by exposure to O₂ and may involve sensitization to Ca²⁺ through the Rho-kinase pathway and alterations in Ca²⁺ handling. Arterial O₂ levels increasing during hatching are most likely the signal by which the O₂-sensitive pathways are stimulated to mature.

Acknowledgements

Sarah Goy Sirsat and two anonymous reviewers provided valuable comments on the manuscript. This study was supported by National Science Foundation Grant No. IOS041720521 awarded to EMD.

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Figure Legends

Figure 1. A) Representative recording of *in vitro* isometric tension from the proximal and distal portions of the left chicken DA during 3-hour exposure to 25% O₂. The bars below the trace indicated the periods of 25% O₂ exposure. The spikes in the proximal DA trace are due to replacement of fresh PSS. B) The bar graph shows O₂-induced tensions in response to acute O₂ changes in day 19, IP, and EP embryos taken from Belanger et al., 2008. C) Nitric oxide is a stronger vasodilator of the pre-term chicken proximal DA than prostaglandins under 3-hour O₂ exposure. Active tension measured from the chicken proximal DA in response to the initial increase in O₂ (25% O₂), after 3-hour exposure to 25% O₂ (3-hr 25% O₂), return back to 4% O₂ (4% O₂), and then back to 25% O₂ (25% O₂). Vessels were either untreated (Control) or exposed to indomethacin (Indo; 5.6 μ M) or L-NAME (L-NAME; 0.1 mM). N = 4-6 vessels. + indicates the only active tension that is not a significant change from the initial baseline tension. Values with different letters are significantly different at $p < 0.05$.

Figure 2. Proximal section of the chicken DA relaxes in response to PGE₂ only after 3-hour O₂ exposure. A) Representative trace showing the response of *in vitro* isometric tension after exposure to three hours of 25% O₂ to increasing levels of PGE₂. The bars below the trace indicated the periods of 25% O₂ exposure. B) Response of the proximal DA to stepwise increases in PGE₂ (n = 5). * = Indicates a significant change in active tension compared with the tension at PGE₂ 10⁻¹⁰ M at $p < 0.05$.

Figure 3. The K_v channel antagonist 4-AP failed to produce a significant contraction in the chicken proximal DA. Contractile response of the pre-term chicken proximal DA to 3-hour O₂ exposure followed by addition of 10 mM 4-AP (n = 6). Values with different letters are significantly different at $p < 0.05$.

Figure 4. Blocking L-type Ca²⁺ channels with nifedipine relaxes the proximal chicken DA. A) Representative trace showing the response of *in vitro* isometric tension following exposure to three hours of 25% O₂ to alterations in Ca²⁺ availability. B) Relaxing response as a result of adding the L-type Ca²⁺ channel blocker, nifedipine (10 μ M), and removal of Ca²⁺ from PSS in 3-hour O₂ constricted day 19 proximal DA (n = 8). Treatments with different letters are significantly different from each other at $p < 0.05$.

Figure 5. Electron transport chain inhibitor rotenone produced relaxation of 3-hour O₂ contracted day 19 proximal DA. Relaxing response of rotenone (10 µM) in 3-hour O₂ constricted DA (n = 8). Treatments with different letters are significantly different at p<0.05.

Figure 6. Inhibitors of Rho kinase (Y-27632 and fasudil) produced significant relaxation of 3-hour O₂ constricted proximal DA from day 19 embryos. A) Representative trace showing the response of *in vitro* isometric tension after exposure to three hours of 25% O₂ to increasing concentrations of fasudil. The bars below the trace indicated the periods of 25% O₂ exposure. B) Relaxing response after the initial increase in O₂, 3-hour O₂ exposure and then return to 4% O₂. C) Relaxing response to stepwise addition of Y-27632 (n = 6) or fasudil (n = 7) in 3-hour O₂ constricted proximal DA of day 19 embryos. * Indicates a significant change in active tension in response to the concentration at p<0.05 for both treatments.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6