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

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ABSTRACT

The ductus arteriosus (DA) are O_2-sensitive, embryonic blood vessels that serve as a right-to-left shunt in developing avian embryos. Prior to internal pipping, the chicken DA produces a weak O_2-induced contraction. During hatching, the O_2-sensitivity of the avian DA vessels increases significantly. To see if we could accelerate the maturation of chicken DA O_2-sensitivity, we exposed the vessel *in vitro* to elevated O_2 (25 kPa) for 3-h prior to internal pipping on day 19 of incubation. The DA initially responded to increasing O_2 with a weak contraction (0.15 ± 0.04 N/m) that significantly increased in strength (0.63 ± 0.06 N/m) during 3-h 25 kPa O_2 exposure. A tonic influence of nitric oxide, not present at low O_2, appeared during the 3-h 25 kPa O_2 exposure. The long-term O_2-induced contraction was mediated by both L-type Ca^{2+} channels and internal Ca^{2+} stores. The Rho-kinase pathway inhibitors Y-27632 and fasudil produced significant relaxation, suggesting a role for Ca^{2+} sensitization in the contractile response to the 3 h of elevated O_2. While the day 19 DA initially exhibited an immature contractile response to O_2, maturation of the pathways regulating O_2-induced contraction was accelerated by exposure to 25 kPa O_2, producing contractions similar in magnitude to those found during the final stage of hatching. This suggests that maturation of O_2-sensitivity may be accelerated *in vivo* by increasing arterial O_2 levels.

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 O_2 sensitive tissues much like the pulmonary arteries and carotid body cells. Initially, avian embryonic DA are insensitive to changes in O_2 (Ågren et al., 2007). Beginning around day 18 or 19 of incubation in the chicken, O_2-sensitive pathways in the DA begin to mature and the vessel constricts weakly in response to increasing O_2 (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 O_2. As hatching progresses to the EP stage, the DA contractile response to O_2 increases significantly (Belanger et al., 2008; Copeland and Dzialowski, 2009) and the vessel begins to constrict (Belanger et al., 2008). This developmental responsive pattern is similar to mammalian species with increases in DA O_2-sensitivity occurring at the end of gestation and at birth in mammals (Coceani et al., 1978, 1979; Kajino et al., 2001).

The increase in O_2-sensitivity of the chicken DA correlates with increases in arterial Po_2 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 Po_2 due to O_2 diffusion limitations of the porous eggshell. Tazawa et al. (1983) found that both arterial and venous Po_2 rise significantly during hatching. The greatest rise in arterial Po_2 occurs during the EP stage of hatching when the embryo begins to respire normoxic air. This increase in blood Po_2 correlates with maturation of O_2-sensitivity of the DA during EP (Belanger et al., 2008). Additionally, we showed that chronic hypoxic (15% O_2) or hyperoxic incubation (30% O_2) of chicken embryos influenced the developmental trajectory of DA O_2-sensitivity (Copeland and Dzialowski, 2009). In contrast, van der Sterren et al. (2014) found that hyperoxic incubation (60% O_2) during the last few days of incubation had no effect on the O_2 induced contraction of the embryonic day 19 DA.

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Between embryonic day 19 (D19) and hatching the DA becomes more sensitive to O_2, producing a greater contraction in response to elevated O_2. The mechanism driving this increase in O_2-sensitivity of the chicken DA during hatching is not well understood. Oxygen levels during incubation may influence the maturation of the O_2-sensitive constriction pathway in the avian DA. Oxygen-sensitivity maturation could be stimulated by increased blood gas Po_2 associated with hatching. Hypoxic incubation delayed the appearance of O_2-sensitivity of the DA, while hyperoxic incubation resulted in earlier maturation of O_2-sensitivity during IP (Copeland and Dzialowski, 2009). We hypothesized that exposing D19 chicken DA to elevated O_2 would accelerate maturation of the O_2-sensitive contractile pathways, leading to increased O_2-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 O_2.

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 h.

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 $MgSO_4$, 1.6 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 20.4 mM $NaHCO_3$, and 10 mM glucose) equilibrated with 95% N_2 and 5% CO_2. 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% N_2, and 5% CO_2 gas mixture resulting in a Po_2 of 4 kPa (4% O_2) and a Pco_2 of 5.3 kPa (5% CO_2). Bath Po_2 and Pco_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 min 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 min to test for the initial acute response to increases in O_2. This was followed by a 3 h exposure to 25% O_2, during which vessels were washed with fresh PSS every 15 min during the first hour and then every 30 min. After exposure for 3 h, O_2 was decreased to 4% for 5 min and returned to 25% to see if baseline tension had changed and determine how the vessel would to respond to O_2. All protocols listed below were run on vessels after a continual 3-h exposure to O_2 (see Fig. 1A for representative trace). In certain cases, to determine the influence of prostaglandins and nitric oxide during the 3-h 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 contracted during the 3-h 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.

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-h 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 E_2, cumulative dose-response curves were constructed for prostaglandin E_2 (10^{-10} to 10^{-5} M; PGE_2) after 3-h exposure. After the addition of each concentration, the vessel was allowed to stabilize before the next concentration was added.

2.5. Drugs

Prostaglandin E_2 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 (± 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 h O_2 exposure

Three hour exposure to 25% O_2 produced a significant increase in tension of the proximal section of D19 DA (see Fig. 1A for representative trace). Initially, O_2 produced a weak proximal DA contraction and distal DA relaxation (Fig. 1A). Although the O_2 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-h exposure to 25% O_2 resulted in a gradual increase in proximal DA tension which was 4 times greater than the O_2 stimulated contraction that occurred within the first 10 min of increasing O_2. After 3-h 25% O_2 exposure, returning O_2 to 4% produced a relaxation of the proximal DA to a new, higher baseline tension. At this point, increasing O_2 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-h 25% O_2 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-h 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-h exposure, indomethacin did not alter DA tension when compared to control. In the presence of L-NAME and