Comparative Biochemistry and Physiology, Part A 191 (2016) 202–208

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Circulatory changes associated with the closure of the ductus arteriosus in hatching emu (*Dromaius novaehollandiae*)

Lauren Shell [a], Warren Burggren [a], David Muirhead [b], Thomas C. Nelson [c,1], Edward M. Dzialowski [a,*]

[a] Developmental Integrative Biology Research Group, Department of Biological Sciences, 1155 Union Circle #305220, University of North Texas, Denton, TX 76203, United States
[b] City of Hope, 1500 East Duarte Road, Duarte, CA 91010, United States
[c] Department of Biological Sciences, Kalamazoo College, Kalamazoo, MI 49008, United States

ARTICLE INFO

Article history:
Received 2 September 2015
Received in revised form 30 October 2015
Accepted 3 November 2015
Available online 5 November 2015

Keywords:
Ductus arteriosus
Hatching
Blood shunt
Vascular remodeling
Apoptosis
Vasoconstriction

ABSTRACT

In developing avian embryos, the right and left ductus arteriosi (DA) allow for a shunt of systemic venous return away from the lungs to the body and chorioallantoic membrane (CAM). Unlike in mammals where the transition from placental respiration to lung respiration is instantaneous, in birds the transition from embryonic CAM respiration to lung respiration can take over 24 h. To understand the physiological consequences of this long transition we examined circulatory changes and DA morphological changes during hatching in the emu (*Dromaius novaehollandiae*), a primitive ratite bird. By tracking microspheres injected into a CAM vein, we observed no change in DA blood flow between the pre-pipped to internally pipped stages. Two hours after external pipping, however, a significant decrease in DA blood flow occurred, evident from a decreased systemic blood flow and subsequent increased lung blood flow. Upon hatching, the right-to-left shunt disappeared. These physiological changes in DA blood flow correspond with a large decrease in DA lumen diameter from the pre-pipped stages to Day 1 hatchlings. Upon hatching, the right-to-left shunt disappeared and at the same time apoptosis of smooth muscle cells began remodeling the DA for permanent closure. After the initial smooth muscle contraction, the lumen disappeared as intimal cushioning formed, the internal elastic lamina degenerated, and numerous cells underwent regulated apoptosis. The DA closed rapidly between the initiation of external pipping and hatching, resulting in circulatory patterns similar to the adult. This response is most likely produced by increased DA constriction in response to increased arterial oxygen levels and the initiation of vessel remodeling.

1. Introduction

In developing mammals and birds, embryonic gas exchange occurs by means of the placenta or chorioallantoic membrane (CAM), respectively. Blood bypasses the lungs via an embryonic vascular shunt known as the ductus arteriosus (DA). The DA is derived from the sixth aortic arch and shunts blood away from the pulmonary artery and into the systemic pathway (Slomp et al., 1992; Bergwerff et al., 1999; Dzialowski et al., 2011; Levin et al., 2005; Belanger et al., 2008; Greyner and Dzialowski, 2008). In birds such as the chicken, the DA consists of two discrete sections. The proximal section lies close to the pulmonary artery and is composed mostly of smooth muscle cells. The distal section connects the vessel directly to the aorta and has more elastin and less smooth muscle than the proximal portion of the DA (Belanger et al., 2008; Greyner and Dzialowski, 2008). In the chicken embryo, 16% of the cardiac output from the right ventricle flows to the lungs, likely to provide nourishment for pulmonary development, while the rest passes through the ductus (Rahn et al., 1985).

The embryo uses the DA throughout its development in utero/in ovo and it must close with birth or hatching. As birth/hatching occurs, the embryonic gas exchanger is superseded by pulmonary respiration, and blood must flow through the lungs as the animal begins to breathe atmospheric air. This transition period is accomplished by the constriction of the DA and subsequent remodeling of the vessel walls (Rabinovitch, 1996; Belanger et al., 2008; Yokoyama et al., 2010; Yokoyama, 2015). The developmental period over which the ductus closes varies interspecifically. For mammals, DA closure occurs over several minutes to a few hours, since the switch from placenta to lungs is immediate. In birds, however, this process occurs over a longer paranatal period of several hours or even days as the embryo transitions from the embryonic in ovo stage to the hatchling ex ovo stage of life (Visschedijk, 1968; Rahn et al., 1985). The morphological and physiological changes that occur over this prolonged period of closure in birds are relatively unknown.

Ductus closure generally occurs in two stages: initial functional closure by smooth muscle constriction of the vessel, followed by morphological remodeling of the ductus arteriosus. During the first stage of DA

* Corresponding author. Tel.: +1 940 565 3631; fax: +1 940 565 3821.
E-mail address: edzial@unt.edu (E.M. Dzialowski).
[1] Current Address: Institute of Ecology and Evolution, 335 Pacific Hall, 5289 University of Oregon, Eugene, OR 97403, United States.

http://dx.doi.org/10.1016/j.cbpa.2015.11.006
1095-6433/

closure, smooth muscles contract in response to an increase in arterial PO_2 (Tristani-Firouzi et al., 1996; Thébaud et al., 2004; Reese et al., 2006; Greyner and Dzialowski, 2008; Yokoyama et al., 2010; Coceani and Baragatti, 2012). The DA from both chicken and emu embryos are sensitive to the increase in blood PO_2 that occurs during pipping, hatching, and the associated switch to pulmonary respiration. This increased PO_2 initiates the contraction that takes place in the DA prior to actual morphological remodeling (Tristani-Firouzi et al., 1996; Imamura et al., 2000; Thébaud et al., 2004; Greyner and Dzialowski, 2008; van der Sterren et al., 2014). In the chicken, proximal DA closure begins with smooth muscle contraction during the last stage of hatching, external pipping (Belanger et al., 2008). This constriction has been shown to be redox sensitive and involved the Rho kinase pathway, as well as influx of Ca^{2+} through L-type calcium channels (Keck et al., 2005; Weir et al., 2008; Greyner and Dzialowski, 2008; Cogolludo et al, 2009; Hong et al., 2013).

The morphological changes that remodel the DA after the initial constriction have been studied mainly in mice, rabbits, and monkeys (Tada and Kishimoto, 1990; Giuriato et al., 1993; Rabinovitch, 1996; Clyman et al., 1999; Imamura et al., 2000; Yokoyama et al., 2010; Coceani and Baragatti, 2012; Yokoyama, 2015), with one study in the birds (Belanger et al., 2008). The proximal DA is the first vascular area to begin anatomical remodeling in the chicken, and does so starting on day 20 of incubation when the embryo is externally pipped (Belanger et al., 2008). Over the next 12 to 24 h, fragmentation of the internal elastic lamina occurs and smooth muscle cells migrate into the tunica intima of the vessel resulting in occlusion of the lumen (Belanger et al., 2008). A decrease in the overall number of smooth muscle cells in the DA occurs in the days following mammalian birth (Tennenbaum et al., 1996). Apoptosis also occurs in the mammalian DA, but its presence and timing in the avian DA are unknown. Lamb, sheep, baboon, and human DA have all exhibited marked levels of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells after birth (Clyman et al., 1999; Goldbarg et al., 2003; Levin et al., 2005, 2006; Kim et al., 2009). When closure occurs, the majority of smooth muscle cells in the lamb DA undergo cell death in the first 24 h after birth (Levin et al., 2005). Immature DA that remain patent, on the other hand, do not display a marked level of TUNEL-positive cells (Levin et al., 2005). Baboon DA show the presence of cell death in the most hypoxic areas of the vessel (Clyman et al., 1999) and other studies confirm that hypoxia, along with ATP depletion and hypoglycemia, contribute to the incidence and amount of TUNEL-positive cells in the mammalian DA (Goldbarg et al., 2003; Levin et al., 2005, 2006).

Hatching in the emu (*Dromaius novaehollandiae*) begins after approximately 49 days of incubation (E49), when the bird breaks through the air cell during internal pipping (IP) and begins to respire with its lungs. External pipping (EP) and hatching follow on day 50 when the bird breaks the eggshell with its beak and breathes normoxic air for the first time. The overall process of the onset of pulmonary respiration is prolonged in birds (hours to days; Visschedijk, 1968; Rahn et al., 1985) compared to mammals (a few minutes). Moreover, the circulatory and morphological changes at the DA are unknown in birds beyond the chicken which, although commonly investigated, is not representative of the great diversity among birds. Emu belong to Palaeognathae, among the most primitive clades of birds (Prum et al., 2015) and when compared with other birds and mammals can provide an understanding of potentially conserved developmental morphological and physiological phenotypes in vertebrate lineages. Thus, the main objective of this study was to examine the DA morphological changes, including apoptosis, and associated blood flow patterns occurring in the hatching emu. We hypothesized that the greatest changes in blood flow and DA morphology would occur during the externally pipped stage of the hatching process. To test this hypothesis, we measured changes in blood flow patterns during hatching and the associated morphological changes in the ductus arteriosus of the emu.

2. Methods

2.1. Eggs and incubation

Emu eggs were obtained from the Cross Timbers Emu Ranch in Pilot Point, Texas. Eggs were incubated in a Hatchrite incubator at a temperature of 36.5 °C, relative humidity of 35%, and automatically turned every 4 h. The University of North Texas Animal Care and Use Committee approved all procedures used in this study.

2.2. Blood flow patterns

The distribution of blood flow from the right atrium to the lungs, heart, brain, and CAM was measured in day 49 internally pipped embryos, externally pipped embryos, and day 0 hatchlings, corresponding to 98%, 99%, and 100% of embryonic development. Relative blood flows were measured by determining the distribution of colored microspheres (15 μm diameter in heparinized 0.09% NaCl saline with 0.05% Tween 80, IMT — Stason Laboratory, Irving, CA) in tissues of interest (see Sbong and Dzialowski, 2007), as briefly described below. Embryonic stages were cannulated by removing a small portion of the eggshell and associated membranes to reveal the chorioallantoic membrane. A chronic cannula was inserted into a small chorioallantoic vein using heat-pulled PE 10 tubing. Externally pipped embryos were cannulated during internal pipping and then were artificially externally pipped by breaking a hole into the shell. The animals were allowed to be externally pipped for two hours prior to injection of microspheres.

Hatchlings were cannulated at either the femoral or jugular vein while anesthetized by inhalation of isoflurane and artificially ventilated. Animals were intubated and ventilated using a Harvard ventilator. 50 microliters of colored microspheres (8000 microspheres/μl) in heparinized saline were injected into the CAM vein or jugular vein, where they passed through the right side of the heart and were then distributed to the systemic or pulmonary circuit. Anesthetized embryos and hatchlings were euthanized by decapitation and the heart, brain, CAM, and lungs were removed and weighed. Each tissue was digested overnight in 10–13 ml of 1 M KOH at 65 °C. Sodium deoxycholic reagent was added to the digested tissue to increase the volume to 14 ml. This solution was then mixed by vortex and centrifuged at 1500 *g* for 30 min. The supernatant was aspirated, and the pellet was re-suspended by sonication into 10 ml 5% Triton X-100 solution. The solution was centrifuged at 1500 *g* for 15 min, and the supernatant was aspirated to a level just above the pellet. The remaining volume was determined with a 200 μl pipette. The number of microspheres in each tissue sample was then counted using a hemocytometer. Data are presented as the number of microspheres counted in the CAM, heart, or brain divided by the number of microspheres counted in the lungs. This measure allows estimation of the blood leaving the right atria and flowing to the tissues through the DA and the interatrial foramina, distinct from blood flowing to the lungs through the pulmonary arteries (Dzialowski et al., 2011; Sbong and Dzialowski, 2007). The greater the ratio, the greater the right-to-left shunt of blood away from the lungs and to that tissue.

2.3. Histology

To examine morphological DA closure during hatching, emus were studied on incubation days 45, 50, and 51, and post-hatching days 0, 1, 2, 3, and 4. Birds were euthanized by inhalation of isoflurane and the right DA was removed and fixed in 4% paraformaldehyde at 4 °C for 24 h before being stored in phosphate buffered saline (pH 7.4).

The fixed vessels were dehydrated in graded methanol, infiltrated with paraffin, and then embedded and oriented in paraffin blocks for sectioning. Using a microtome, 5 μm thick sections of DA were sectioned and mounted on microscope slides. Slides were deparaffinized in xylene and rehydrated in graded ethanol. The samples were stained for further morphological analysis with hematoxylin and eosin (H&E).