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The actions of the renin–angiotensin system on cardiovascular and osmoregulatory function in embryonic chickens (*Gallus gallus domesticus*)

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ABSTRACT

Using embryonic chickens (*Gallus gallus domesticus*), we examined the role of the renin–angiotensin system (RAS) in cardiovascular and osmotic homeostasis through chronic captopril, an angiotensin-converting enzyme (ACE) inhibitor. Captopril (5 mg kg^{-1} embryo wet mass) or saline (control) was delivered via the egg air cell daily from embryonic day 5–18. Mean arterial pressure (MAP), heart rate (f_H), fluid osmolality and ion concentration, and embryonic and organ masses were measured on day 19. Exogenous angiotensin I (ANG I) injection did not change MAP or f_H in captopril-treated embryos, confirming ACE inhibition. Captopril-treated embryos were significantly hypotensive, with MAP 15% lower than controls, which we attributed to the loss of vasoconstrictive ANG II action. Exogenous ANG II induced a relatively greater hypertensive response in captopril-treated embryos compared to controls. Changes in response to ANG II following pre-treatment with phentolamine (α-adrenergic antagonist) indicated a portion of the ANG II response was due to circulating catecholamines in captopril-treated embryos. An increase in MAP and f_H in response to hexamethonium indicated vagal tone was also increased in the absence of ACE activity. Captopril-treated embryos had lower osmolality, lower Na^+ and higher K^+ concentration in the blood, indicating osmoregulatory changes. Larger kidney mass in captopril-treated embryos suggests disrupting the RAS may stimulate kidney growth by decreasing resistance at the efferent arteriole and increasing the fraction of cardiac output to the kidneys. This study suggests that the RAS, most likely through ANG II action, influences the development of the cardiovascular and osmoregulatory systems.

1. Introduction

The regulation of blood pressure and osmotic balance during embryonic/fetal development, particularly in non-mammalian animals, is understudied. As a result, we have little understanding of how integrated, coordinated interactions between the cardiovascular and renal systems develop during early life stages. In adults, the renin–angiotensin system (RAS) plays a vital role in coordinating cardiovascular and renal function (Guyton and Hall, 1996; Nguyen Dinh Cat and Touyz, 2011). The RAS hormonal cascade begins with the release of renin from the kidneys, which catalyzes the splitting of angiotensin I (ANG I) from angiotensinogen. In turn, ANG I is cleaved by angiotensin-converting enzyme (ACE) to form angiotensin II (ANG II), a powerful vasoconstrictor that acts via ANG II AT receptors in the vasculature (Bottari et al., 1993; J hren et al., 2004). ANG II stimulates catecholamine release from the sympathetic nervous system, which adds to its vasoconstrictor action (Nishimura et al., 1981; Nishimura et al., 1982; Farrell et al., 2001; Dendorfer et al., 2002). ANG II also stimulates the release of aldosterone from the adrenal cortex and arginine vasotocin from the pituitary, both of which promote proximal tubular sodium reabsorption (Vander, 1980; Harrison-Bernard, 2009).

Components of the RAS, such as renin, ACE, ANG II and its receptors, are present early in vertebrate ontogeny (Siegel and Fisher, 1980; Robillard and Nakamura, 1988; Nishimura, 2001; Nishimura et al., 2003; Savary et al., 2005; Crossley et al., 2010; Tate et al., 2012). During avian development, ANG II is a trophic factor and a tonic regulator of cardiovascular function (Baker and Aceto, 1990; Le Noble et al., 1993; Nishimura, 2001; Nishimura et al., 2003; Savary et al., 2005; Crossley et al., 2010). ANG II recoeptor mRNA concentrations, plasma hormone concentrations, and the cardiovascular response to acute ANG II injection have been characterized over the second half of embryonic chicken development (Crossley et al., 2010). ANG II levels are elevated in chicken embryos compared to adult birds (Crossley et al., 2010), which attenuates baroreflex control of heart rate on day 19 (Mueller et al., 2013). A previous attempt to inhibit the action of ANG II in chicken embryos using a receptor antagonist was unsuccessful until hatching

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(Crossley et al., 2010). However, we have previously demonstrated that reduced arterial blood pressure and increased baroreflex sensitivity following chronic ACE inhibition were reversed by infusion of ANG II in chicken embryos (Mueller et al., 2013).

We examined the role of the RAS, ANG II in particular, in cardiovascular and osmoregulatory function in embryonic chickens (*Gallus gallus domesticus*). During avian development the maturing kidney and extra-embryonic structures, including the chorioallantoic membrane (CAM) and allantois, work in concert to regulate ion and water balance (Mueller et al., 2015). The allantois, which acts as a repository for kidney excretions, decreases in volume during the second half of incubation as water is absorbed by the embryo (Romanoff and Hayward, 1943; Hoyt, 1979). Avian kidney development progresses through three overlapping structural stages: the pronephros, mesonephros and metanephros. The mesonephros is functional for the first half of incubation but regresses so that the metanephros is the functional form at hatch (Wideman, 1989; Carretero et al., 1995). We hypothesized that chronic disruption the RAS with captopril would result in relative hypotension during chicken embryonic development. Due to the interactions between cardiovascular and osmoregulatory systems in establishing blood pressure, we hypothesized that RAS disruption would decrease sodium (Na^+) reabsorption into the blood, lowering blood osmolality and Na^+ concentration. The hypertensive response to exogenous ANG II in adult chickens is partly due to catecholamine release (Nishimura et al., 1982), thus we also hypothesized that pretreatment with adrenergic and ganglionic pharmacological blockers would attenuate the pressure response to exogenous ANG II in embryonic chickens by removing the catecholamine contribution, and that responses would be altered by ACE inhibition. Lastly, we hypothesized that disruption of the RAS would reduce embryo, heart and metanephric kidney mass via a decrease in ANG II-induced growth.

2. Materials and methods

2.1. Egg source and incubation

Fertilized white leghorn chicken eggs (*G. gallus domesticus*) were purchased from Texas A&M University (College Station, TX, USA) and shipped to the University of North Texas (Denton, TX, USA). All procedures were approved by the University of North Texas Animal Care and Use Committee (Projects 09-009 and 11-007). Eggs were weighed to ±0.1 g on an electronic balance (Denver Instrument Company, USA) and placed in an incubator (model 1502, G.Q.F. Manuf. Co., Savannah, GA, USA). Temperature was maintained at 38 ± 0.5 °C at a relative humidity of approximately 55%, and eggs were turned automatically every 3 h.

2.2. Captopril administration

Captopril, an ACE inhibitor (MP Biomedicals, Solon, OH, USA), was injected into eggs daily at 15:00 from day 5 to 18 of incubation, following the protocol of our previous study (Mueller et al., 2013). Briefly, viability was checked via candling on day 5, the air cell was marked and the shell surface over the air cell was wiped with 80% EtOH. A small hole was made through the shell using a 20 G needle and a solution of captopril (5 mg kg^{-1} embryo wet mass) dissolved in 0.9% NaCl sterile saline was injected into the air cell. The shell was again wiped with 80% EtOH and the hole sealed with silicone gel (DAP Products, Baltimore, MD, USA). On subsequent injection days, egg mortality was recorded and injections were administered through the silicone seal and the egg surface wiped with 80% EtOH.

Injection volume of the captopril solution was maintained between 10 and 100 μL, using 0.1 and 1 mg mL^{-1} concentrations, depending on embryo mass. The correct volume to achieve a 5 mg kg^{-1} dosage was based on estimated embryo wet mass for each developmental day (Romanoff, 1960). Control eggs were injected with identical volumes of 0.9% NaCl sterile saline solution. Egg mass immediately prior to incubation did not vary between control and captopril treatments.

2.3. Embryo and organ masses

Day 19 eggs were placed in a desiccator for 10 min with cotton gauze saturated with isoflurane (Isoflo, Abbott Laboratories, North Chicago, IL, USA) to induce anesthesia. The embryo was removed from the shell and extra-embryonic membranes, blotted with tissue paper to remove excess fluid, and weighed on an electronic balance to ±0.01 g (XD-800, Denver Instrument, Bohemia, NY, USA). The embryo was decapitated, and the heart, metanephric kidneys, lungs and liver removed. Each organ was carefully blotted on Kimwipes® (Kimtech Science, Roswell, GA, USA) to remove any surface fluids, and weighed on an electronic balance (Ohaus Explorer® E12140, Pine Brook, NJ, USA) to ±0.1 mg. Organs and remaining embryonic tissue were then placed in a 60 °C oven (Isotemp 100 series model 106G, Fisher Scientific, Asheville, NC, USA) and dried for 72 h before being weighed again.

2.4. Vascular catheterization and experimental set up

On day 19, eggs were candled to locate a tertiary chorioallantoic (CAM) artery. An egg was placed in a thermostatically controlled chamber at 38 ± 0.5 °C, and a small portion of the egg shell at the site of the artery removed. The exposed artery was catheterized with PE-50 tubing (heat-pulled tip to narrow diameter) filled with heparinized 0.9% NaCl saline under a dissecting microscope (Leica M60, Leica Microsystems, Waukegan, IL, USA), as described previously (Crossley et al., 2003; Mueller et al., 2013). The occlusively implanted catheter was glued to the egg shell (Duro Quick Gel®) and the egg placed in a multi-chambered water-jacketed stainless steel experimental apparatus (one egg per chamber) to stabilize for 1 h. The experimental apparatus was maintained at 38 ± 0.5 °C via a constant temperature circulator (Julabo F32, Seelbach, Germany). Each chamber was fitted with a lid containing small openings for the catheter and air flow (200 mL min^{-1}), which was pre-warmed to 38 ± 0.5 °C via flow through a copper pipe.

The arterial catheter from each egg was attached to a pressure transducer (ADInstruments disposable transducer, Colorado Springs, CO, USA) connected to a bridge amplifier (ML228 octal bridge, ADInstruments) and the pressure signal recorded using a PowerLab data acquisition system (ADInstruments) and Chart software (version 7, ADInstruments). The system was calibrated using a vertical column of saline set at the top of the chamber. Distance from the catheter entry in the egg to the top of the chamber was measured and the pressure reading was corrected for this distance. Heart rate was continuously derived from the pressure signal.

For all pharmacological studies, drugs were administered via a Y connector in the arterial catheter line. Each drug injection was followed by a saline flush that was twice the volume of the drug mixture. Total drug injection volumes were 150 μL, which was less than 5% of total blood volume (Romanoff, 1967).

2.5. ANG I and ANG II responses

The effect of the chronic captopril treatment on cardiovascular function was assessed by measuring mean arterial pressure (MAP, kPa) and heart rate (f_H, beats min^{-1}) during acute injections of chicken ANG I ([Asp^1,Val^5,Ser^9]ANG I, 2 μg kg^{-1} of embryo wet mass, Bachem) and chicken ANG II ([Asp^1,Val^5]ANG II, 2 μg kg^{-1}, Bachem) in the first group of captopril-treated and control embryos. The dose of ANG II was selected based on the reported maximal cardiovascular effects in embryonic chickens at this developmental point (Crossley et al., 2010).