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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).

2.6. ANG II mechanism of action

Possible adrenergic receptor mediated ANG II cardiovascular response, and source of catecholamines, was examined by injecting a second and third group of embryos with 2 μg kg^{-1} ANG II before and after either injecting the α-adrenergic receptor blocker phentolamine (3 mg kg^{-1}, Sigma; to examine α-adrenergic contribution), or the ganglionic blocker hexamethonium (25 mg kg^{-1}, Sigma; to examine catecholamine source). An initial dose of ANG II was administered, and MAP and f_H were allowed to return to pre-injection levels. Either phentolamine or hexamethonium was then injected and approximately 30 min after blockade, when MAP and f_H had stabilized for a minimum of 10 min, the second ANG II dose was given.

2.7. Osmolality and ion concentration

On embryonic day 19, eggs were exposed to isoflurane in a desiccator for 5 min to induce light anesthesia in the embryo. The shell and outer membranes above the egg air cell were removed to reveal the underlying CAM. A small area of the CAM that contained no large vessels was peeled back on itself. This prevented any blood from broken vessels from contaminating the underlying allantoic fluid. Approximately 500 μL of allantoic fluid was sampled with a 26 G needle and syringe. The amniotic sac was then pierced with a needle and syringe and approximately 200 μL of amniotic fluid sampled. The embryo was removed from the egg, the yolk removed and 20 μL of cloacal fluid sampled. To sample blood, eggs were exposed to isoflurane for 5 min to anesthetize the embryo, the egg opened and the embryo removed without detaching it from the CAM or yolk. The chest was opened to expose the heart and approximately 250–1000 μL of blood was sampled from the ventricles using a 26 G needle and heparinized syringe. All fluids were transferred to a microcentrifuge tube and osmolality and ion concentrations measured within 5 min. Osmolality (mmol kg^{-1}) was measured by a vapor pressure osmometer (5520 Vapro, Wescor Inc., South Logan, UT, USA) and Na^+, K^+ and Cl^- concentrations ($[Na^+]$, $[K^+]$ and $[Cl^-]$) were measured using a blood chemistry analyzer (StatProfile, pHOx Plus L, Nova Biomedical, Waltham, MA, USA).

2.8. Data analysis

Differences in survival, masses, MAP, diastolic pressure (P_d), systolic pressure (P_s) and f_H between the control and captopril-treated embryos were assessed with Student's *t* test. To assess differences in MAP and f_H before and after drug injections and between treatments, a two-way repeated measures ANOVA, with treatment and before/after injection as the main affects, was used. Post hoc Tukey HSD multiple pairwise comparisons were run when main effects were significant. A two-way repeated measures ANOVA and post hoc Tukey HSD, with treatment and before/after blockade as the main effects, was used to assess pre and post injection MAP and f_H, as well as the absolute (ΔMAP or Δf_H) and relative (%) changes in MAP and f_H in response to ANG II before and after phentolamine or hexamethonium blockade. A two-way ANOVA and post hoc Tukey HSD was used to test for differences in osmolality and ion concentration between fluids and treatments. Data were ranked transformed when they did not meet the parametric assumptions of normality and equal variances. The relative (%) changes were normally distributed and therefore not transformed. Statistics were carried out in SigmaStat 3.5 (Systat Software Inc., Chicago, IL, USA) using a significance level of $P < 0.05$.

3. Results

3.1. Survival and masses

Twelve separate embryo groups containing 6–10 control embryos (total of 101) and 6–10 captopril-treated embryos (total of 103) were used across the experiments. Chronic captopril treatment had no effect on embryonic survival. Survival to day 19 averaged 81 ± 5% in captopril-treated embryos, statistically identical to the 87 ± 4% survival rate of controls (N = 12 embryo groups for controls and captopril-treated embryos, Student's *t*, $P = 0.36$).

The chronic captopril treatment had no effect on embryo wet mass (control: 24.8 ± 0.3 g, captopril: 24.2 ± 0.5 g, $P = 0.58$) or dry mass (control: 4.9 ± 0.2 g, captopril: 4.8 ± 0.3 g, $P = 0.77$). Chronic captopril treatment also had no effect on wet masses of the heart (control: 147.7 ± 2.6 mg, captopril: 141.9 ± 4.8 mg, $P = 0.29$), lungs (control: 231.6 ± 7.1 mg, captopril: 210.7 ± 7.7 mg, $P = 0.052$) and liver (control: 570.9 ± 15.7 mg, captopril: 580.2 ± 15.7 mg, $P = 0.55$). Likewise, dry masses of the heart, lungs and liver were not significantly different between captopril-treated embryos and controls (Fig. 1a). However, metanephric kidney wet mass was 11% larger in the captopril-treated embryos (181.5 ± 5.9 mg) compared to controls (163.7 ± 3.8 mg, $P = 0.017$). Likewise, the ratio of kidney wet mass to embryo wet mass was 14% larger in the captopril-treated embryos ($P = 0.004$, Fig. 1b). The metanephric kidney dry mass of captopril-treated embryos was also 9% larger (Fig. 1a, $P = 0.013$) and the ratio of kidney dry mass to embryo dry mass was 11% larger than controls (Fig. 1b, $P = 0.03$).

3.2. MAP and f_H

Resting MAP of captopril-treated embryos was 15% lower than controls (Student's *t*, $P = 0.005$, Table 1). Captopril-treated embryos also had a significantly lower P_s ($P = 0.004$) and P_d ($P = 0.017$). Resting f_H was not significantly different between treatments ($P = 0.051$).

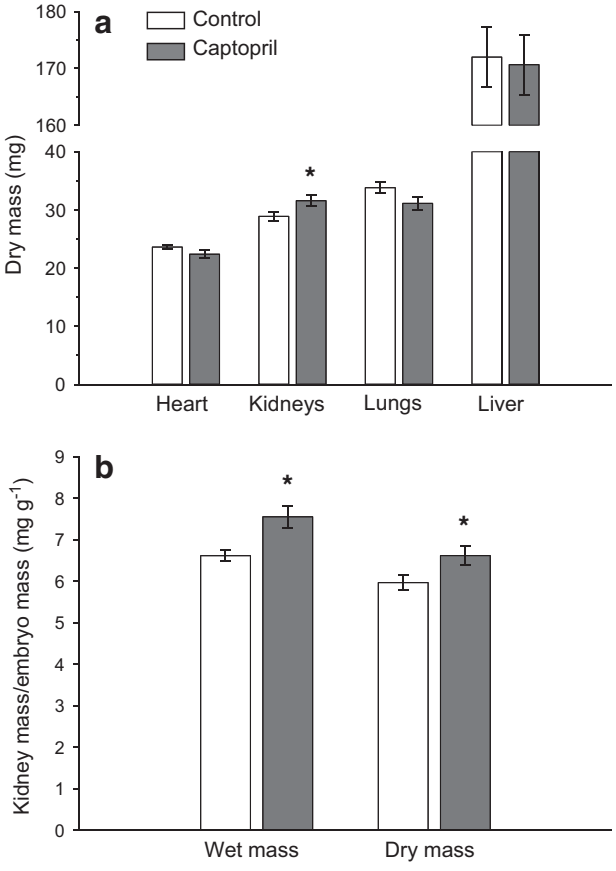

Fig. 1. a) Dry organ masses and b) wet and dry ratios between kidney mass and embryo mass of day 19 control embryos and embryos chronically treated with captopril. Data presented as mean ± S.E. An * indicates a significant difference ($P < 0.05$) from control values.

Table 1
Resting mean arterial pressure (MAP, kPa), systolic pressure (P_{sys}, kPa), diastolic pressure (P_{dia}, kPa), heart rate (f_H, beats min^{-1}) and the % differences between each parameter in day 19 control embryos and embryos chronically treated with captopril. Data presented as mean ± S.E.

	Control	Captopril	Difference (%)
N	14	15	
MAP (kPa)	2.35 ± 0.08	1.99 ± 0.09*	15
P_{sys} (kPa)	3.24 ± 0.10	2.71 ± 0.13*	16
P_{dia} (kPa)	1.90 ± 0.08	1.63 ± 0.07*	14
f_H (beats min^{-1})	256 ± 4	245 ± 4	11

* Indicates a significant difference ($P < 0.05$) from control values (Student's *t* test).

3.3. ANG I and ANG II responses

Control injections of saline equivalent to the drug injection volume (150 μL) had no effect on MAP or f_H in either treatment (paired *t* tests, $P > 0.05$). ANG I injection caused a significant hypertension ($P < 0.001$, two-way repeated measures ANOVA, Tukey HSD, Fig. 2a) and bradycardia ($P = 0.002$) in control embryos, but not in captopril-treated embryos ($P > 0.05$, Figs. 2b, 3a,b). Therefore, MAP of captopril-treated embryos was significantly lower than controls before and after ANG I injection ($P < 0.05$). Despite the bradycardia in controls, f_H did not differ between the treatments before or after ANG I injection ($P > 0.05$).

ANG II injection caused a significant hypertension in both control and captopril-treated embryos ($P < 0.001$, two-way repeated measures ANOVA, Tukey HSD, Figs. 2c,d, 3c,d). The intensity (% change) of this response in captopril-treated embryos (92 ± 3%), was significantly higher than controls (80 ± 5%, Student's *t*, $P = 0.035$), due to the relative resting hypotension in this group compared to controls. The ANG II hypertensive response corresponded to a significant bradycardia in control embryos only ($P = 0.01$). Captopril-treated embryos responded with significant tachycardia approximately 5 min after the ANG II injection (paired *t*, $P = 0.001$, Fig. 2d), which was not observed in control embryos ($P = 0.32$).

3.4. ANG II mechanism of action

ANG II induced similar responses prior to phentolamine or hexamethonium injection in the second and third group of embryos as the first group described above (Fig. 3c,d). Injection of the α-adrenergic receptor blocker phentolamine caused a significant hypotension and bradycardia in both control and captopril-treated embryos, with similar responses between the groups (two-way repeated measures ANOVA, Tukey HSD,

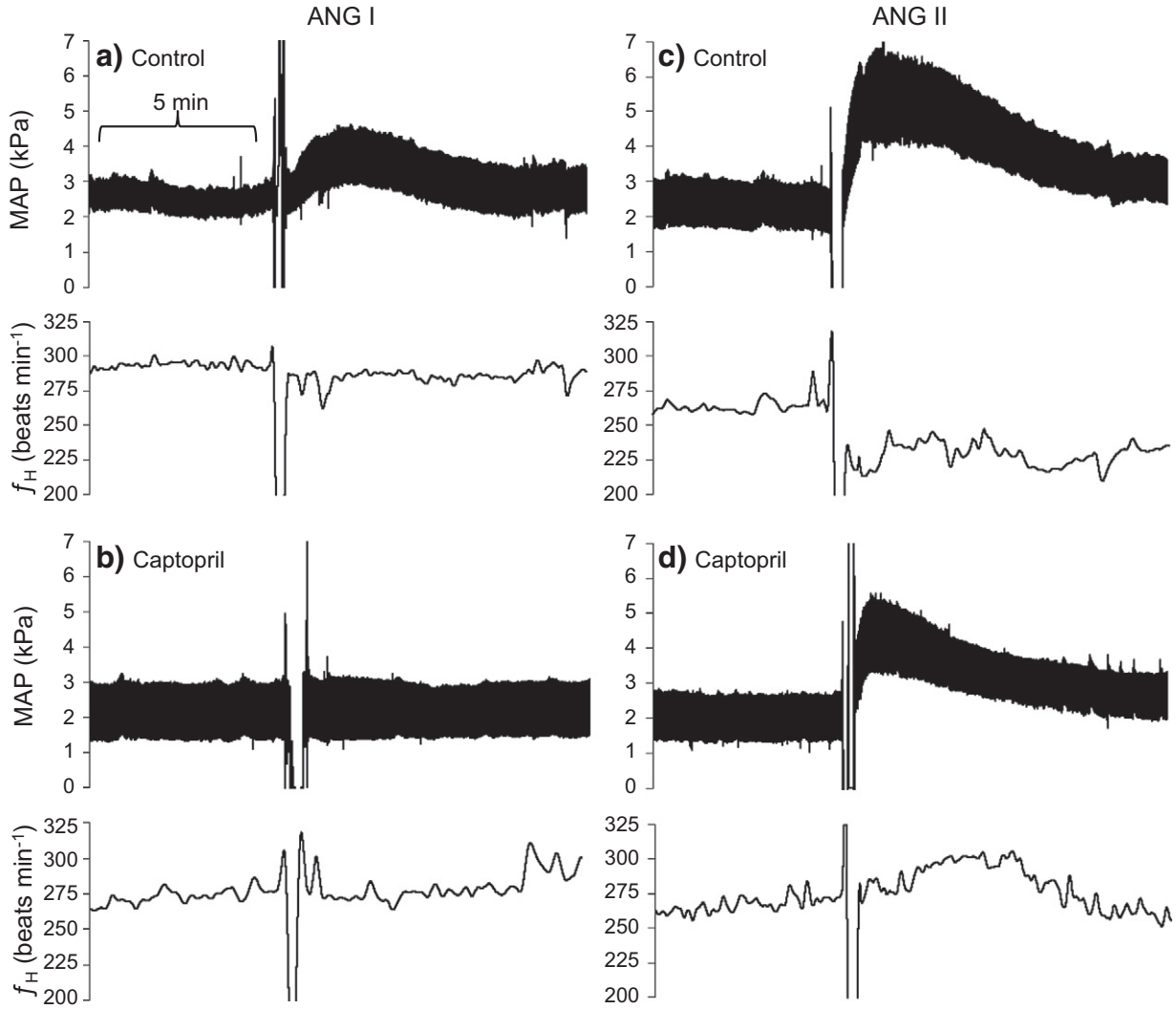

Fig. 2. Representative traces of day 19 control embryos and embryos chronically treated with captopril. Traces illustrate the mean arterial pressure (MAP, kPa) and heart rate (f_H, beats min^{-1}) response to angiotensin I (ANG I, 2 μg kg^{-1}) in a control (a) and a captopril-treated (b) embryo and angiotensin II (ANG II, 2 μg kg^{-1}) in a control (c) and a captopril-treated (d) embryo. The bracket indicates 5 min and the arrows indicate injection.

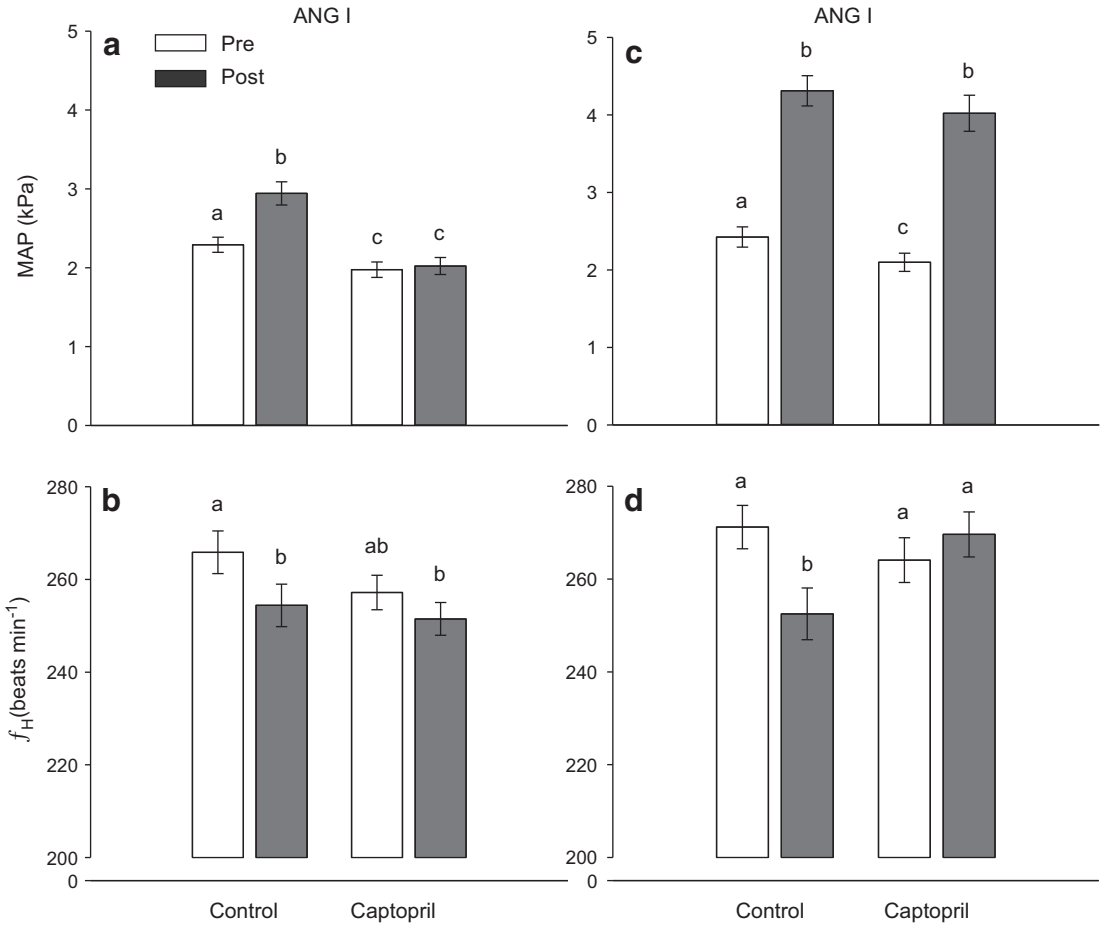

Fig. 3. Pre and post injection values for a) mean arterial pressure (MAP, kPa) and b) heart rate (f_H, beats min^{-1}) in response to angiotensin I (ANG I, 2 μg kg^{-1}) and c) MAP and d) f_H in response to angiotensin II (ANG II, 2 μg kg^{-1}) in day 19 control embryos and embryos chronically treated with captopril. Data presented as mean ± S.E. Pre: before injection, Post: ~1 min after injection. Different letters indicate significant differences ($P < 0.05$) across treatments and pre and post injection values (two-way repeated measures ANOVA, Tukey HSD).

Table 2). Following phentolamine, the absolute change in MAP (ΔMAP) induced by ANG II injection decreased in both control and captopril-treated embryos, with no differences between treatments (two-way repeated measures ANOVA, Tukey HSD, Table 3, Fig. 4a). The intensity of the ANG II response was unchanged in controls (before block: 89 ± 6%, after block: 75 ± 4%, $P = 0.13$), but significantly decreased from 116 ± 11% to 79 ± 3% after blockade in captopril-treated embryos ($P = 0.002$). Therefore, the intensity of the response in captopril-treated embryos was no longer significantly greater than controls after blockade ($P = 0.64$). Neither treatment showed a significant change in f_H in response to ANG II before blockade ($P > 0.05$, Table 3, Fig. 4b), but after blockade both experimental groups showed significant tachycardia immediately after ANG II injection that persisted for 5 min post injection ($P < 0.05$).

Captopril-treated embryos showed a significant hypertension ($P = 0.005$, two-way repeated measures ANOVA, Tukey HSD) and tachycardia ($P < 0.001$) in response to hexamethonium that was absent in control embryos (Table 2). Blockade with hexamethonium did not change the MAP response to ANG II in either control or captopril-treated embryos ($P > 0.05$, Table 3). Hexamethonium eliminated the bradycardic response to ANG II in both treatment groups, and, instead a significant tachycardia occurred in both groups ($P < 0.05$, Table 3).

Table 2
Mean arterial pressure (MAP, kPa) and heart rate (f_H, beats min^{-1}) before and after injection of phentolamine (3 mg kg^{-1}) or hexamethonium (25 mg kg^{-1}) in day 19 control embryos and embryos chronically treated with captopril. All data presented as mean ± S.E. Pre: before injection, Post: ~30 min after injection.

		Phentolamine		Hexamethonium	
		Control	Captopril	Control	Captopril
	n	14	15	5	6
MAP (kPa)	Pre	2.35 ± 0.13^{a}	2.22 ± 0.14^{a}	2.58 ± 0.17^{a}	2.20 ± 0.14^{b}
	Post	1.88 ± 0.15^{a,*}	1.64 ± 0.12^{a,*}	2.65 ± 0.18^{a}	2.43 ± 0.19^{a,*}
f_H (beats min^{-1})	Pre	270 ± 6^{a}	262 ± 4^{a}	258 ± 6^{a}	246 ± 6^{a}
	Post	253 ± 8^{a,*}	248 ± 7^{a,*}	258 ± 7^{a}	262 ± 5^{a,*}

Different letters indicate significant differences ($P < 0.05$) between treatments before and after each blockade.
* Indicates a significant difference ($P < 0.05$) from pre-injection levels (two-way repeated measures ANOVA, Tukey HSD).

Table 3
Mean arterial pressure (MAP, kPa) and heart rate (f_H, beats min^{-1}) before and after injection of angiotensin II (ANG II, 2 μg kg^{-1}), prior to and following either α-adrenergic blockade with phentolamine (3 mg kg^{-1}) or ganglionic blockade with hexamethonium (25 mg kg^{-1}) in day 19 control embryos and embryos chronically treated with captopril. Data presented as mean ± S.E. Pre: before injection, Post: ~1 min after injection.

		Pre-phentolamine		Post-phentolamine	
		Control	Captopril	Control	Captopril
	n	8	7	8	7
MAP (kPa)	Pre	2.14 ± 0.10^{a}	1.63 ± 0.18^{bc}	1.73 ± 0.09^{b}	1.29 ± 0.21^{c}
	Post	4.04 ± 0.19^{a,*}	3.44 ± 0.29^{ab,*}	3.00 ± 0.13^{bc,*}	2.34 ± 0.40^{c,*}
f_H (beats min^{-1})	Pre	258 ± 4^{a}	255 ± 6^{a}	259 ± 6^{a}	221 ± 18^{b}
	Post	245 ± 9^{a}	260 ± 8^{a}	273 ± 9^{a,*}	245 ± 22^{a,*}
		Pre-hexamethonium		Post-hexamethonium	
		Control	Captopril	Control	Captopril
	n	5	6	5	6
MAP (kPa)	Pre	2.46 ± 0.09^{a}	2.17 ± 0.11^{b}	2.58 ± 0.18^{a}	2.42 ± 0.11^{a}
	Post	4.57 ± 0.22^{a,*}	4.37 ± 0.22^{a,*}	4.86 ± 0.21^{a,*}	4.64 ± 0.28^{a,*}
f_H (beats min^{-1})	Pre	254 ± 9^{a}	256 ± 4^{a}	259 ± 7^{a}	261 ± 6^{a}
	Post	230 ± 4^{a,*}	233 ± 3^{a,*}	272 ± 8^{b,*}	281 ± 5^{b,*}

Different letters indicate significant differences ($P < 0.05$) between treatments and before and after blockade within pre and post injection levels (two-way repeated measures ANOVA, Tukey HSD).
* Indicates a significant response ($P < 0.05$, two-way repeated measures ANOVA, Tukey HSD).

3.5. Osmolality and ion concentration

Whole blood osmolality on embryonic day 19 was significantly lower in captopril-treated embryos compared to controls ($P = 0.03$, two-way ANOVA, Tukey HSD, Fig. 5). [Na^+] was lower, [K^+] was higher and [Cl^-] was unchanged in the whole blood of captopril-treated embryos compared to controls (Table 4). Allantoic, amniotic and cloacal fluid osmolality and [Na^+], [K^+] and [Cl^-] of the allantoic and amniotic fluids of captopril-treated embryos were not significantly different from control values. Allantoic fluid osmolality was significantly lower than whole blood ($P < 0.001$) and amniotic fluid ($P = 0.03$) osmolality in control embryos, but these gradients disappeared in captopril-treated embryos.

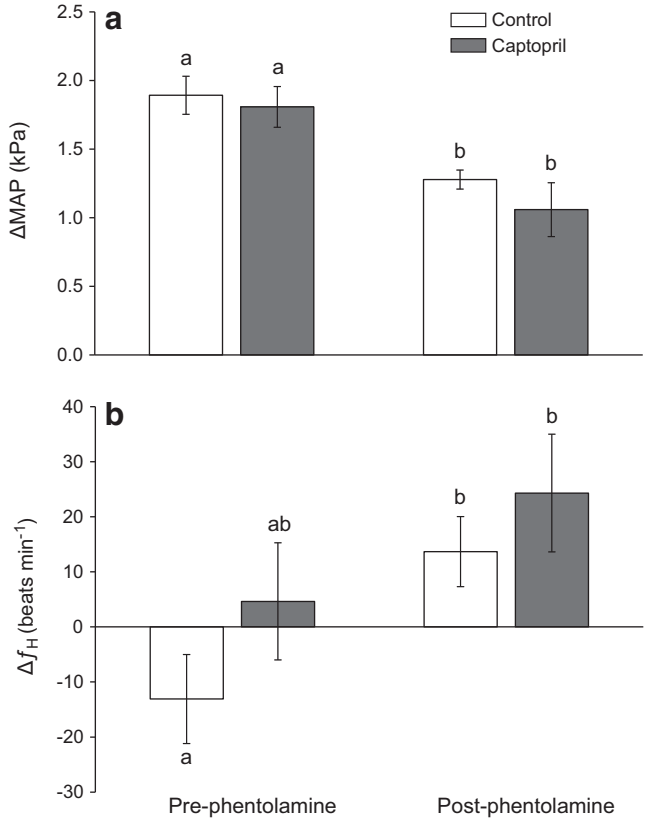

Fig. 4. Absolute change in a) mean arterial pressure (ΔMAP, kPa) and b) heart rate (Δf_H, beats min^{-1}) in response to angiotensin II (ANG II, 2 μg kg^{-1}) before and after α-adrenergic blockade with phentolamine (3 mg kg^{-1}) in day 19 control embryos and embryos chronically treated with captopril. Data presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) across treatments and pre and post injection values (two-way repeated measures ANOVA, Tukey HSD).

4. Discussion

This study establishes that the RAS regulates both the developing cardiovascular and osmoregulatory systems in an avian embryo. Chronic treatment, from embryonic day 5 to 18, with the ACE inhibitor captopril resulted in an embryo that was relatively hypotensive on day 19. This direct effect on blood pressure was coupled to changes in osmoregulation as well as metanephric kidney mass, indicating the multiple actions of the RAS during embryogenesis.

4.1. MAP, f_H, ANG I and ANG II responses

Chronic ACE inhibition resulted in significantly hypotensive embryos on day 19 compared to controls (Table 1), confirming previous findings that the RAS contributes to the maintenance of embryonic baseline MAP

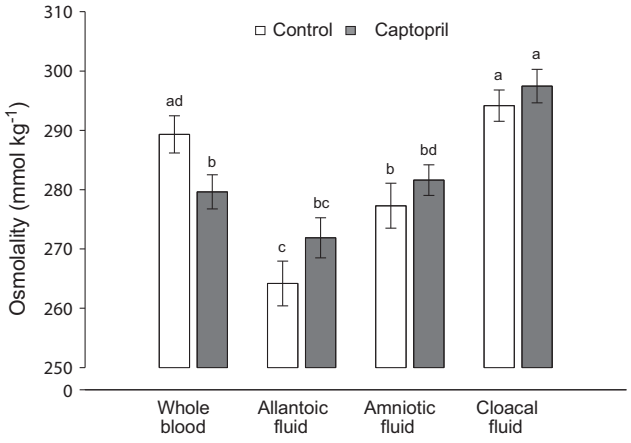

Fig. 5. Whole blood, allantoic, amniotic and cloacal fluid osmolality (mmol kg^{-1}) in day 19 control embryos and embryos chronically treated with captopril. Data presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) across treatments and fluids (two-way ANOVA, Tukey HSD).

Table 4
Ion concentration (mmol L^{-1}) in whole blood, allantoic and amniotic fluid in day 19 control embryos and embryos chronically treated with captopril. Data presented as mean ± S.E. Different letters indicate significant differences ($P < 0.05$) between treatments and fluids for each ion (two-way ANOVA, Tukey HSD). [Na^+] of the allantoic fluid was too low to be detected by the blood analyzer.

	Whole blood		Allantoic fluid		Amniotic fluid	
	Control	Captopril	Control	Captopril	Control	Captopril
N	10	10	11	9	13	13
mmol L^{-1}						
[Na^+]	138.6 ± 2.1[a]	132.7 ± 1.5[b]	–	–	135.4 ± 4.2[ab]	123.4 ± 6.0[b]
[K^+]	4.5 ± 0.1[a]	5.0 ± 0.1[b]	56.1 ± 6.1[c]	48.0 ± 6.4[c]	15.4 ± 1.7[d]	22.5 ± 5.3[d]
[Cl^-]	97.2 ± 2.7[a]	99.2 ± 2.2[b]	83.8 ± 6.7[a]	87.2 ± 5.5[a]	124.5 ± 3.8[b]	117.1 ± 4.6[b]

(Mueller et al., 2013). Indeed, the 15% decrease in MAP of day 19 captopril-treated chicken embryos is comparable to the 6–13% decrease determined in fetal sheep treated with captopril (Robillard et al., 1983; Lumbers et al., 1992) or the ANG II receptor antagonist, Sar^1-Ala^8-angiotensin II (Iwamoto and Rudolph, 1979). ANG I is an inactive peptide, so the observed pressor response and concurrent bradycardia in control embryos was mediated by the conversion of ANG I into the active ANG II catalyzed by ACE — all hallmarks of a functioning RAS in these embryos (Figs. 2a, 3a). The lack of a significant response in MAP or f_H to ANG I in captopril-treated embryos confirmed that chronic treatment with 5 mg kg^{-1} of captopril completely inhibited ACE. ACE also degrades the vasodilator bradykinin (Yang et al., 1971; Schmaier, 2003), thus, ACE inhibition can increase bradykinin in the plasma, which contributes to hypotension (Hornig et al., 1997; Gainer et al., 1998). However, in our previous study, MAP returned to control levels during infusion of ANG II over a 4 h period (Mueller et al., 2013). The return of MAP to control levels following ANG II infusion indicated that the hypotension was most likely due to the absence of the vasoconstrictive action of endogenous plasma ANG II in chicken embryos (Mueller et al., 2013).

Acute exogenous ANG II produced a pronounced increase in MAP in both control and captopril-treated embryos (Figs. 2c,d, 3c,d). The potency of the ANG I pressor response, calculated on a dose weight basis, in control embryos was only 35% of that of ANG II. This potency was lower than the weight basis 47% difference between ANG I and ANG II in adult chickens (Nishimura et al., 1982), possibly indicating that ACE is a limiting factor in the embryonic RAS, and that additional exogenous ANG II overcomes this limiting step. A prior study in embryonic chickens reported a similar ANG II hypertensive response coupled to a reflexive bradycardia, with the bradycardic response appearing at a lower concentration (0.25 μg kg^{-1} vs 2 μg kg^{-1}) on day 19 (Crossley et al., 2010). However, both these studies demonstrated an absence of immediate hypotension that is characteristic of an ANG II response in adult chickens (Nakamura et al., 1982; Nishimura et al., 1982).

The intensity of the MAP response to ANG II was altered in captopril-treated embryos that conceivably developed without circulating endogenous plasma ANG II. The relative MAP change (% change) was greater in captopril-treated embryos, indicating that the chronic absence of endogenous ANG II, possibly coupled to other ACE-dependent systems, increased the sensitivity to an acute exogenous source. Furthermore, captopril-treated embryos did not show a reflex bradycardia in response to ANG II, instead responding with tachycardia ~5 min post-injection. The transient tachycardia as pressure returned to baseline was possibly due to an increase in venous return as a result of the hypertension or a rapid resetting of the baroreflex set point to a higher pressure (Figs. 2d, 3d). Such a response was only evident in captopril-treated embryos and may be attributed to their increased baroreflex sensitivity (Mueller et al., 2013).

4.2. ANG II mechanism of action

The response to phentolamine was consistent with a previous embryonic chicken study (Crossley and Altimiras, 2000), and inhibition of ACE had no influence on basal α-adrenergic tone. The hypotensive and bradycardic response to α-adrenergic receptor blockade with phentolamine was similar between the treatment groups (Table 2). Therefore, hypotension in captopril-treated embryos did not cause a change in α-adrenergic tone. Prior studies of the embryos of chickens, American alligators (*Alligator mississippiensis*) and snapping turtles (*Chelydra serpentina*) have also reported that the chronic developmental hypoxic-induced hypotension was not accompanied by a change in α-adrenergic tone (Eme et al., 2011; Crossley and Altimiras, 2012; Eme et al., 2013). Collectively these studies suggest that adrenergic receptor mediated tone on the vasculature may lack plasticity during the ontogeny of these amniotes.

The absolute change in MAP (ΔMAP) in response to ANG II was attenuated after α-adrenergic blockade in both control and captopril-treated embryos (Table 3, Fig. 4). The decreased ANG II pressor response was a result of vessel dilation caused by α-adrenergic blockade, which reduced the effects of the vasoconstrictor action of ANG II. α-Adrenergic blockade did not alter the relative response intensity (% change) to ANG II in controls. Therefore, our hypothesis that the ANG II response would include an α-adrenergic component was refuted. However, in support of our hypothesis, the intensity of the relative ANG II response was reduced following α-adrenergic blockade in captopril-treated embryos (116% increase in MAP before blockade, 79% increase in MAP after blockade). This indicated that α-adrenoreceptor stimulation due to ANG II-induced catecholamine release partially contributed to the ANG II pressor response in captopril-treated embryos. Catecholamines similarly contributed to ANG II responses in adult chickens, American alligators, lumpfish (*Cyclopterus lumpus*), bullfrogs (*Rana catesbeiana*), red-eared turtles (*Chrysemys scripta elegans*) and ducks (*Anus platyrhynchos*) (Zehr et al., 1981; Carroll and Opdyke, 1982; Nishimura et al., 1982; Butler et al., 1986; Silldorff and Stephens, 1992). Thus, the vasoconstrictive response to ANG II in captopril-treated embryos, which included the action of catecholamines, was more representative of the ANG II response in adult chickens.

In an effort to further isolate the source of the adrenergic stimulatory response to ANG II in captopril-treated embryos, embryos were treated with the ganglionic/cholinergic blocker hexamethonium to eliminate acetylcholine-stimulated release of catecholamines via the adrenal medulla and sympathetic nerves. Hexamethonium had no effect on resting MAP or f_H of control embryos, as previously found in day 19 chicken embryos (Tazawa et al., 1992; Crossley and Altimiras, 2000). However, captopril-treated embryos responded to hexamethonium with a moderate, but significant, increase in MAP and f_H (Table 2), which suggests increased cholinergic tone on the heart. ANG II has been reported to decrease vagal tone on the heart in mammals via a central nervous system inhibition (Reid, 1992; Segar, 1997) and plasma concentrations of ANG II are significantly elevated in embryos compared to adult chickens (Crossley et al., 2010). Thus, chronic ACE inhibition may release the central inhibition of vagal tone on the heart due to ANG II, resulting in the tachycardic response to ganglionic blockade.

We hypothesized that hexamethonium would eliminate ANG II-induced central catecholamine release in captopril-treated embryos, thus reducing the ANG II pressor response. Hexamethonium pretreatment eliminated the bradycardic response to ANG II, and ANG II

injection following hexamethonium caused significant tachycardia in both treatments (Table 3). Therefore, in both groups a hypertensive baroreflex response was mediated by parasympathetic stimulation following ANG II injection. However, in opposition to our hypothesis, the ANG II MAP response did not change (Table 3). This indicated that the α-adrenoreceptor stimulation in captopril-treated embryos most likely originated from ANG II induced release of catecholamines from peripheral sources and was not mediated via the central nervous system. The use of additional pharmacological approaches, such as the blockade of catecholamine release from nerve endings with bretylium, would potentially confirm if peripheral catecholamines contributed to the ANG II response (McCarty and Kopin, 1979). Determining whether the catecholamine contribution is via an increased production of catecholamines or by amplifying the receptor response in captopril-treated embryos also requires additional investigation.

4.3. Osmoregulation

ACE inhibition lowered osmolality and $[Na^+]$ and increased $[K^+]$ of whole blood on day 19 (Fig. 5). In addition, ACE inhibition eliminated the osmolality gradient between the plasma, allantoic and amniotic fluids, demonstrating a change in osmoregulation within the chicken egg. Under ACE inhibition, the plasma concentration, and by extension the extracellular fluid, was relatively dilute. In chicken embryos, allantoic fluid volume peaks at ~7 mL on day 13, decreasing to ~1 mL on day 19 as the embryo absorbs water (Romanoff and Hayward, 1943). While we did not measure allantoic fluid in this study, it is possible that the loss of osmolality gradient between the plasma and allantoic fluid in captopril-treated embryos was in part due to a change in the rate of allantoic fluid movement throughout development.

The reduction in $[Na^+]$ and concurrent increase in $[K^+]$ of the blood suggests that ACE inhibition, and the assumed decrease in plasma ANG II, likely decreased aldosterone levels as well as other hormones involved in osmoregulation. ACE inhibition can reduce aldosterone release and cause natriuresis, even in the presence of hypotension (Atlas et al., 1979; Bakris et al., 2000). Complex interactions exist between a number of hormonal factors, such as ANG II, bradykinin, arginine vasotocin and atrial natriuretic peptide, which influence water and ion balance (McCormick and Bradshaw, 2006). Future studies are required to isolate the basis for the osmoregulatory changes found in this study, as it is unclear if the changes are blood pressure independent. Concurrent treatments with captopril and aldosterone, for example, would help determine if decreased osmolality of the blood was related to hypotension, aldosterone deficiency or both.

4.4. Embryo and organ masses

Our finding that chronic captopril treatment did not affect embryonic, heart, lung or liver mass refuted our hypothesis that the disruption of the RAS would reduce embryo and heart mass. The similar heart mass was surprising, given that ANG II has been shown to have hypertrophic effects on embryonic chicken myocytes (Baker and Aceto, 1990), and suggested that a fully functional RAS was not vital to total cardiac growth. However, cardiac cellular growth, such as increased cell number, could have been affected by ACE inhibition in embryonic chickens, a change that would not have been detected by mass measurements alone. ACE inhibition during development has been associated with reduced body and kidney mass, via the inhibition of ANG II-induced growth (Friberg et al., 1994; Tufro-McReddie et al., 1995; Mezzano et al., 2001). However, we found an increase in metanephros mass (Fig. 1). There are reported instances in which RAS antagonism produced enlarged kidneys related to renal abnormalities, such as overproduction of glomeruli, tubular and interstitial inflammation and thickening of arterial and arteriolar walls, in human (Pryde et al., 1993; Martinovic et al., 2001; Daïkha-Dahmane et al., 2006) and rat (Friberg et al., 1994) fetuses. Such renal damage was likely a secondary effect caused by hemodynamic and fluid balance changes (Friberg et al., 1994).

ANG II primarily acts on the efferent renal arteriole, increasing resistance, and therefore the elimination of plasma ANG II may have alternatively increased renal perfusion in embryonic chickens. Infusion of ANG II decreased the fraction of cardiac output delivered to the kidneys in adult dogs (Heyndrickx et al., 1976; Quillen and Reid, 1988), and thus ACE inhibition in chicken embryos may do the opposite, increasing cardiac output to the kidneys. A greater cardiac output fraction distributed to the kidneys may have stimulated growth and this could account for the metanephric kidney enlargement documented here. During chicken embryonic development, in which the allantois is the major osmoregulatory organ, high levels of plasma ANG II may antagonize renal growth. The chicken metanephric kidney begins to function around incubation day 12 (Carretero et al., 1995), and continues to develop post-hatch (Wideman, 1989). Once the embryo hatches, and metanephric kidney function becomes vital, ANG II levels may drop to adult levels so that growth is intensified. Chronic ACE inhibition during embryonic incubation could be analogous to this process, accelerating the developmental trajectory of the metanephros. Mammalian studies suggest that inhibition or overexpression of the RAS can program changes in the developing kidney (Moritz et al., 2003), and this may also occur in chicken embryos.

5. Conclusions

This study demonstrated that the RAS has a role in maintaining baseline cardiovascular function in embryonic chickens. Furthermore, osmoregulatory changes and increased metanephric kidney mass after chronic ACE inhibition indicate the RAS also functions in osmoregulation. Additional studies are required, however, to tease apart the role of ANG II and other hormones in these observations. Disruption of the RAS altered the vasoconstrictor action of exogenous ANG II, released central inhibition of vagal tone on the heart, and possibly increased renal perfusion. Direct examination of pressure changes in renal arterioles and glomerular filtration would help elucidate how the RAS influences growth and function of the renal system. Additionally, the onset of the RAS contribution to basal cardiovascular and osmoregulatory function remains to be studied. With the knowledge that we can successfully block ACE, and remove ANG II, a series of experiments in which captopril is administered at particular developmental time points, or critical windows, will allow us to determine when during embryogenesis the RAS first has an active role in cardiovascular and renal development and maintenance.

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