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ANG II and baroreflex control of heart rate in embryonic chickens (*Gallus gallus domesticus*)

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Mueller CA, Burggren WW, Crossley II DA. ANG II and baroreflex control of heart rate in embryonic chickens (*Gallus gallus domesticus*). *Am J Physiol Regul Integr Comp Physiol* 305: R855–R863, 2013. First published September 4, 2013; doi:10.1152/ajpregu.00298.2013.—ANG II alters the short-term blood pressure buffering capacity of the baroreflex in many adult animals. In embryonic chickens, high plasma ANG II levels contribute to baseline mean arterial pressure (MAP, kPa) without changing heart rate (f_H, beats/min). We hypothesized, on the basis of these features, that an ANG II-induced reduction in baroreflex sensitivity is present in embryonic chickens as in adults. We examined baroreflex function in *day 19* embryonic chickens (*Gallus gallus domesticus*) after chronic depletion of endogenous ANG II via angiotensin-converting enzyme (ACE) inhibition with captopril (5 mg/kg) from *days 5–18* of incubation. The correlation between MAP and f_H was assessed using increasing doses of sodium nitroprusside, a vasodilator, and phenylephrine, a vasoconstrictor. We used two analytical methods to evaluate baroreflex function: a conventional "static" method, in which maximal MAP and f_H responses were examined, and a "dynamic" method that assessed beat-to-beat changes during the response to pharmacological manipulation. Captopril-treated embryos were hypotensive by 19% with baroreflex slopes ~40% steeper and normalized gains ~50% higher than controls, and differences across treatments were similar using either analytical method. Furthermore, reintroduction of ANG II via infusion raised MAP back to control levels and decreased the baroreflex gain in captopril-treated embryos. Therefore, during typical chicken development, ANG II dampens the baroreflex regulatory capacity and chicken embryos can be used as a natural model of elevated ANG II for studying developmental cardiovascular function. This study is the first to demonstrate that reduction of embryonic ANG II alters normal baroreflex function.

angiotensin-converting enzyme inhibitor; blood pressure; ANG II infusion; sodium nitroprusside; phenylephrine

THE BAROREFLEX IS AN IMPORTANT compensatory mechanism that mitigates short-term changes in arterial pressure. Peripheral limb motor outputs adjust vascular resistance, and cardiac limb motor outputs adjust arterial pressure via heart rate (f_H, beats/min) (37). In the case of the cardiac limb, this negative feedback loop produces an inverse relationship between arterial pressure and f_H. Barostatic reflexes are functional during the fetal and adult phases of life, with clear function demonstrated from ~60% of development in fetal sheep (5, 32, 46, 49), from ~80% of incubation in embryonic chickens (1, 15), and from ~70% of incubation in embryonic American alligators (*Alligator mississippiensis*) (12). The common conclusion from each of these investigations is that baroreflex function is an important homeostatic mechanism during fetal or embryonic life. However, modulation of baroreflex function via systemic hormones and central neuropeptides, such as ANG II, has been unexplored in most species.

In adult mammals, ANG II alters the baroreflex by decreasing vagal tone, possibly modifying sympathetic output and elevating arterial pressure with no change in f_H (39). ANG II enacts these changes by acting centrally (16, 21, 34, 40) without directly affecting baroreceptors (20, 31). In mammals, ANG II infusion resets the baroreflex to a higher arterial pressure, as evident by a right shift of the baroreflex curve relating mean arterial pressure (MAP; kPa) and f_H (7, 20, 29, 31, 33, 34, 40). Likewise, treatment with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor (AT_1) antagonists can reset the baroreflex at a lower pressure, i.e., a left shift of the baroreflex curve (13, 16, 17, 21, 51). A key metric of baroreflex function is the slope of arterial pressure change plotted against f_H. This function is termed "gain", which can decrease during ANG II infusion or increase with the use of renin-angiotensin system antagonists (16, 20, 29).

ANG II is a key regulatory hormone that contributes to cardiovascular regulation during ontogeny in mammals and birds (10, 24, 30, 42; also Mueller CA, Crossley II DA, Burggren WW, unpublished data). However, the interaction between ANG II and baroreflex function during development has only been investigated in fetal sheep (22, 47). In embryonic chickens, exogenous ANG II produces a dose-dependent increase in MAP that increases in intensity over the final 50% of development (10). In addition, plasma ANG II concentration in *day 19* chicken embryos is ~115 pg/ml (10), which is three to four times that found in adult birds (18, 28). Elevated ANG II levels have previously been implicated in fetal programming of hypertension in mammalian studies (36, 45). Thus, the embryonic chicken may be a natural model for the chronic effects of elevated ANG II levels on the development of central nervous system regulatory capacity of the cardiovascular system. In a previous study, we found that chronic ACE inhibition lowered MAP with no significant change in f_H, and ACE inhibition eliminated the bradycardic response to ANG II, while the ANG II-mediated release of catecholamines from peripheral sympathetic nerve terminals was retained (Mueller CA, Crossley II DA, Burggren WW, unpublished data). These observations suggest that ANG II may modify the cardiac limb of baroreflex control in embryonic chickens.

We investigated the role of the innate elevated ANG II plasma concentration on baroreflex function at *day 19* of chicken embryonic development. Utilizing chronic ACE inhibition with captopril, we separated baroreflex ontogeny from the influence of ANG II. The central nervous system actions of ANG II on baroreflex function were verified via a "rescue study" in which ANG II was acutely increased to control plasma concentrations in *day 19* embryos that were chronically ACE inhibited. This manipulation was conducted to answer

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two questions: *1*) was MAP increased back to resting control levels? and *2*) was the baroreflex reset to control conditions? We hypothesized that ANG II interacts with embryonic baroreflex function and that the absence of ANG II would result in a baroreflex reset at a lower pressure and an increase in the gain of the reflex. We also predicted that infusing embryos with ANG II on *day 19* after chronically removing the hormone would raise the pressure and reset the baroreflex to control levels. We assessed baroreflex function using two methods: a conventional static method that examined the maximal f_H response associated with a pharmacologically induced change in MAP and a dynamic method that examined beat-to-beat changes during the MAP change.

MATERIALS AND METHODS

Egg source and incubation. Fertilized white leghorn chicken eggs (*Gallus gallus domesticus*) were purchased from Texas A&M University (College Station, TX) and shipped to the University of North Texas (UNT; Denton, TX). Eggs were weighed (± 0.1 g; Denver Instrument, Denver, CO) and placed in an incubator (model 1502; G.Q.F. Manufacturing, Savannah, GA) maintained at 38 ± 0.5°C and 55% relative humidity, and eggs were turned automatically every 3 h. All experiments were carried out according to approved UNT Institutional Animal Care and Use Committee protocols 909 and 1212–20.

Captopril administration. Captopril, an ACE inhibitor (MP Biomedicals, Solon, OH), was injected into eggs daily at 1500 from *days 5* to *18* of incubation. Prior to injection on *day 5*, embryo viability was checked via candling, the air cell was marked, and the eggs were then moved to a bench-top incubator (model 1602N; Hova-bator; G.Q.F. Manufacturing). The shell surface over the air cell was wiped with 80% EtOH, and a small hole (0.2-mm diameter) was made through the shell using a 20 G needle. A solution of captopril [5 mg/kg (23 μmol/kg) embryo wet mass] dissolved in 0.9% NaCl sterile saline was injected into the air cell using a glass syringe (Hamilton, Reno, NV). The shell was again wiped with 80% EtOH and the hole sealed with silicone gel (DAP Products, Baltimore, MD). The egg was returned to the bench top incubator until the silicone had dried (~1 h), before being returned to the main incubator. On subsequent injection days, eggs were candled, and mortality was recorded. Injections were administered through the silicone seal, and the egg surface was wiped with 80% EtOH.

Injection volume of the captopril solution was maintained between 10 and 100 μl, using 0.1 and 1 mg/ml concentrations, depending on embryo mass. The correct volume to achieve a 5 mg/kg dosage was based on estimated embryo wet mass for each developmental day (43). 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.

Vascular catheterization and experimental setup. On *day 19*, eggs were candled to locate a tertiary chorioallantoic membrane (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 heat-pulled PE-50 tubing filled with heparinized 0.9% NaCl saline under a dissecting microscope (Leica M60, Leica Microsystems, Waukegan, IL), as described previously (11, 50). The occlusively implanted catheter was glued to the egg shell (Duro quick gel), and the egg was placed in a multichambered water-jacketed stainless-steel experimental apparatus (one egg per chamber) and left to stabilize for 1 h. The experimental apparatus was maintained at 38 ± 0.5°C via a constant temperature circulator (Julabo F32, Julabo, Seelbach, Germany). Each chamber was fitted with a lid containing small openings for the catheter and air flow (200 ml/min), which was prewarmed 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; ADInstruments, Colorado Springs, CO) connected to a bridge amplifier (ML228 octal bridge; ADInstruments), and the pressure signal was recorded using a PowerLab data acquisition system (ADInstruments) and LabChart 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. f_H was continuously derived from the pressure signal. Two experiments were conducted to establish the impact of chronic ACE inhibition on baroreflex function and to determine whether the chronically ACE-inhibited embryos could be "rescued" via continuous ANG II infusion.

ACE inhibition and baroreflex. The cardiac limb of the baroreflex was examined using the Oxford method of pharmacological manipulation of MAP in control and captopril-treated embryos. A series of increasing doses [25, 50, 100, 150, and 200 μg/kg (0.08, 0.17, 0.34, 0.50, and 0.67 μmol/kg) of wet embryo mass] of sodium nitroprusside (SNP; Sigma), a vasodilator that acts via the release of nitric oxide, was used to assess baroreflex response to a decrease in systemic pressure. This was followed by a series of doses [25, 50, 100, 150, and 200 μg/kg (0.12, 0.25, 0.49, 0.74, and 0.98 μmol/kg)] of a vasoconstrictor, phenylephrine (PE; Sigma), to assess baroreflex response to an increase in pressure. Drugs were administered via a Y connector in the arterial catheter line. Each drug injection was followed by a saline flush comprising twice the volume of the drug mixture. Total drug injection volumes were <5% of total blood volume, which was ~3 ml on *day 19* (44). Cardiovascular variables were allowed to return to preinjection levels (at least 1 h) between each dose.

ANG II rescue and baroreflex. For the ANG II infusion study on *day 19*, control embryos and chronically captopril-treated embryos were instrumented with both an arterial and a venous catheter in CAM vessels, as described above. The arterial line was connected to the pressure transducer and data acquisition system, as previously described. The venous catheter was used for the infusion via PE-50 tubing connected to a 250-μl Hamilton glass syringe housed on an infusion pump (PhD Ultra 70–3007; Harvard Apparatus, Holliston, MA). The infusion pump housed four syringes, allowing four eggs to be infused simultaneously.

In a preliminary experiment to assess the effects of infused fluid volume on resting MAP and f_H, untreated *day 19* embryos were infused with 0.9% heparinized NaCl sterile saline. The venous catheter was first flushed at a rate of 2.5 μl/min for 15 min before a constant infusion of 0.5 μl/min was run for 6 h.

For the ANG II infusion, the venous catheter was first flushed with chicken ANG II (Bachem) in 0.9% heparinized NaCl sterile saline at 131 ng·kg^{-1}·min^{-1} (0.13 nmol·kg^{-1}·min^{-1}) for ~12 min to clear the heparinized saline, as calculated on the basis of the internal diameter and length of the PE tubing. The infusion was then set at a constant rate of 26 ng·kg^{-1}·min^{-1} (0.025 nmol·kg^{-1}·min^{-1}). With the knowledge that the chronic captopril treatment completely blocked ACE (Mueller CA, Crossley II DA, Burggren WW, unpublished data), we assumed that the captopril-treated embryos had no ANG II in their blood. Accordingly, the concentration of ANG II infused was calculated on the basis of plasma ANG II levels from untreated *day 19* embryos from Crossley et al. (10).

After 1 h of infusion, the baroreflex injection protocol was begun with 25 μg/kg of SNP administered via the arterial line. Subsequently, 100 and 200 μg/kg SNP and 25, 100, and 200 μg/kg PE were injected, with MAP and f_H allowed to return to preinjection levels (at least 1 h) between each dose. Once the baroreflex protocol was completed, the infusion was stopped, and MAP and f_H were allowed to stabilize for 1 h. An injection of 2 μg/kg (1.94 μmol/kg) ANG II was given via the venous catheter to verify that venous injections would produce a change in MAP similar to that following arterial injection. The ANG

II was stored under the same bench top conditions (20°C) as the ANG II being infused.

Data analysis. Differences in resting MAP and f_H between noninfused and ANG II-infused control and captopril-treated embryos were tested using a one-way ANOVA with Tukey highly significant difference (HSD) in SigmaStat 3.5 (Systat Software, Chicago, IL). Changes in MAP and f_H during the ANG II infusion in control and captopril-treated embryos were tested using two-way repeated-measures ANOVA with Tukey HSD, with time and treatment as the main effects.

To assess baroreflex function, two methods were used: a conventional "static" method and a "dynamic" method. To employ the static method, the maximum/minimum MAP and corresponding maximum reflex change in f_H after each drug injection were extracted from the data. The MAP and f_H values obtained across all of the SNP and PE injections were plotted against each other for each individual embryo. Only injections that showed a reflex response were used in analyses. The corresponding MAP and f_H were analyzed for each embryo using a four-variable sigmoidal logistic function (38):

$$f_H = \frac{(\max - \min)}{(1 + (\mathrm{MAP}/C)^B)} + \min$$

where max and min are the maximum and minimum f_H (beats/min), respectively, attained by the baroreflex, B is the maximum slope of the linear portion of the curve (beats·min^{-1}·kPa^{-1}), and C is the MAP (kPa) when f_H is at the midpoint of its range. The best fit was determined, as previously described (2) using the quasi-Newton iterative method in the nonlinear estimation module of Statistica 10 (StatSoft, Tulsa, OK).

The dynamic method examined reflex responses within the pressure change after each injection. A section of the data trace in which MAP increased or decreased and f_H reflexively decreased or increased was selected. Beat-to-beat changes within this range were visualized by plotting cycle height (kPa) against cycle duration (s) in the pressure module in LabChart Pro 7 (ADInstruments). The four-variable sigmoidal logistic function (described above) was fitted to the dynamic changes in MAP and f_H. The min and max f_H, B, and C values were determined for each injection response and were then averaged across the injections to produce mean values for each embryo. This method represented a simple derivative of previous studies that used mathematical modeling to examine dynamic beat-to-beat changes in f_H (52) and sympathetic nerve activity (25–27) in adult mammals.

Gain (G_{50}) of the baroreflex when MAP = C was calculated for both the static and dynamic method as

$$G_{50} = \mathrm{ABS}\left(\frac{-B(\max - \min)}{4C}\right)$$

For comparison between treatments, the gain was normalized as a percentage change in f_H per unit change in MAP (G; %/kPa), and adapted to the four-variable sigmoid model (2, 4):

$$G = \frac{100B}{\min}$$

Differences in baroreflex variables were tested with a two-way ANOVA with Tukey HSD, with treatment and baroreflex method as the main effects. $P < 0.05$ was used for all statistics.

RESULTS

Survival, resting MAP, and f_H. Chronic captopril treatment had no effect on embryonic survival. Survival to *day 19* averaged 74 ± 7% in captopril-treated embryos and 68 ± 8% survival in controls (Student's *t test*, $P = 0.59$).

In the noninfused group, MAP of the captopril-treated embryos was 15% lower than controls ($P = 0.032$, one-way ANOVA, Tukey HSD, Table 1). In the ANG II rescue group prior to the infusion, MAP of the captopril-treated infused embryos was 1.74 ± 0.06 kPa, which was also significantly hypotensive (19%) compared with the controls (2.16 ± 0.15 kPa) for this study group ($P = 0.015$). Prior to infusion, resting MAP did not differ between the two control treatments or the two captopril treatments. f_H of the noninfused captopril-treated embryos was significantly lower than the controls ($P = 0.015$, one-way ANOVA, Tukey HSD, Table 1). Prior to the infusion, f_H of the infused captopril-treated embryos (225 ± 11 beats/

Table 1. *Mean arterial pressure, heart rate, and baroreflex variables in day 19 chicken control embryos and embryos chronically treated with captopril without and with an acute infusion of ANG II*

	Non-ANG II-Infused		ANG II-Infused	
	Control	Captopril	Control	Captopril
n	10	11	6	6
MAP, kPa	2.27 ± 0.11^{ab}	1.93 ± 0.11^{c}	2.43 ± 0.09^{a}	2.08 ± 0.14^{bc}
f_H, beats/min	284 ± 6^{a}	257 ± 6^{b}	249 ± 7^{b}	241 ± 10^{b}
Static baroreflex variables				
max f_H, beats/min	307 ± 9^{a}	283 ± 7^{ab}	285 ± 4^{ab}	273 ± 6^{b}
min f_H, beats/min	259 ± 5^{a}	245 ± 8^{a}	250 ± 6^{a}	244 ± 3^{a}
B, beats·min^{-1}·kPa^{-1}	5.0 ± 0.5^{a}	7.0 ± 0.7^{b}	6.6 ± 0.9^{ab}	5.6 ± 1.0^{ab}
C, kPa	2.3 ± 0.2^{a}	1.9 ± 0.1^{a}	2.2 ± 0.1^{a}	1.9 ± 0.1^{a}
gain, G_{50}, beats·min^{-1} ·kPa^{-1}	136 ± 25^{a}	122 ± 16^{a}	122 ± 21^{a}	78 ± 18^{a}
normalized gain, G, %/kPa	1.9 ± 0.2^{a}	2.9 ± 0.3^{b}	2.6 ± 0.4^{ab}	2.4 ± 0.4^{ab}
Dynamic baroreflex variables				
max f_H, beats/min	291 ± 7^{a}	277 ± 6^{ab}	269 ± 6^{ab}	260 ± 11^{b}
min f_H, beats/min	278 ± 8^{a}	264 ± 6^{a}	256 ± 5^{a}	249 ± 11^{a}
B, beats·min^{-1}·kPa^{-1}	52.1 ± 6.5^{a}	72.5 ± 6.6^{b}	35.4 ± 2.1^{a}	35.9 ± 6.3^{a}
C, kPa	2.4 ± 0.2^{a}	2.2 ± 0.2^{a}	2.4 ± 0.1^{a}	2.2 ± 0.2^{a}
gain, G_{50}, beats·min^{-1}·kPa^{-1}	386 ± 60^{a}	535 ± 82^{a}	275 ± 48^{ab}	193 ± 38^{b}
normalized gain, G, %/kPa	18.8 ± 2.3^{a}	27.8 ± 2.7^{b}	13.8 ± 0.9^{a}	14.1 ± 2.0^{a}

Baroreflex variables were determined by two methods: static and dynamic (see METHODS for details). Mean arterial pressure (MAP) and heart rate (f_H) for ANG II-infused treatments were taken 1 h into infusion. min and max f_H: minimum and maximum f_H attained by the baroreflex. B: maximum slope of the linear portion of the curve. C: MAP (kPa) when f_H is at the midpoint of its range. Data are presented as means ± SE. [a,b,c]Different letters indicate significant differences [$P < 0.05$, two-way ANOVA, Tukey highly significant difference (HSD) test between treatments].

min) was not different from the controls (234 ± 8 beats/min). f_H was significantly lower in both the ANG II rescue control and captopril-treated embryos prior to infusion compared with the noninfused groups ($P < 0.01$).

ANG II rescue. The total fluid volume of the preliminary 0.9% heparinized NaCl sterile saline infusion was 0.22 ml, 7% of total blood volume. This fluid load had no effect on MAP or f_H at any time point during the 6-h infusion ($P > 0.05$, one-way repeated-measures ANOVA). Similarly, the total fluid volume of the ANG II infusion was 0.23 ml, <8% of blood volume. Infusion with ANG II induced significant changes in MAP and f_H in both control and captopril-treated embryos. After 1 h of infusion, MAP had significantly increased by 14% in control embryos and 16% in captopril-treated embryos compared with preinfusion levels ($P < 0.05$, two-way repeated-measures ANOVA; Tukey HSD, Fig. 1*A*). The captopril-treated embryos were still significantly hypotensive compared with controls ($P < 0.05$). f_H did not significantly increase in either treatment 1 h into the infusion ($P > 0.05$), and f_H did not differ between the treatments ($P = 0.54$; Fig. 1*B*).

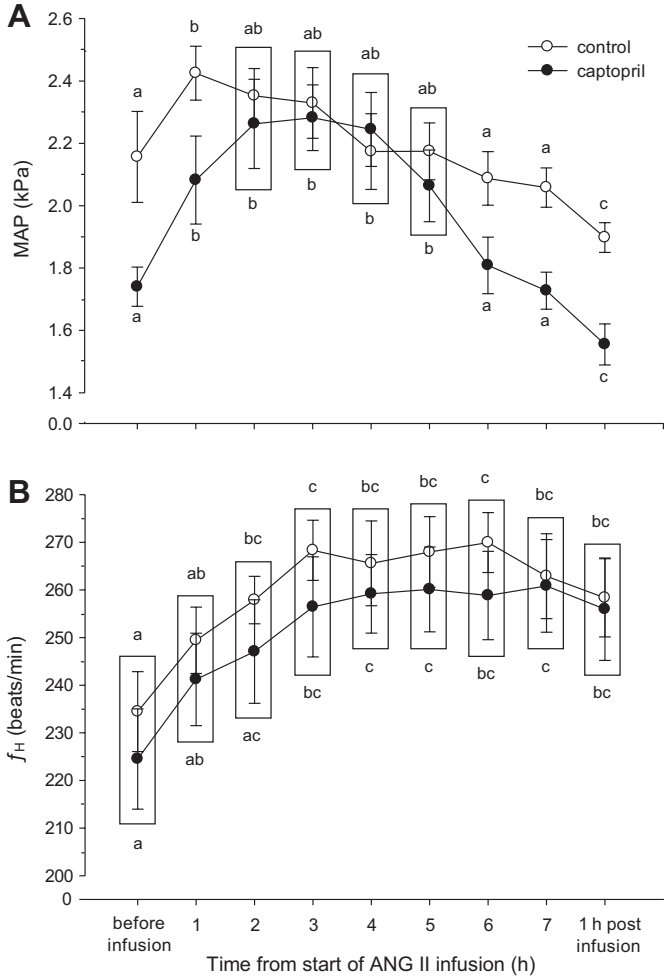

Fig. 1. Changes in mean arterial pressure (MAP; kPa) (*A*) and heart rate (f_H, beats/min) (*B*) of *day 19* control chicken embryos and embryos chronically treated with captopril during infusion with ANG II. Symbols represent mean values. Error bars indicate 1 SE. [a,b,c]Different letters indicate significant differences ($P < 0.05$) between times within each treatment. Treatment means at each exposure time that are not significantly different are grouped within boxes.

The ANG II rescue increased resting MAP in the infused captopril-treated embryos, so that it was not significantly different from the resting MAP of noninfused controls ($P = 0.67$; Table 1). However, the ANG II infusion did not significantly raise MAP of the infused control embryos higher than the noninfused controls ($P > 0.05$). f_H in the noninfused control embryos remained significantly higher than the other three treatments ($P > 0.05$).

During the infusion, MAP remained elevated in captopril-treated embryos, reaching a peak elevation from preinfusion levels of 24% at 3 h. However, at 6 h, MAP was no longer higher than preinfusion levels ($P = 0.84$, two-way repeated-measures ANOVA, Tukey HSD, Fig. 1*A*). Controls were no longer significantly different from their preinfusion level after 2 h ($P = 0.34$). From 2 to 5 h, MAP of the treatments did not differ, but at 6 and 7 h, the captopril-treated embryos were once again relatively hypotensive ($P = 0.024$). After 2 h, f_H was significantly elevated from preinfusion levels in both control and captopril-treated embryos, with no difference between the treatments (Fig. 1*B*). At the end of the infusion (7 h), f_H was still significantly elevated by 12 and 17% in control and captopril-treated embryos, respectively ($P < 0.001$; Fig. 1*B*).

One hour after the infusion was stopped, MAP dropped by 11% in both control and captopril-treated embryos ($P < 0.05$). Captopril-treated embryos were still hypotensive compared with controls ($P = 0.019$). There was no significant change in f_H in either treatment 1 h postinfusion. Baseline MAP prior to each baroreflex dose did not differ in either treatment until the last PE injection (200 μg/kg) given at 6 h (Fig. 1*A*). Therefore, this injection response was excluded from the baroreflex analyses. An acute injection of ANG II after the infusion produced a characteristic significant increase in MAP in control and captopril-treated embryos (paired *t*-tests; $P < 0.001$). However, f_H did not significantly change in either treatment ($P > 0.05$).

Baroreflex. Control saline injections equivalent to the baroreflex drug injection volume (150 μl) had no effect on MAP or f_H in any of the treatments, either with or without the ANG II infusion ($P > 0.05$, paired *t*-tests).

SNP induced a reciprocal increase in f_H in all treatments with and without the ANG II infusion (Fig. 2*A*). The number of embryos that responded was consistent across treatments and increased with increasing dose, from 20% at 25 μg/kg to 71% at 200 μg/kg. MAP after injection of 25 μg/kg SNP was higher in the infused control embryos than the other groups, due to the higher baseline MAP in this group (Fig. 3*A*). However, this effect was washed out over the higher SNP doses. PE induced a reciprocal decrease in f_H that occurred in an average of 38% of embryos in all treatments at all doses (Fig. 2*B*). The maximum MAP reached in response to PE did not vary between treatments, despite differences in baseline MAP (Fig. 3*B*). The percentage of embryos that showed reflex responses was similar to the percentage previously reported for embryonic chickens on *days 19* and *20* of incubation (1).

The static method for assessing baroreflex indicated that the baroreflex variables differed significantly between the four treatments. Maximum f_H was significantly higher in the noninfused control group compared with the infused captopril-treated group ($P = 0.02$, two-way ANOVA, Tukey HSD, Table 1), but minimum f_H did not differ between the treatments ($P > 0.05$). The slope of the steepest part of the

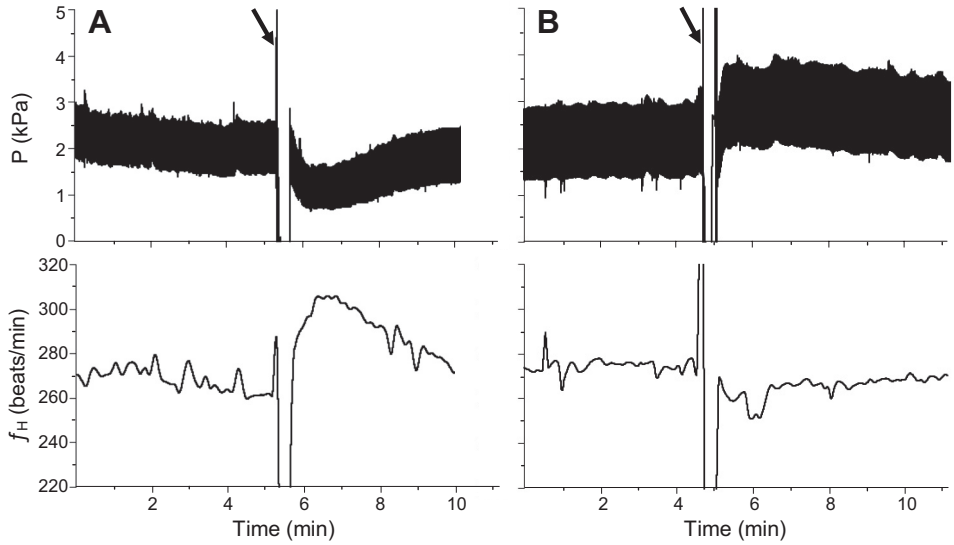

Fig. 2. Representative traces demonstrating the pharmacologically induced change in pressure (P, kPa) and resultant reflex response in heart rate (f_H, beats/min) after injection of sodium nitroprusside (200 μg/kg embryo wet mass) (*A*) and phenylephrine (50 μg/kg) (*B*) in a *day 19* control embryo. The bracket indicates 5 min, and the arrows indicate injection.

baroreflex curve, *B*, was significantly higher in noninfused captopril-treated embryos compared with noninfused controls ($P = 0.032$). This difference disappeared with the infusion study, and *B* in both ANG II infusion groups was intermediate between the noninfused groups (Fig. 4). MAP at the midpoint of the curve, *C*, did not differ between the four treatments ($P = 0.100$). The gain of the baroreflex when MAP is equal to *C*, G_{50}, also did not differ between the treatments ($P = 0.267$). However, normalized gain, *G*, was significantly higher in the noninfused captopril-treated embryos compared with noninfused controls ($P = 0.017$), but not different from the two infused treatments, which had intermediate *G* values.

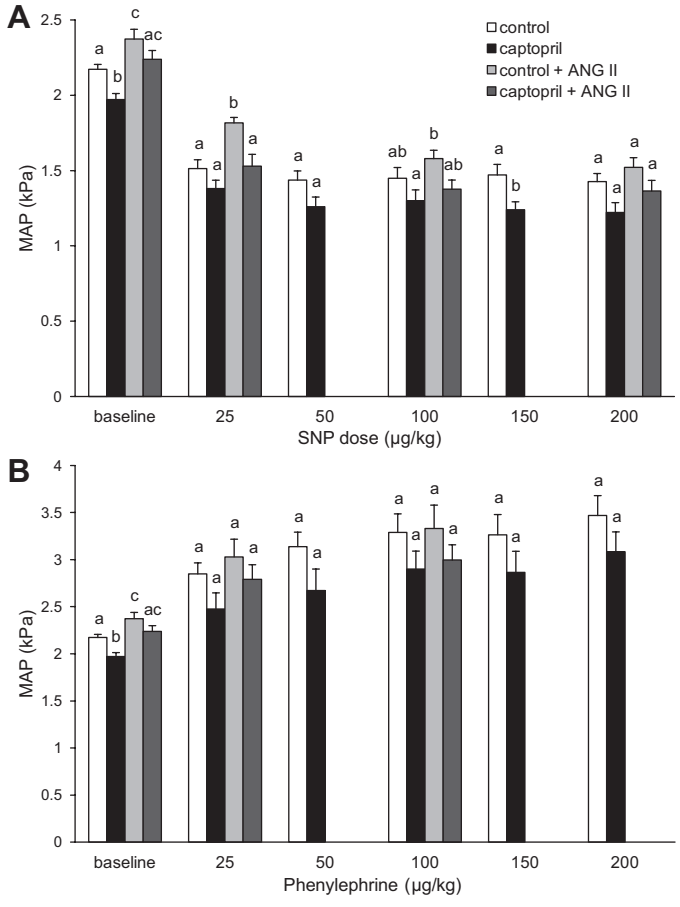

Fig. 3. Baseline MAP and responses to increasing doses of sodium nitroprusside (*A*) and phenylephrine (*B*) in *day 19* control embryos and embryos chronically treated with captopril with and without an infusion of ANG II. Baseline MAP did not differ prior to each dose and so was combined for all doses. Bars represent means ± SE. [a,b,c]Different letters indicate significant differences ($P < 0.05$) between treatments within each dose.

The dynamic method for assessing baroreflex also indicated significant differences in baroreflex variables between the four treatments. Maximum f_H was significantly higher in the noninfused control embryos compared with the infused captopril-treated embryos ($P = 0.04$; two-way ANOVA, Tukey HSD, Table 1), but minimum f_H and *C* did not differ between the treatments ($P > 0.05$). However, *B* was significantly higher in the noninfused captopril-treated embryos compared with the other treatments ($P < 0.05$). G_{50} was significantly lower in the infused captopril-treated group compared with the two noninfused groups ($P < 0.05$). Normalized gain, *G*, was significantly higher in the noninfused captopril-treated embryos compared with the other three treatments ($P < 0.05$), which did not differ.

Comparing the two baroreflex analytical methods, maximum f_H did not differ in any treatments, but minimum f_H determined by the dynamic method was significantly higher than the static method in the noninfused captopril-treated group (two-way ANOVA, Tukey HSD, Table 2). *C* did not differ between the two methods in any treatments. However, the dynamic method produced significantly higher *B*, G_{50}, and *G* values compared with the static method in all treatments (Table 2).

DISCUSSION

ANG II plays an integral role in both acute and long-term adjustments to deviations in central arterial pressure in adult organisms. Here, we demonstrated that during embryonic development, this regulatory hormone has similar actions on the cardiovascular system. Our findings indicated that the innate environment of elevated plasma ANG II during chicken embryonic development instills a chronic adjustment of baroreflex function, dampening its regulatory capacity. Overall, in *day 19*

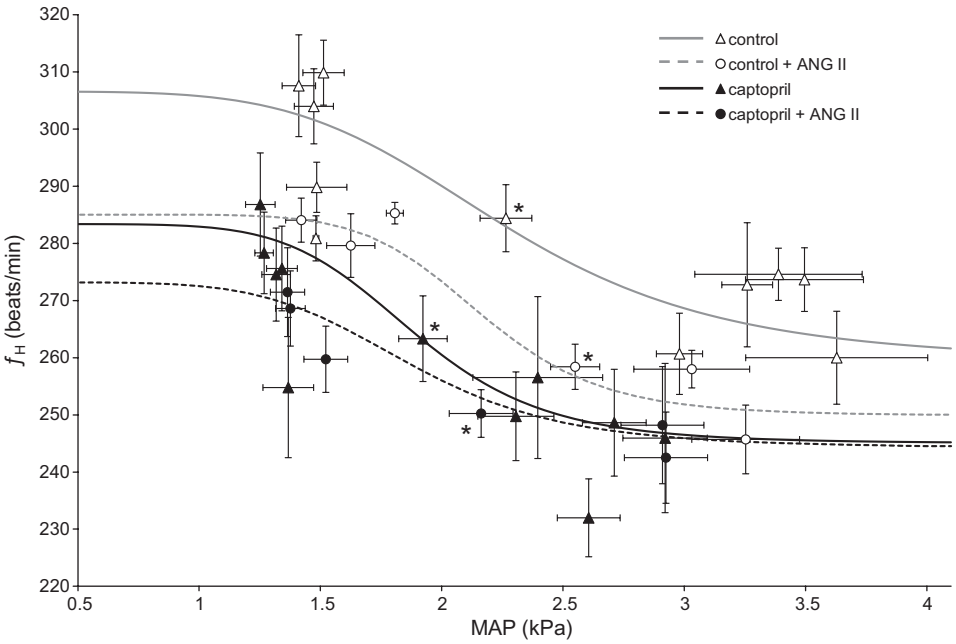

Fig. 4. Baroreflex curve estimation for the relationship between mean arterial pressure (MAP; kPa) and heart rate (f_H; beats/min) in *day 19* control embryos and embryos chronically treated with captopril with and without an infusion of ANG II. Data are presented as the means ± SE for the maximal response in MAP and f_H after injection of sodium nitroprusside or phenylephrine. Data points with asterisks indicate resting values. Curves represent a four-variable sigmoidal logistic function (see MATERIALS AND METHODS for details) determined from the calculated mean static baroreflex parameters of each treatment.

chicken embryos, ANG II reduced baroreflex sensitivity and increased resting MAP, and, therefore, the baroreflex operating point. Thus, as documented during mammalian growth restriction models, ANG II contributes to vascular tone in embryonic chickens. This is achieved, in part, through ANG II modulating baroreflex function, resulting in an increase in MAP without accompanying adjustments in f_H.

ANG II rescue. ANG II infusion successfully increased MAP in both control and captopril-treated embryos on *day 19*. The increase in MAP during infusion negated the 19% difference between the control and captopril-treated embryos prior to the infusion, bringing the captopril-treated embryos up to control pressure (Table 1). The captopril-treated embryos responded to the ANG II infusion with a greater, sustained increase in MAP compared with controls (Fig. 1*A*). The stronger response of captopril-treated embryos is likely due to the absence of endogenous ANG II, a result of ACE inhibition. Likewise, captopril-treated embryos showed a greater relative increase in MAP in response to an acute dose of exogenous ANG II in our previous study (Mueller CA, Crossley II DA, Burggren WW, unpublished data).

Table 2. *Differences in baroreflex variables determined by the dynamic method compared to the static method in day 19 control embryos and embryos chronically treated with captopril without and with an acute infusion of ANG II*

	Non-ANG II-Infused		ANG II-Infused	
	Control	Captopril	Control	Captopril
max f_H, beats/min	□	□	□	□
min f_H, beats/min	□	↑	□	□
B, beats·min^{-1}·kPa^{-1}	↑	↑	↑	↑
C, kPa	□	↑	□	□
gain, G_{50}, beats·min^{-1}·kPa^{-1}	↑	↑	↑	↑
normalized gain, *G*, %/kPa	↑	↑	↑	↑

↑ indicates that the value predicted by the dynamic method is significantly higher ($P < 0.05$) than the value determined by the static method (two-way ANOVA, Tukey HSD test), and □ indicates no difference ($P > 0.05$). min and max f_H, minimum and maximum f_H attained by the baroreflex; *B*, maximum slope of the linear portion of the curve; *C*, MAP (kPa) when f_H is at the midpoint of its range.

The decline in MAP that occurred after 1 h into the infusion in control embryos and from 5 h in captopril-treated embryos may be the result of a number of effects. First, the catheterized vein may have closed off and blocked the infusion. This is highly unlikely, as all embryos showed the same response, and an acute dose of ANG II given through the venous catheter at the end of the infusion produced a normal pressor response in all embryos. Second, the ANG II may have degraded. However, the acute dose of ANG II was stored under the same laboratory bench conditions as the ANG II being infused, and the normal pressor response indicated it did not degrade during the 7-h experiment. Third, the increase in f_H, and maintenance of tachycardia throughout the infusion, may have created a reflex response, driving MAP back to preinfusion levels. A secondary messenger from the central nervous system, such as nitric oxide, may have altered vascular tone and caused vasodilation. MAP of both treatments decreased further once the infusion was stopped. This may have represented a continued decline in MAP, or it may have indicated that ANG II was still causing vasoconstriction. Despite a slow decline in MAP during the infusion, baseline pressure did not vary significantly in either treatment until 6 h (Fig. 1*A*). Therefore, the infusion maintained MAP for the majority of the baroreflex protocol.

Static vs. dynamic baroreflex. This study utilized two analytical methods for assessing reflex responses in f_H, a conventional static method and a dynamic method. The widely used static method uses the maximal response in MAP and f_H after each injection to create a curve across the series of doses given (Fig. 4). This method assumes that a change in MAP is associated with a single response in f_H (52). The static method is also reliant upon each increasing dose of SNP or PE inducing a greater MAP response, so that the baroreflex curve can be formed over a range of pressure, which did not always occur in these embryos (Fig. 3). In comparison, the dynamic method

examines beat-to-beat changes that occur during pharmacological manipulation of pressure. The dynamic method has the potential advantage over the static method in that it yields more data points, not just maximal responses to each injection, potentially resulting in a more accurate curve fit for the relationship between MAP and f_H. However, each drug dose did not consistently yield a greater change in MAP, and a curve could not be built from that data. Instead, the sigmoidal logistic function was fitted to the changes in MAP and f_H within each dose, and the resultant dynamic baroreflex variables were averaged across multiple doses for each embryo. This had consequences for the dynamic baroreflex variables generated, as discussed below.

The most notable differences in the baroreflex variables determined by the dynamic method compared with the static method were the much higher slope (B), gain (G_{50}), and normalized gain (G) values (Tables 1 and 2). These higher values were due to the smaller MAP and f_H range over which the four-variable sigmoidal logistic function was calculated. Additionally, while the maximum f_H did not statistically differ between the two methods, and minimum f_H only differed in the noninfused captopril treatment, the f_H range determined by the dynamic method was smaller. For example, in the noninfused control group, the average f_H range determined by the static method was 48 beats/min, but the range using the dynamic method was 13 beats/min (Table 1). This may indicate that the dynamic method overestimated the slope of the curve and, therefore, the normalized gain. Despite the order-of-magnitude higher values, the pattern of the slope and normalized gain between treatments was similar when determined by either method.

ANG II and baroreflex control. Chronic removal of ANG II resulted in a reset baroreflex at *day 19* in captopril-treated embryos. Both the slope of the steepest part of the curve (B) and the normalized gain (G) were higher in captopril-treated embryos compared with controls (Table 1), indicating that captopril-treated embryos had increased baroreflex sensitivity. Therefore, ANG II appears to have an attenuating effect on normal baroreflex control of f_H in the developing chicken embryo. The calculated MAP when f_H was at the midpoint of its range, and the slope of the curve was steepest, C, was not significantly shifted. This was due to variability in the f_H ranges of the treatments, which influenced the f_H midpoint. However, ANG II did affect the operating point of the baroreflex, as evidenced by the significantly lower resting MAP and the left-shifted curve of the captopril-treated embryos (Table 1, Fig. 4).

ANG II rescue, which reset hypotensive captopril-treated embryos back to the control pressure state, also influenced baroreflex function. In the presence of the ANG II infusion, the slope and normalized gain of the captopril-treated embryos were no longer significantly higher than control embryos (Table 1). Therefore, ANG II infusion decreased baroreflex sensitivity in captopril-treated embryos, as similarly documented during ANG II infusion in mammals (7, 20, 33, 40). Furthermore, the finding that ANG II rescue could somewhat reverse the changes in baroreflex function induced by ACE inhibition was consistent with previous studies in which baroreflex changes were observed when ANG II was infused and then subsequently stopped in adult mammals (3, 6).

ACE inhibition increased baroreflex sensitivity, and ANG II rescue of captopril-treated embryos decreased sensitivity. Consequently, we also expected a decrease when control embryos were infused with additional ANG II. However, the slope and normalized gain of ANG II-infused control embryos were not significantly different from the noninfused control embryos (Table 1). This may be due to the fact that the MAP of control embryos did not remain elevated during the entire infusion. Furthermore, control embryos already have high levels of plasma ANG II compared with adult birds (10, 18, 28), and, therefore, additional ANG II may not have attenuated baroreflex sensitivity further.

The changes in baroreflex sensitivity reported in the current study are somewhat different to previously published reports on the influence of ANG II on baroreflex regulation. Many studies in adult animals found that ANG II altered the operating point but did not change baroreflex sensitivity (7, 13, 17, 21, 33, 34, 40), while some found a change in sensitivity (16, 20, 29). It is important to note that this study dealt with developing animals that, because of their naturally high plasma ANG II, may be more strongly influenced by ANG II than adults. Therefore, the removal of ANG II significantly increased baroreflex sensitivity, despite the relative immaturity of this reflex at incubation *day 19* in these animals (1, 15).

In comparison to embryonic chickens, no changes in either baroreflex sensitivity or operating point were found in fetal sheep after ACE inhibition (47). However, this difference may be due to the high plasma ANG II of chicken embryos compared with fetal sheep (10, 23, 41), which lowers baroreflex gain in control embryos compared with both adult chickens and fetal sheep (1). The difference in ANG II concentration may also account for the late onset of baroreflex function in chicken embryos compared with fetal sheep (46, 49). Furthermore, ANG II plasma levels are also elevated during hypoxemia (8, 19), a chronic condition in growth-restricted fetal sheep, and ANG II is implicated in the maintenance of fetal MAP and programming of adult hypertension in fetal growth restriction models (14, 48). Therefore, the elevated plasma ANG II of chicken embryos can be viewed as analogous to a hypertensive state, and chronic ACE inhibition removed the attenuating effect of ANG II on embryonic baroreflex regulatory capacity.

There are a number of possible mechanisms behind the effect of ANG II on baroreflex control. Most adult studies suggest withdrawal of vagal tone on the heart rather than an increase in sympathetic output as the primary mechanism of ANG II's action (22, 31, 40). We have previously shown in chronically captopril-treated embryos that sympathetic activity contributed to the ANG II pressor response (Mueller CA, Crossley II DA, Burggren WW, unpublished data). However, ganglionic blockade with hexamethonium indicated that this increased sympathetic activity was from a peripheral rather than a central source, and ANG II is believed to act centrally to reset baroreflex control (39). Furthermore, endogenous ANG II had no influence on α-adrenergic tone in chicken embryos and f_H of captopril-treated embryos increased in response to an acute injection of hexamethonium. These data suggested ACE inhibition released central inhibition of vagal tone on the heart (Mueller CA, Crossley II DA, Burggren WW, unpublished data). Our results support the speculation that ANG II may

alter the baroreflex without changes in sympathetic output, while suppressing vagal activity. If ANG II suppresses vagal tone and natural plasma levels in embryos are high, ANG II may contribute to the general lack of vagal tone in chicken embryos (9).

Perspectives and Significance

This study is the first to examine the effect of ANG II on baroreflex function in a nonmammalian embryo. Using chronic ACE inhibition, we demonstrated that the elevated plasma ANG II of chicken embryos decreased the sensitivity and increased the operating pressure of baroreflex control in *day 19* chicken embryos, indicating an impact of ANG II on the central control of the reflex. Furthermore, we illustrated how an ANG II rescue, via infusion of ANG II into the CAM vasculature, raised resting MAP and altered the baroreflex of chronically captopril-treated embryos back to control conditions. Therefore, it is via its influence on short-term baroreflex regulation that ANG II enacts some of its long-term influence on baseline embryonic MAP without altering f_H. The dampened baroreflex regulatory capacity in the presence of the natural high-plasma ANG II levels of chicken embryos is similar to the hypertensive states created in mammalian models. The high plasma levels create an interaction between ANG II and baroreflex regulation in chicken embryos not found in mammalian fetuses. Therefore, plasma concentration may influence whether a relationship is detected between ANG II and baroreflex function and must be a consideration of future baroreflex studies. The high-plasma ANG II of the chicken embryo provides a unique opportunity to examine developmental cardiovascular function in a natural ANG II-induced hypertension model.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.A.M., W.W.B., and D.A.C. conception and design of research; C.A.M. and D.A.C. performed experiments; C.A.M. analyzed data; C.A.M. and D.A.C. interpreted results of experiments; C.A.M. prepared figures; C.A.M. drafted manuscript; C.A.M., W.W.B., and D.A.C. edited and revised manuscript; C.A.M., W.W.B., and D.A.C. approved final version of manuscript.

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