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Prenatal hypoxia programs changes in β -adrenergic signaling and postnatal cardiac contractile dysfunction

I. Lindgren and J. Altimiras

AVIAN Behavioural Genomics and Physiology, IFM Biology, Linköping University, Linköping, Sweden

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Lindgren I, Altimiras J. Prenatal hypoxia programs changes in β -adrenergic signaling and postnatal cardiac contractile dysfunction. *Am J Physiol Regul Integr Comp Physiol* 305: R1093–R1101, 2013. First published October 2, 2013; doi:10.1152/ajpregu.00320.2013.—Prenatal hypoxia leads to an increased risk of adult cardiovascular disease. We have previously demonstrated a programming effect of prenatal hypoxia on the cardiac β -adrenergic (β AR) response. The aim of this study was to determine 1) whether the decrease in β AR sensitivity in prenatally hypoxic 5-wk old chicken hearts is linked to changes in β_1 AR/ β_2 ARs, $G_{\alpha i}$ expression and cAMP accumulation and 2) whether prenatal hypoxia has an effect on heart function in vivo. We incubated eggs in normoxia (N, 21% O₂) or hypoxia from day 0 (H, 14% O₂) and raised the posthatchlings to 5 wk of age. Cardiac β_1 AR/ β_2 ARs were assessed through competitive binding of [³H]CGP-12177 with specific β_1 AR or β_2 AR blockers. $G_{\alpha s}$ and $G_{\alpha i}$ proteins were assessed by Western blot and cAMP accumulation by ELISA. Echocardiograms were recorded in anesthetized birds to evaluate diastolic/systolic diameter and heart rate and tissue sections were stained for collagen. We found an increase in relative heart mass, β_1 ARs, and $G_{\alpha s}$ in prenatally hypoxic hearts. cAMP levels after isoproterenol stimulation and collagen content was not changed in H compared with N, but in vivo echocardiograms showed systolic contractile dysfunction. The changes in β AR and G protein subtypes may be indicative of an early compensatory stage in the progression of cardiac dysfunction, further supported by the cardiac hypertrophy and systolic contractile dysfunction. We suggest that it is not the changes in the proximal part of the β AR system that causes the decreased cardiac contractility, but Ca²⁺ handling mechanisms further downstream in the β AR signaling cascade.

β -adrenergic receptors; G proteins; developmental programming; systolic dysfunction; heart failure

THE CONNECTION between low birth weight and increased risk for adult disease is well recognized, and many studies have confirmed the phenomenon of developmental (or fetal) programming of adult cardiovascular disease (1–3, 36). Cardiovascular disease is closely related to changes in β -adrenoceptor (β AR) signaling in the heart (26), but not much is known about how the β AR system is affected by prenatal stress such as hypoxia. We previously showed that the response to vascular β AR is enhanced in embryos incubated in chronic prenatal hypoxia and that chronic prenatal hypoxia decreases β AR sensitivity to epinephrine stimulation in the 5-wk postnatal chicken heart, without changes in receptor density (18). This in

vitro evidence indicates that prenatal hypoxia has a programming effect on β AR signaling in the adult, but the molecular background of the decreased β AR sensitivity in the heart and the in vivo implications of this loss of function are not known.

Classic β AR signaling starts with a conformational change of the receptor in response to catecholamine stimulation. This enables intracellular association of stimulatory G proteins to the receptor that upon the interaction dissociates into an α -subunit ($G_{\alpha s}$) and a $\beta\gamma$ complex. The $G_{\alpha s}$ subunit in turn stimulates adenylyl cyclase (AC) to convert ATP to cAMP and cAMP act on kinases responsible for both altering calcium flux in the cell (leading to increased Ca²⁺ and subsequent contraction in cardiomyocytes) and phosphorylation of the receptor itself, causing desensitization and eventually downregulation of the receptor. Desensitization and downregulation is necessary in the normal heart to control the cardiac response to catecholamine stimulation and protect the cell from overstimulation in case of catecholamine surges. However, the failing heart is subject to increased and prolonged sympathetic stimulation, and the same mechanisms that normally protect the heart may turn to adverse due to the extended loss of cardiac responsiveness (28).

The predominant β AR subtypes in the heart are β_1 ARs and β_2 ARs with an approximate ratio of 70/30 in humans (28). In the failing heart, the β_1 AR/ β_2 AR subtype ratio decreases since β_1 ARs desensitize to a larger extent than β_2 ARs (5, 7). This has implications for the sensitivity of the heart to adrenergic stimulation, since β_2 ARs do not contribute considerably to cardiac contractility (35). Furthermore, increased expression of inhibiting G proteins ($G_{\alpha i}$), that block cAMP production when activated, has been demonstrated in heart failure (26), and there is strong evidence that β_2 ARs are able to cross signal through $G_{\alpha i}$ (11, 14, 34). An increase in both β_2 ARs and $G_{\alpha i}$ could thereby increase the probability of β_2 ARs signaling through $G_{\alpha i}$ and further dampen the cardiac response to β AR stimulation.

The chicken is an excellent model for studying the effects of prenatal conditions on adult cardiovascular function since the chicken embryo, in contrast to mammalian models, is free from any confounding maternal effects.

Our aim with this study was to further investigate the molecular mechanisms behind the previously observed decrease in β AR sensitivity in the heart of 5-wk-old chickens exposed to prenatal hypoxia and to evaluate if the β AR sensitivity decrease is reflected in the in vivo cardiac function. We hypothesized that the β_1 AR/ β_2 AR ratio would be de-

Address for reprint requests and other correspondence: I. Lindgren, IFM Biology, Linköping Univ., SE-58183 Linköping, Sweden (e-mail: isali@ifm.liu.se).

creased due to an increase in β_2 AR in prenatally hypoxic animals, whereas the $G_{\alpha i}$ expression would be increased, resembling the characteristic β AR changes of a failing human heart. As a result of this, we also expected the cAMP concentration to be lower after β AR stimulation and the *in vivo* cardiac response to β AR stimulation to be impaired in the prenatally hypoxic hearts. Additionally, we studied cardiac function in prenatally hypoxic 5-wk animals by echocardiography to see if the decreased contractility and responsiveness to β AR stimulation *in vitro* has any functional (i.e., programming) effects in the postnatal animal.

MATERIALS AND METHODS

Incubation and animal maintenance. All animal procedures were approved by the local Ethical Committee (Linköping, Sweden) diary numbers 22–07 and 26–10. Fertilized Ross 308 broiler eggs were obtained from a local hatchery (Lantmännen SweHatch, Väderstad, Sweden). The eggs were kept at 18°C for no longer than 10 days before incubation. At *day 0*, eggs were set in a commercial incubator (model 25 HS, Masalles Comercial, Barcelona, Spain) at 37.8°C and 45% humidity in either normoxic (N, 21% O₂) or hypoxic (H, 14% O₂) conditions. The oxygen levels were continuously monitored with fuel cell oxygen sensors (MAX-250, Maxtec, Salt Lake City, UT) steering an integrated control unit and gas pump (ROXY-4, Sable Systems International, Las Vegas, NV), which pumped nitrogen into the incubator to keep oxygen levels constant. Chicks hatched on *day 21* in the same environment they were incubated in. All hatchlings were reared in normoxic conditions as previously described (18) until 35 days (5 wk) of age.

At 35 ± 2 days of age, all animal weights were recorded. Some of the animals from both experimental groups were anesthetized with isoflurane for recording of *in vivo* echocardiograms. Birds aimed for molecular analysis of the cardiac tissue were euthanized by decapitation and the ventricles were excised, rinsed in modified Ringer buffer (composition in mM: 138 NaCl, 3 KCl, 1.8 CaCl₂, 1.8 MgCl₂, and 10 HEPES, using Tris-HCl to adjust pH to 7.4), and weighed. For receptor binding and Western blot the left ventricle was dissected, divided into pieces, and flash frozen in liquid nitrogen. Tissue was stored in –80°C for no longer than 6 mo before used. For cAMP measurements, intact hearts were dissected from the carcass and used for cardiomyocyte isolation.

β AR binding assay. We used a radioligand binding assay on intact cardiac tissue punches as previously described (9). Briefly, frozen cardiac tissue ($N = 10$ in all experiments) was embedded in cryomedium and sectioned in 350- μ m slices in a cryostat (Microm HM550, Thermo Fisher Scientific, Waltham, MA). Slices were put in a Petri dish filled with Ringer buffer to obtain tissue punches with a 2-mm diameter sample corer (Fine Science Tools, Heidelberg, Germany). Punches were assayed for total receptor density (saturation assay) or specific β_1 AR/ β_2 AR blocking (inhibition assay) to determine the inhibition concentration at 50% of the binding sites (IC₅₀) of β_1 ARs and β_2 ARs. For saturation binding, punches were incubated with increasing concentrations [³H]CGP-12177 (β AR agonist, 0–2 nM). For the subtype-specific inhibition assay, tissue punches were incubated in 1.5 nM of [³H]CGP-12177 and increasing concentrations (10 nM to 100 μ M) of the β_2 AR blocker ICI-118,551 or β_1 AR blocker CGP-20712A. Nonspecific binding (NSB) was assessed by incubating one set of punches in 1.5 nM [³H]CGP-12177 with a 10 mM addition of the nonspecific β AR antagonist timolol. All incubations took place in darkness for 2 h on ice. After being washed and incubated overnight in scintillation vials with Optiphase Hi-Safe 3 scintillation cocktail (Both from Perkin Elmer, Upplands Väsby, Sweden), punches were counted in a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA) with 60% efficiency for ³H. For every individual heart, tissue punches were set aside for determination of protein

content with a standard protein assay kit (Pierce BCA Protein Assay kit, Pierce Biotechnology, Rockford, IL).

SDS-PAGE and Western blot for assessing $G_{\alpha s}$ and $G_{\alpha i}$ protein content. Frozen pieces of heart tissue ($N = 20$ for both N and H) were lysed in presence of Halt Protease Inhibitor Cocktail and 0.5 mM EDTA (Pierce Biotechnology) and mechanically homogenized on ice. An aliquot of each supernatant was used for protein analysis as specified above for the receptor binding experiment. Samples were diluted to 2.5 μ g/ μ l (suitable concentration was determined in pilot trials), and 15 μ l of each sample was separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and separating gels contained 36% urea and 6% and 14% acrylamide, respectively. A prestained protein ladder (PageRuler, Fermentas/Thermo Fisher Scientific) was loaded in the first lane, and an internal standard consisting of tissue homogenate from chicken brain (aliquots of the same sample used in all runs, same protein concentration as heart samples) was loaded in the last lane. The brain homogenate was used as both positive control for the G protein antibodies and as an internal standard with which the heart samples were compared.

Blotting was performed according to a protocol modified from Towbin et al. (32). The gels were blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ) using a semidry blotting machine (2117 Electrophore II, LKB instruments, Mt Waverley, Victoria, Australia). After blocking was completed, membranes were incubated overnight in 15°C with anti- $G_{\alpha s}$ or anti- $G_{\alpha i}$ primary antibody (1:10,000). The lower part of the membrane containing the band for cytochrome *c* oxidase (COX) was cut off, incubated with an antibody against COX and used as a housekeeper for loading control. After the membranes were washed, they were incubated in a 1:3,000 dilution of secondary antibody at RT. The membranes were developed with the Amersham ECL Plus Western Blot Detection System (GE Healthcare) and visualized with a LAS-4000mini CCD camera (Fujifilm, Tokyo, Japan).

Antibodies. Rabbit anti- $G_{\alpha s}$ and anti- $G_{\alpha i}$ antibodies were purchased from CalbioChem (product nos. 371732-50UL and 371723-50UL, respectively, Merck, Darmstadt, Germany). The rabbit anti-COX IV polyclonal antibody used for loading control was purchased from AbCam (product no. ab14744, Cambridge, UK). The secondary antibody was an ECL Donkey Anti rabbit IgG, peroxidase linked whole antibody and was purchased from GE Healthcare.

Cardiomyocyte isolation, stimulation, and cAMP assay. At 5 wk of age, chickens were weighed and injected intravenously with a bolus of 2,500 IU heparin (LEO 5,000 IU/ml, Apoteket, Sweden). After 10 min the chickens were euthanized by decapitation and the intact heart was removed and weighed. All arteries except for the aorta were tied off with surgical silk to ensure unidirectional flow of the perfusion buffer through the coronary vasculature. Prewarmed calcium-free Tyrode buffer (38°C; composition in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 10 glucose, 10 HEPES, NaOH to pH 7.35) was pumped through the perfusion needle into the coronary vasculature in a retrograde manner using a peristaltic pump (Miniplus 3, Gilson) adjusted to a pumping speed of 3.5 ml·min⁻¹·g⁻¹ to wash out excess blood. When the perfusion buffer exiting the heart was clear of erythrocytes, the perfusion solution was switched to an enzymatic solution composed of Tyrode solution containing 160 U/ml Type II collagenase (Collagenase Type 2, 270 U/mg, Worthington Biochemical, Lakewood, NJ) and 0.78 U/ml protease type XIV (Protease from *Streptomyces griseus*, 3.5 U/mg, Sigma-Aldrich, Stockholm, Sweden), and the heart was perfused for another 10 min. After perfusion, the left ventricle was dissected and cut to small pieces in the same enzyme solution supplemented with 13% bovine serum albumin and was gently pipetted up and down to dissociate cells from the tissue. The slurry was filtered through a 200- μ m mesh and centrifuged, after which the supernatant was discarded and the cells were resuspended in Tyrode solution with 200 μ M Ca²⁺. The centrifugation/washing procedure was repeated with 400 μ M Ca²⁺ before the pellet was finally resuspended in cell culture medium containing 1 mM Ca²⁺. This

stepwise reintroduction of Ca^{2+} has been shown to yield better quality cardiomyocytes when isolating from adult tissue (30). The medium containing the resuspended cells was layered on top of a 32% colloidal Percoll solution and centrifuged at 450 g for 30 min. The pellet obtained by this method contains a purified fraction of cardiomyocytes based on cell size (25). The pellet was resuspended in 1 mM Ca^{2+} Tyrode solution, and the cells were let to rest for 1 h before stimulation. While resting, the viable cell concentration was determined by counting cells stained with Trypan blue in a Bürkner chamber, and the suspension was diluted to 5 million cells/ml. One-hundred microliter aliquots (500,000 cells) were stimulated with forskolin (Forsk, 10 mM), epinephrine (Epi, 10 mM) or isoproterenol (Iso, 0.1 mM) for 30 min at 38°C. Control aliquots were treated the same as stimulated aliquots without addition of drug. After stimulation, cell aliquots were centrifuged at 4°C, the pellet was washed with ice-cold Tyrode solution without disrupting it, and cells were lysed with the sample diluent included in the kit used for the cAMP assay and frozen at -80°C.

At the time of cAMP analysis, samples were thawed and centrifuged at 4°C. The supernatant was removed and kept on ice until used for determination of cAMP content using a plate-based ELISA kit following the kit manufacturer's instruction (DetectX Cyclic AMP Direct Immunoassay kit, Arbor Assays, Ann Arbor, MI).

Echocardiography. The volatile anesthetic isoflurane (Tec 3 vaporizer, e-Vet, Haderslev, Denmark) was used to attain a level of anesthesia that allowed echocardiographic monitoring. Ventilation frequency was monitored throughout the procedure using impedance pneumography (model 2991 Impedance Meter, UFI, Morro Bay, CA). All echocardiograms were recorded at a ventilation frequency of ~20 breaths/min, which corresponds to a medium depth of anesthesia in broiler chickens as evaluated in pilot trials. The heart was imaged in supine animals using a 9-MHz linear probe (HL9.0/40/64D, Teled, Vilnius, Lithuania) in a right parasternal short-axis view (LogicScan 64 FLT-IT, Teled). To ensure that M-mode sequences were recorded at the same cross-sectional level for all hearts, the probe was moved from the base of the heart toward the apex and the transsectional point where the right ventricle ends was used as a landmark for where to carry out the measurements (see Fig. 4A, top). Recordings were performed before and 0, 2, 5, and 10 min after an intravenous bolus injection of 5 $\mu\text{g}/\text{kg}$ Iso. The end-diastolic and end-systolic diameters were determined from the recordings by averaging measurements of maximal and minimal lumen diameter and systolic peak-to-peak measurement for heart rate in three consecutive beats at four different sections of the recorded sequence (see Fig. 4A, bottom). Fractional shortening was calculated as $[(\phi\text{Dia} - \phi\text{Sys})/(\phi\text{Dia} \times 100)]$.

Basal metabolic rate. Basal metabolic rate (BMR) was obtained measuring oxygen consumption in 16 normoxic and 18 hypoxic animals at 4–5 wk of age. Average body mass in the postabsorptive state was matched between groups to avoid allometric effects on BMR (725 \pm 84 g and 744 \pm 84 g in N and H, respectively). Animals were individually placed in a respiration chamber with a volume of 10 liters in a temperature-controlled chamber at 22°C. A total of four animals could be measured at the same time, and all animals were measured twice, at least 48 h apart. Air flow through the respiration chambers was 2.5 l/min (Flow Bar, Sable Systems, Las Vegas, NV). Gas from the four respiration chambers was analyzed using an oxygen analyzer (FOXBOX II, Sable Systems) for 3 min each, resulting in one measurement for each animal every 15 min (a baseline measurement of chamber air was taken in each full cycle). Before it entered the gas analyzer, the air passed through Drierite (calcium sulfate with cobalt chloride; Hammond Drierite, Xenia, OH) to remove water vapor. Oxygen consumption was obtained continuously between 17:00 and 11:00 on a 12:12 photoperiod (lights off at 19:00) but only postabsorptive values (i.e., those between 5:00 and 7:00) were considered representative of BMR. Temperature in the chamber was gradually decreased during the night to 14°C and raised again (at a rate of 1.6°C per hour) to simulate a slight temperature change during the night.

Metabolic rates at the time the chamber temperature was lowest (00.00–2.00, 14°C) are presented for comparison purposes. All values are presented as mass-specific oxygen consumption ($\text{ml O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$).

Histology. Left ventricular heart tissue was snap frozen in isopentane, embedded in cryomedia, and set in -80°C for 10 min. The tissue was sectioned in 10- μm sections in a cryostat (Microm HM550, Thermo Fisher Scientific), caught on room-tempered polylysine slides, and air-dried. Sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 1 h followed by Bouin's fixative (Sigma-Aldrich) overnight. Fixed sections were stained with a Masson's Trichrome kit (Sigma-Aldrich), which stains collagen in blue and surrounding muscle tissue red.

Analysis. All results are presented as means \pm SD. Data sets were compared between N and H using Student's *t*-test, and the level of significance was set to $P < 0.05$. For the radioligand receptor binding saturation assay, the counts per minute (CPM) values were plotted against the [^3H]CGP-12177 concentrations including a linear subtraction of NSB. The corrected values were transformed to femtomole per milligram of protein based on protein content. For the inhibition assay, NSB was subtracted and the CPM values were plotted against the logged concentrations of blocker and fitted to a nonlinear regression competition curve (GraphPad Prism, GraphPad Software, La Jolla, CA). The fitted curves were normalized (0–100%), the IC_{50} was extracted, and pIC_{50} results within the same inhibition treatment were compared between the N and H group. Analysis of the Western blot densitometry results was done by expressing the intensity of the bands as a percentage of the corresponding band in the internal brain standard (Fujifilm Multi Gauge software v3.2). Histological preparations were analyzed for collagen content in Masson's trichrome-stained sections by detecting blue pixels in micrographs at $\times 200$ magnification (NIS-elements AR 3.21.01, Nikon, Shinjuku, Tokyo, Japan) and expressing them as percentage area covered.

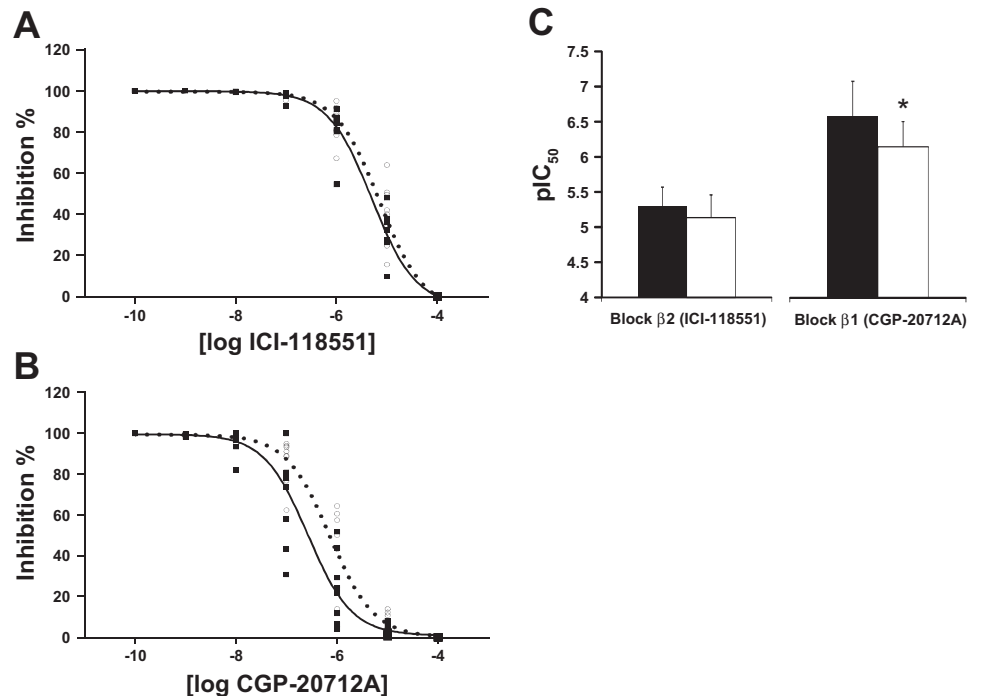
RESULTS

Growth is impaired and relative heart mass increased in 5-wk-old chickens exposed to prenatal hypoxia. Body mass of the hatchlings differed significantly (48.4 ± 2.0 vs. 47.2 ± 2.2 g, N and H respectively, $P < 0.05$). The difference in body mass of the hatchlings was maintained and enhanced in the 5-wk old birds (1260.9 ± 51.3 vs. 1048.7 ± 35.8 g, N and H, respectively, $P < 0.05$), although the absolute ventricular mass was similar between the groups (5.7 ± 0.2 vs. 5.5 ± 0.3 g, N and H, respectively). Because of the lower body mass and equal heart mass in H, the heart-to-body ratio in the prenatally hypoxic group was significantly higher (0.45 ± 0.01 vs. 0.53 ± 0.02 , N and H, respectively, $P < 0.01$).

$\beta_1\text{AR}$ and G_{α_s} expression is increased in the hearts of juvenile chickens prenatally exposed to hypoxia. Saturation binding curves confirmed our previous finding (18) that the absolute density of surface expressed receptors is around 35 fmol/mg protein (34.13 ± 10.14 vs. 35.87 ± 14.09 fmol/mg protein, N vs. H, respectively). Competitive binding curves with $\beta_1\text{AR}$ or $\beta_2\text{AR}$ specific antagonists (CGP-20712A and ICI-118,551, respectively) revealed a right shift in the normalized inhibition curve for $\beta_1\text{AR}$, but not $\beta_2\text{AR}$, for the group treated with prenatal hypoxia (Fig. 1, A and B). The right shift in the $\beta_1\text{AR}$ inhibition curve translates into a significantly lower pIC_{50} in hearts from prenatally hypoxic chickens and indicates an increased protein expression of $\beta_1\text{AR}$ (Fig. 1C).

Western blots for G_{α_s} showed that both the long and short isoforms are present in the brain homogenate used as internal

Fig. 1. Assessment of β -adrenoceptor (β AR) subtype changes in cardiac tissue from 5-wk-old broiler chickens prenatally developed in either normoxia (N, solid symbols/bars) or chronic hypoxia (H, 14% O_2 , open symbols/bars). In the average normalized competition curves to β_2 AR-specific blocker ICI-118551 (A) no difference is apparent between prenatally hypoxic samples (dashed line) and normoxic controls (solid line), while the hypoxic curve to β_1 AR-specific blocker CGP-20712A (B) demonstrate a right shift compared with the normoxic control. C: average of pIC_{50} for competition binding with β_2 AR-specific blocker (ICI-118551) and β_1 AR-specific blocker (CGP-20712A) of increasing concentration. * $P < 0.05$ between N and H. $n = 8-9$ (N and H, respectively).



standard and control (Fig. 2A, top). Two splice variants of the short isoform of G_{α_i} were seen in the brain homogenate, whereas only one of the splice variants was found in the heart (Fig. 2A, bottom). The two splice variants of G_{α_i} detected in the internal standard were identified as $G_{\alpha_{i1}}$ and $G_{\alpha_{i2}}$ (23), but only the $G_{\alpha_{i2}}$ was found in considerable amounts in the heart (Fig. 2A, bottom). When comparing G-protein protein expression between control and prenatally hypoxic hearts (densitometry values normalized against the internal standard), we found that G_{α_s} was increased, but not G_{α_i} (Fig. 2B).

cAMP production in response to β AR stimulation is not changed with prenatal hypoxia. cAMP production was measured in lysates of 500,000 freshly isolated cardiomyocytes. Nonstimulated cells (control) contained 0.48 ± 0.29 and $0.34 \pm$

0.24 pmol/ml cAMP (N vs. H, respectively). As expected, direct AC stimulation with Forsk (3.08 ± 1.82 and 3.78 ± 1.00 pmol/ml, N vs. H, respectively) increased the cAMP content more than β AR stimulation with both Epi and Iso, but none of the stimulations evoked a difference in the cAMP produced between the groups (Fig. 3).

Prenatal hypoxia causes in vivo systolic contractile dysfunction in the 5-wk-old chicken heart. Echocardiography showed a normal diastolic contraction (Fig. 4B) but a larger systolic diameter in H than in N (Fig. 4C). This systolic contractile dysfunction was present already before a bolus injection of Iso but was further enhanced by the selective β AR stimulation, something that is made even clearer when looking at the fractional shortening (Fig. 4E). Heart rate was increased after

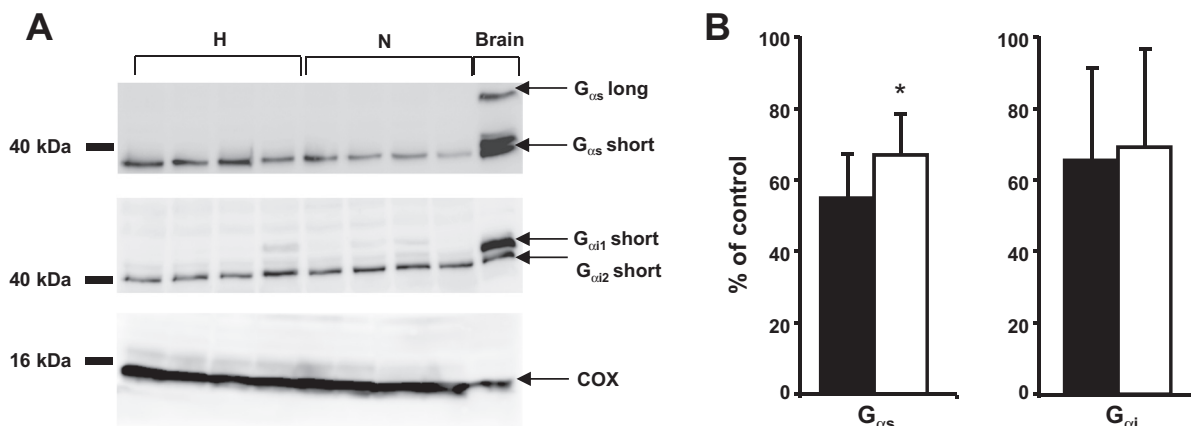


Fig. 2. Expression of stimulatory (G_{α_s}) and inhibitory (G_{α_i}) G protein α -subunit subtypes in cardiac tissue from 5-wk-old broiler chickens prenatally developed in either N or chronic H (14% O_2). In the Western blots (representative blots shown in A), chicken brain homogenate at the same protein concentration as samples was used as positive control for the antibodies and an internal standard. Although several isoforms and splice variants of $G_{\alpha_s}/G_{\alpha_i}$ can be seen in the brain, only one G_{α_s} short isoform (A, top) and the $G_{\alpha_{i2}}$ short splice variant (A, bottom) can be seen in the heart. COX, cytochrome *c* oxidase. In B, the densitometry values of the G protein subtypes (normalized against the internal standard) in normoxic (solid bars) and hypoxic (open bars) hearts show that G_{α_s} , but not G_{α_i} , is increased in prenatally hypoxic animals. * $P < 0.05$ between N and H. $n = 20$.

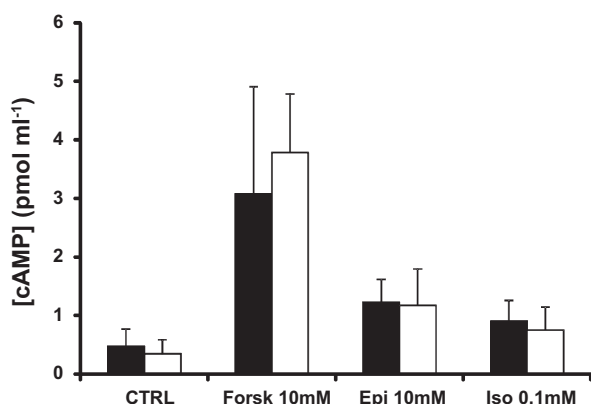


Fig. 3. cAMP accumulation in 500,000 isolated cardiomyocytes from normoxic (solid bars) and prenatally hypoxic (open bars) 5-wk-old chicken hearts stimulated with forskolin (Forsk), epinephrine (Epi), or isoproterenol (Iso). The concentration of each drug used is shown next to the individual drug in graph. $n = 10$.

the Iso injection, but there was no difference between the N and the H group (Fig. 4D).

Basal metabolic rate is not changed in prenatally hypoxic chickens. Prenatal stress often causes fetal growth retardation and may cause alterations in metabolic rate (31). An altered metabolic rate would in turn affect the circulatory requirements of the animal, so to rule out changes in BMR as the cause for the systolic alteration observed in the echocardiograms from hypoxic animals, we performed BMR measurements in mass-matched N and H birds. The BMR was significantly increased during 14°C compared with 20°C, but there was no difference between N and H for any of the two temperatures (Fig. 5). Thus chronic prenatal hypoxia did not alter the postnatal BMR.

Fibrosis is absent in hearts from 5-wk-old chickens exposed to prenatal hypoxia. Histological preparations treated with Masson's trichrome stained positive for collagen surrounding the cardiac blood vessels on both N and H hearts (Fig. 6A, right) but did not show any abnormal increase of collagen in the myocardial tissue from prenatally hypoxic chickens (Fig. 6A, left) at visual inspection. Digital analysis of the area covered by blue pixels (collagen) showed no difference in collagen content between N and H (Fig. 6B).

DISCUSSION

Prenatally hypoxic animals display signs of cardiac hypertrophy. Cardiac hypertrophy is caused by an increase in cardiomyocyte length and/or width and is clinically diagnosed as an enlarged heart (12, 21). Prenatal hypoxia can cause cardiac hypertrophy in the fetus (33, 36), but because of the overall stunted growth, the heart might not be enlarged in absolute terms. The heart-to-body ratio is therefore an index that can indicate cardiac hypertrophy relative to the overall growth. The stunted growth seen in the hypoxic hatchlings was maintained at 5 wk and there was no difference in heart mass in the hypoxic group compared with the control. This translated into a significantly higher heart-to-body ratio in H, indicating cardiac hypertrophy.

Prenatal hypoxia programs changes in the β AR pathway. Our previous results show that prenatal hypoxia decreases β AR sensitivity in the 5-wk-old chicken without changes in receptor density (18). Cardiac β_2 ARs produce significant amounts of cAMP when stimulated, but the cAMP increase

from β_2 AR signaling does not have an impact on cardiac inotropy (20). Therefore, our initial hypothesis was that a decrease in β_1 AR/ β_2 AR ratio due to an increase in β_2 ARs will contribute to an impaired contractile response of the heart and be the cause behind the decreased β AR sensitivity observed in our previous study. This is not the case according to the present results. We confirm that there is no change in total receptor density between N and H, and we also show that there is an increase in β_1 ARs in the chickens prenatally exposed to hypoxia, but no change in β_2 ARs. In human heart failure the β_1 AR/ β_2 AR ratio decreases and, because it is a ratio, this decrease could be caused by either an increase in β_2 AR expression or a loss in β_1 ARs (5, 7). In humans the decreased β_1 AR/ β_2 AR ratio is indeed due to an actual loss of receptor numbers, mainly β_1 ARs (5, 7), while we know that the total receptor density in the chicken heart is unchanged. Thus, even if the curves to specific β AR-subtype blocking will not give us absolute receptor numbers, we can draw the conclusion that the change in the β_1 AR/ β_2 AR is increased in the prenatally hypoxic chicken because of an increase in β_1 ARs.

There is a debate around whether β AR desensitization is adaptive or maladaptive in heart failure (4, 10). Most cases of human heart failure are associated with an increase in sympathetic activity (7, 28) and, in the case of β AR overstimulation, β_1 AR signaling through G_{α_s} is reported to increase apoptosis, arrhythmia, cardiac remodeling, and hypertrophy (11, 14, 34), while β_2 AR signaling through G_{α_i} seems to be protective and has an antiapoptotic effect (5, 27, 35). At the same time, the intrinsic ability of β_2 ARs to switch from G_{α_s} signaling to G_{α_i} also inhibits cAMP production and can further impede the cardiac response to β AR stimulation. Desensitization of the cardiac β AR system connected to an increase in G_{α_i} expression has indeed been demonstrated in heart failure (26) and led us to hypothesize that this would also be the case in the chicken. However, our result was the opposite and there was instead an increase in G_{α_s} . It is known that mice overexpressing G_{α_s} (despite initially increasing cardiac contractility) develop cardiomyopathy (10), so one could therefore speculate that there is a time aspect in the progress of cardiomyopathy; an increase of G_{α_s} is an initial adaptation of the heart to increase contractility, but prolonged stimulation increases G_{α_s} -mediated apoptosis and thus upregulating G_{α_i} as a protective measure. Following this argument, the G_{α_s} increase in our 5-wk experimental group together with the increase in β_1 AR/ β_2 AR ratio could be a sign of early cardiac remodeling/failure and the fact that it is the opposite to what is seen in human heart failure may simply be because it is in an early stage of pathology progression. Additionally, the mere increase in G_{α_s} proteins does not assure their participation in receptor signaling; functional activity of the G_{α_s} is impaired in the adult rat exposed to hypoxia and is not regained with normoxic recovery (15, 16). The G_{α_s} proteins may also be in the cytosolic fraction where they are unavailable for signaling (15). The functional activity of G_{α_i} in heart failure is also increased (8), which would make the inhibitory effect higher despite the lack of an increase in actual protein levels. Our cAMP measurements further argue that the sole increase of β_1 AR and G_{α_s} is not enough to enhance cAMP production, since β AR stimulation in our study did not produce higher levels of cAMP in the hearts expressing more G_{α_s} .

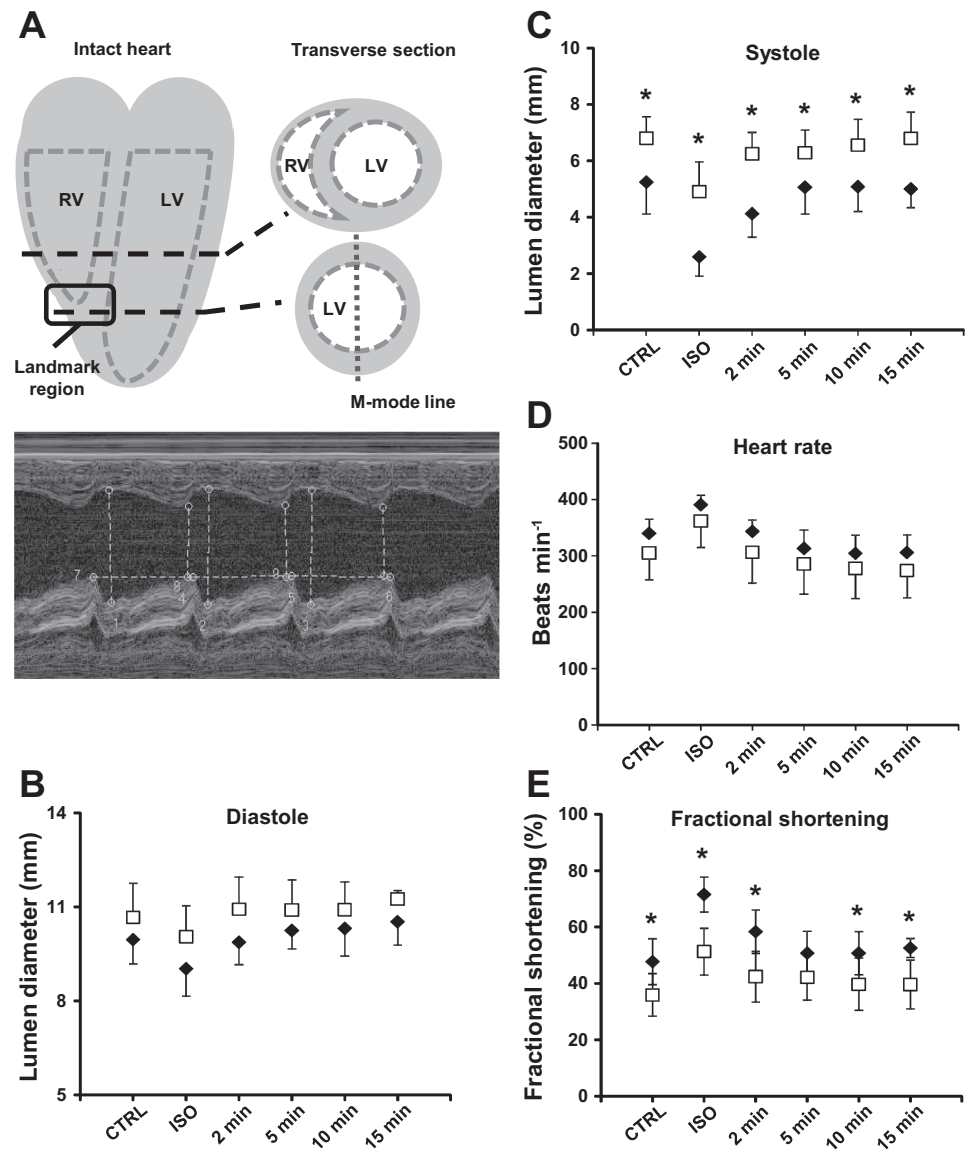


Fig. 4. Echocardiographic measurements of diastolic/systolic left ventricular (LV) lumen diameter and heart rate from 5-wk-old broiler chickens prenatally developed in either normoxia (solid symbols) or chronic hypoxia (14% O₂, open symbols). *A*: schematic of the level of echocardiogram recordings in the heart (*top*) and how the systolic/diastolic diameter and heart rate measurements were done in the recorded sequences (*bottom*). RV, right ventricle. Diastolic diameter (*B*), systolic diameter (*C*), and heart rate (*D*) were obtained from recorded sequences before and 0, 2, 5, 10, and 15 min after a 5 μ g/kg bolus injection of Iso. Fractional shortening (*E*) was calculated as $[(\phi\text{Dia} - \phi\text{Sys})/(\phi\text{Dia} \times 100)]$. * $P < 0.05$ between N and H. $n = 7$ and 6 (N and H, respectively).

Chickens exposed to prenatal hypoxia display systolic dysfunction that is not caused by cardiac fibrosis. Chickens exposed to prenatal hypoxia had a larger left ventricular systolic lumen diameter as measured by echocardiography. We interpret these results as a sign of contractile dysfunction of the heart. An alternative explanation could be an increase of venous return or an increase in peripheral vascular resistance increasing afterload. Fetal sheep exposed to chronic high-altitude hypoxia indeed display an increased peripheral vascular sensitivity to norepinephrine (NE) and increased vascular resistance that is still present postnatally (13), together with a decreased ventricular output (17). In contrast to the sheep, however, peripheral vascular sensitivity to NE in the chronically hypoxic chicken embryo is decreased (29), the β AR sensitivity in peripheral vasculature is increased (counteracting α AR-mediated vasoconstriction by β AR-mediated vasodilatation) (19), and they are hypotensive instead of hypertensive (19). Supported by this knowledge, the scenario that the larger left ventricular systolic lumen diameter observed in this study is caused by increased peripheral resistance is unlikely. Another plausible contributing factor to the larger left ventricular

systolic lumen diameter is an altered metabolic demand in the hypoxic birds. A decrease in metabolic rate in the hypoxic birds would also mean a reduced circulatory demand, leading to a lower cardiac output caused by decreased ventricle contraction. This was ruled out by measuring basal metabolic rate in size-matched normoxic and prenatally hypoxic animals and finding no difference in oxygen consumption between the groups. We therefore suggest that the larger left ventricular systolic lumen diameter observed is a systolic contractile dysfunction, likely originating in the innate cardiac contractile apparatus and/or its coupled signaling pathways.

Interestingly, the systolic dysfunction was apparent before β AR stimulation but was further enhanced with Iso. There are many known molecular and cellular changes in heart failure that influence the systolic contractile function of the heart. Alterations in extracellular collagen architecture has been shown to impair cardiac contractility (6), but this causation was ruled out for the observed systolic dysfunction by finding no difference in collagen deposits between N and H hearts. According to Davies et al. (6), altered Ca²⁺ handling is the

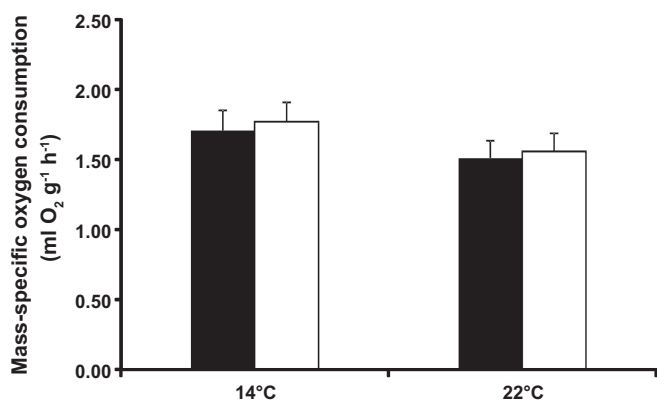


Fig. 5. Basal metabolic rate (BMR) in 4- to 5-wk-old mass-matched normoxic (solid bars) and prenatally hypoxic (open bars) chickens. The graph shows the postabsorptive BMR during the night at 14°C and 22°C. Values are presented as mass-specific oxygen consumption (ml O₂.g⁻¹.h⁻¹). n = 18 and 16 (N vs. H, respectively).

predominant contributor to contractile dysfunction. The main observed disturbance is a reduced Ca²⁺ uptake rate of the sarcoplasmic reticulum, which results in a prolonged calcium transient and thus a decrease in relaxation rate (22). In our previous study of concentration-response curves to Epi in myocardial strips from 5-wk-old chickens in vitro (18), we did

indeed find a significantly lower pEC₅₀ in concentration-relaxation rate curves in H compared with N, while the contraction rate curves did not differ. This further connects our previous in vitro results with the present in vivo results. The study of Ca²⁺ handling mechanisms in the myocardium was not in the scope of this study, but our results suggests that it is indeed on this level the changes lie, causing the functional changes observed in 5-wk-old chicken hearts exposed to prenatal hypoxia.

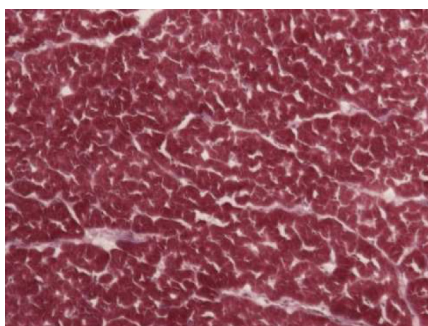
In conclusion, exposure to prenatal hypoxia programs a change in βAR signaling and systolic dysfunction in the 5-wk-old chicken heart without affecting cAMP production. The changes in βAR and G protein subtypes are opposite to those observed in fully developed human heart failure and may instead be indicative of an early compensatory stage in the progression of cardiac dysfunction. This is further supported by the cardiac hypertrophy, display of systolic contractile dysfunction, and decreased relaxation rate in in vitro preparations. Our results suggest that it is not the changes in the proximal part of the βAR system that causes the decreased cardiac contractility, but Ca²⁺ handling mechanisms further downstream in the βAR signaling cascade.

Perspectives and Significance

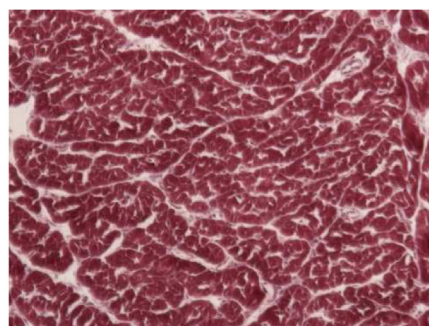
The disparate physiological responses to β₁AR and β₂AR stimulation has largely been ascribed to the fact that the two

A

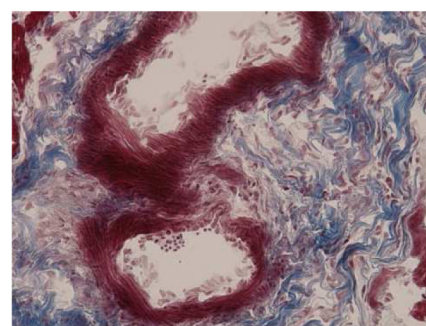
N 200x magnification



H 200x magnification



BV 400x magnification



B

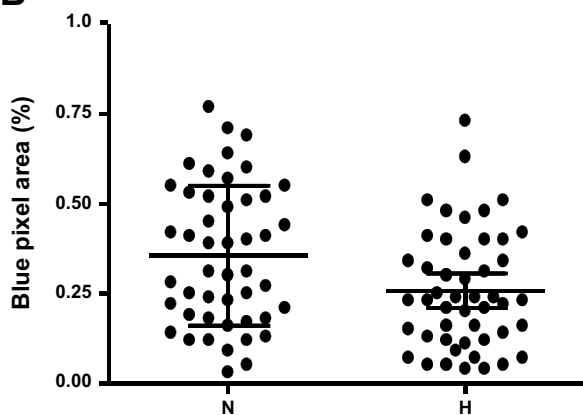


Fig. 6. Assessment of collagen content in cardiac tissue from 5-wk-old broiler chickens prenatally developed in either normoxia (N) or chronic hypoxia (14% O₂, H). 10-μm sections of cardiac tissue were stained with Masson’s trichrome, which stains collagen blue and myocardial tissue red. A: neither normoxic (left) nor hypoxic (middle) heart tissue showed a significant myocardial collagen content. Connective tissue around blood vessels was used as a positive control for the staining (right). Analyzing blue pixel area coverage showed no difference in collagen content between N and H (B).

subtypes are heterogeneously distributed in the cell (35). The receptors and all proteins involved in the immediate signaling pathway, such as G_i/G_o proteins, arrestins, and phosphodiesterases, are colocalized in cellular microdomains which are different for β_1 ARs and β_2 ARs (35). The β_2 ARs have been shown to be confined exclusively to deep T-tubules in the healthy cardiomyocyte while β_1 ARs are dispersed throughout the cell surface (24). However, in cardiomyocytes from a heart failure model β_2 ARs are redistributed and spread over the cell surface, thus losing their microdomains and enabling a change in their signaling pattern (24). This may play a role in the progression of heart failure. Chicken cardiomyocytes do not have T-tubules, and the distribution of β AR subtypes in avian heart cells is not known. This is an interesting avenue for further research on differences in the effects in β AR signaling between complex mammalian and more primitive avian cardiomyocytes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: I.M.L. and J.A. conception and design of research; I.M.L. performed experiments; I.M.L. analyzed data; I.M.L. and J.A. interpreted results of experiments; I.M.L. prepared figures; I.M.L. drafted manuscript; I.M.L. and J.A. edited and revised manuscript; I.M.L. and J.A. approved final version of manuscript.

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