Chronic in ovo hypoxia decreases pulmonary arterial contractile reactivity and induces biventricular cardiac enlargement in the chicken embryo

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Villamor, Eduardo, Carolina G. A. Kessels, Karin Ruijtenbeek, Robert J. van Suylen, Jaques Belik, Jo G. R. De Mey, and Carlos E. Blanco. Chronic in ovo hypoxia decreases pulmonary arterial contractile reactivity and induces biventricular cardiac enlargement in the chicken embryo. Am J Physiol Regul Integr Comp Physiol 287: R642–R651, 2004. First published April 29, 2004; 10.1152/ajpregu.00611.2003.—Although chronic prenatal hypoxia is considered a major cause of persistent pulmonary hypertension of the newborn, experimental studies have failed to consistently find pulmonary hypertensive changes after chronic intrauterine hypoxia. We hypothesized that chronic prenatal hypoxia induces changes in the pulmonary vasculature of the chicken embryo. We analyzed pulmonary arterial contractility and structure and heart morphology of chicken embryos maintained from days 6 to 19 of the 21-day incubation period under normoxic (21% O2) or hypoxic (15% O2) conditions. Hypoxia increased mortality (0.46 vs. 0.14; P < 0.01) and reduced the body mass of the surviving 19-day embryos (22.4 ± 0.5 vs. 26.6 ± 0.7 g; P < 0.01). A decrease in the response of the pulmonary artery to KCl was observed in the 19-day hypoxic embryos. The contractile responses to endothelin-1, the thromboxane A2 mimetic U-46619, norepinephrine, and electrical-field stimulation were also reduced in a proportion similar to that observed for KCl-induced contractions. In contrast, no hypoxia-induced decrease of response to vasoconstrictors was observed in externally pipped 21-day embryos (incubated under normoxia for the last 2 days). Relaxations induced by ACh, sodium nitroprusside, or forskolin were unaffected by chronic hypoxia in the pulmonary artery, but femoral artery segments of 19-day hypoxic embryos were significantly less sensitive to ACh than arteries of control embryos [pD2 = − log EC50: 6.51 ± 0.1 vs. 7.05 ± 0.1, P < 0.01]. Pulmonary vessel density, percent wall area, and periarterial sympathetic nerve density were not different between control and hypoxic embryos. In contrast, hypoxic hearts showed an increase in right and left ventricular wall area and thickness. We conclude that, in the chicken embryo, chronic moderate hypoxia during incubation transiently reduced pulmonary arterial contractile reactivity, impaired endothelium-dependent relaxation of femoral but not pulmonary arteries, and induced biventricular cardiac hypertrophy.

pulmonary hypertension; endothelium; cardiac hypertrophy

THE PRINCIPAL CONSEQUENCE of chronic postnatal hypoxia in humans and experimental animals is pulmonary hypertension, which occurs as a result of increased vasomotor tone, structural remodeling of the pulmonary vascular bed, and polycythemia (26). Pulmonary endothelial and vascular smooth muscle dys-

function contributes to the pathogenesis of hypoxia-induced pulmonary hypertension (26). Hypoxia affects endothelial cellular physiology in a number of ways, including the transcriptionally regulated expression of vasoactive substances and matrix proteins involved in modulating vascular tone or remodeling the vasculature and surrounding tissue (1, 43). Chronic hypoxia may also have direct effects on pulmonary vascular smooth muscle cells, modulating receptor populations, ion channel activities, or signal transduction pathways (1, 43).

Successful adaptation to postnatal life requires a dramatic transition of the pulmonary circulation from a prenatal high-resistance state to a low-resistance state after birth. Some infants fail to achieve the normal decrease in pulmonary vascular resistance at birth, which leads to severe respiratory distress and hypoxemia, referred to as persistent pulmonary hypertension of the newborn (PPHN) (1, 19, 20). This condition is characterized by failure of postnatal vascular remodeling, impaired endothelium-dependent and -independent relaxation, and an excess of vasoconstrictor agents (1, 19, 20). Although chronic prenatal hypoxia is considered a major cause of PPHN (1, 20, 57), several experimental studies have failed to consistently find functional or morphological evidences of hypertensive changes in fetal or neonatal pulmonary arteries after chronic intrauterine hypoxia (11, 39).

The “fetal origins” hypothesis proposes that adverse intrauterine environment results in developmental adaptations that permanently change structure, physiology, and metabolism, thereby predisposing individuals to cardiovascular, metabolic, and endocrine disease in adult life (36, 46, 51, 58). The chicken embryo appears as an interesting animal model to study mechanisms in the intriguing new field of developmental physiology that deals with the prenatal programming of cardiovascular pathology (36, 46). As the embryo develops outside the mother, effects of selected external stresses on cardiovascular development can be studied without interferences of maternal hormonal, metabolic, or hemodynamic alterations (36, 46).

Hypoxia of the chicken embryo is easily induced by incubating the egg in a low-oxygen environment (36, 45, 47–49).

In the present study, we hypothesized that chronic prenatal hypoxia might produce alterations in the pulmonary vascular system of the chicken embryo. We tested this hypothesis by
evaluating vascular reactivity, lung vessel morphometry, and sympathetic innervation in pulmonary arteries of chicken embryos maintained in hypoxic (15% O₂) or normoxic (21% O₂) conditions from 0.3 to 0.9 incubation. Femoral arteries of the same embryos were used as positive controls because we have demonstrated, in previous studies, that chronic moderate hypoxia in the chicken embryo led to functional and structural alterations of systemic femoral arteries, namely impairment of endothelium-dependent relaxation (47, 48) and increased periarterial sympathetic innervation (49). In addition, we also tested the effects of chronic hypoxia on heart morphometry.

METHODS

Experiments were performed in accordance with Dutch law for animal experimentation. Fertile Lohman-selected White Leghorn eggs incubated at 38°C and relative air humidity of 60% were transferred on embryonic day 6 to an incubator (Salvis Biocenter 2001) maintained at an O₂ level of 21 or 15% (47–49). On embryonic day 19 of the 21-day incubation, the embryo was removed from the egg, immediately decapitated, and weighed, and the lungs and heart were removed. The 19-day embryos were defined as non-internally pipped embryos, as verified by candling (63). In a second set of experiments, hypoxic and normoxic 19-day non-internally pipped embryos were incubated under 21% O₂ until the establishment of external pipping and then killed. With the aid of a dissecting microscope, rings of 1.7–2 mm of length of the main axial intrapulmonary artery (between the first major branching and a distal symmetrical branching point) were carefully dissected. The rings taken from the main axial intrapulmonary artery had a branching order of three. Ring segments of the femoral artery of some embryos were also dissected.

Recording of arterial reactivity. The isolated arteries were mounted between an isometric force transducer (Kistler Morce DSC 6, Seattle, WA) and a displacement device in a myograph (model 610M, JP Trading, Aarhus, Denmark) using two stainless steel wires (diameter 40 μm). During mounting and experimentation, the myograph organ bath (5-ml vol) was filled with Krebs-Ringer bicarbonate (KRB) buffer maintained at 39°C and aerated with 95% O₂-5% CO₂. Each artery was stretched to its individual optimal lumen diameter, i.e., the diameter at which it developed the strongest contractile response to 125 mM K⁺, using a diameter-tension protocol as previously described (63).

Contractile agonists were evaluated under optimal basilar passive tension. Concentration-response curves to K⁺ (5.95–125 mM), the thromboxane A₂ mimetic 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin F₂α (U-46619, 10⁻⁷–10⁻³ M), endothelin-1 (ET-1, 10⁻⁹ M–3 × 10⁻⁷ M), and norepinephrine (NE, 10⁻⁸–10⁻⁴ M) were constructed by increasing the organ chamber concentration of the drug, by cumulative increments after a steady-state response had been reached with each increment. Time to reach steady-state responses varied between 1–2 min (response to NE) and 5–10 min (responses to ET-1 and U-46619). Sympathetic neuroeffector mechanisms were studied using electrical field stimulation (EFS, 0.25–16 Hz, 2 ms, 85 mA) via two platinum electrodes that were placed in the axial direction of the blood vessel. Constant-current pulses were delivered by a stimulator (Technical Services, University Maastricht, Maastricht, The Netherlands).

Relaxing agonists were evaluated during contraction induced by 62.5 mM K⁺. Concentration-response curves for ACh (10⁻⁹–10⁻⁴ M), the nitric oxide (NO) donor sodium nitroprusside (SNP, 10⁻⁷–10⁻⁴ M), and the adenyl cyclase activator forskolin (10⁻⁷ M–3 × 10⁻⁵ M) were constructed. Some experiments were performed in the presence of the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME, 10⁻³ M). Pulmonary vascular responses to ACh were also studied under lower oxygenation conditions (5% instead of 95% O₂). In these experiments, arteries were mounted and stabilized under 95% O₂, and bubbling gas was switched to 5% O₂-5% CO₂-90% N₂, 15 min before 62.5 mM K⁺ precontraction and maintained during the concentration-response curve to ACh (63). The PO₂ values were measured by a blood gas analyzer (ABL 510 Radiometer, Copenhagen, Denmark).

Staining of perivascular nerves. Whole mount preparations of pulmonary artery segments were incubated, while in the organ bath, in 2% glyoxylic acid and 10% sucrose in phosphate buffer for 10 min at room temperature. After this, the segments were air-dried (90 s), stretched at 100°C for 4 min, and enclosed with entellan and a coverslip. Glyoxylic acid-induced fluorescence was visualized with fluorescent microscopy (microscope objective Fluor 10×, Nikon Diaphot, BA-470-DM 455 filter, Nikon FE2 camera). Nerve density was quantified by counting intersections of nerve fibers with a Merz grid (distance 50 μm, radius 35 μm) within a selected area of the image, as previously described (49).

Assessment of pulmonary vessel density and percent wall area. Lungs from hypoxic and normoxic embryos were excised and fixed for 24 h in 10% phosphate-buffered formalin (pH 7.4). Afterward, lungs were paraffin embedded, and 4-μm sections were stained with a monoclonal antibody against α-smooth muscle actin (diluted 1:300; Dako, Carpinteria, CA) using diaminobenzidine as the chromagen and hematoxylin as the counterstain. Microscopic images (using a ×40 objective) were analyzed using a computerized morphometric system (Quantimet 570; Leica, Cambridge, UK). The number of actin-positive vessels that were <100 μm in diameter was counted for 10 randomly selected fields per specimen. The medial circumference (assessed from the outer margin of the external elastic lamina), luminal circumference, and vessel diameter were obtained for analysis. Percent arterial wall area was determined by subtracting the calculated area circumscribed by the luminal circumference from that area within the medial circumference and then dividing by the total area of the vessel. Vessels were categorized depending on the external diameter. Within each category 6–12 arteries were measured per animal. The average of the values obtained was used for calculations. Analysis was performed by a single observer (C. G. A. Kessels), who was unaware of the experimental conditions (60).

Morphometric analysis of the heart. Hearts were weighed and fixed in 10% phosphate-buffered formalin for 24 h. Afterward, hearts were paraffin embedded, and three coronal 4-μm sections, at the level of aorta lumen, were stained with von Gieson’s stain. Slices were digitally recorded and analyzed by a computerized morphometry system (Quantimet 570, Leica) to determine right ventricle (RV) and left ventricle (LV) wall area and RV and LV and septum maximal thickness.

To determine whether cardiomyocyte hypertrophy was present, the total number of nuclei (including myocyte as well as nonmyocyte nuclei) and the total nuclear surface area were determined by averaging counts of three separated fields from corresponding areas (RV, LV, and septum) from hypoxic and control hearts. These measurements were performed on digitized photographs (using a ×40 objective) of hematoxylin- and eosin-stained histological sections with the assistance of an automated image analysis software (SigmaScan Pro, Jandel Scientific, San Rafael, CA).

Data analysis. Results are shown as means ± SE of measurements in n embryos. Contractile responses are expressed in terms of active wall tension (N/m) or as a percentage of the individual contraction induced by 125 mM KCl. Relaxations are expressed as a percentage of the preexisting tone. Sensitivity to ACh, SNP, and forskolin (expressed as P_D = −log EC₅₀) were determined for each artery by fitting individual concentration-response data to a nonlinear sigmoid regression curve and interpolating (Graphpad Prism version 2.01; Graphpad Software, San Diego, CA). Comparisons between hypoxic and normoxic groups were made by an unpaired t-test. Proportions were compared using a z-test. Differences were considered significant at P < 0.05. All analyses were performed using a commercially
available statistics package (SigmaStat, Jandel Scientific, San Rafael, CA).

Drugs and solutions. KRB buffer contained (in mmol/l) 118.5 NaCl, 4.75 KCl, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, 25.0 NaHCO₃, 2.5 CaCl₂, and 5.5 glucose. Solutions containing different concentrations of K⁺ were prepared by replacing part of the NaCl by an equimolar amount of KCl. Arterenol bitartrate (NE), l-NAME, forskolin, endothelin-1, and U-46619 (9,11-dideoxy-11β,9α-epoxymethano-prostaglandin F₂α methyl acetate solution) were obtained from Sigma Chemical (St. Louis, MO), ACh chloride from Janssen Chimica (Beersen, Belgium), and SNP from Acros (Geel, Belgium). All the drugs were dissolved initially in distilled deionized water (except for forskolin that was dissolved in DMSO) to prepare a 10⁻¹ or 10⁻² M stock solution, and further dilutions were made in KRB.

RESULTS

Exposure of chicken embryos to 15% instead of 21% O₂ from day 6 to day 19 of incubation reduced embryonic survival (54 vs. 86%; n = 32 in each group, P < 0.01). In the surviving embryos, body mass at day 19 of incubation was significantly reduced (22.4 ± 0.5 vs. 26.6 ± 0.7 g; P < 0.01). In contrast, body mass of 21-day externally pipped embryos was not significantly different (hypoxia 42.3 ± 1.2 g, n = 12; normoxia 44.1 ± 1.1 g, n = 12). The relative number of embryos (alive at embryonic day 19) that initiated external pipping and the time to external pipping were not significantly affected by in ovo hypoxia. In addition, the Hamburger-Hamilton stages (16) of the 19-day embryos at the time of experiments were similar in hypoxic and normoxic embryos, indicating absence of apparent developmental retardation.

Pulmonary arteries isolated from chicken embryos at day 19 of incubation responded to depolarizing high-K⁺ solution with a tonic contraction. The diameter at which maximal responses was obtained (hypoxia 222 ± 12.5 μm, n = 32; control 273 ± 14 μm, n = 34; P < 0.01) and the amplitude of the response (Fig. 1A) were significantly reduced in the hypoxic embryos. Optimal diameters of femoral arteries of embryos exposed to chronic hypoxia were not different from those of normoxic control embryos. The amplitudes of the contractile responses induced by NE (Fig. 1B), U-46619 (Fig. 1C), endothelin-1 (Fig. 1D), or electrical-field stimulation (not shown) in pulmo-

![Fig. 1. Concentration-dependent contractile effects of KCl (A), norepinephrine (NE; B), the thromboxane A₂ mimetic U-46619 (C), and endothelin 1 (ET-1; D) in endothelium-intact pulmonary arteries of non-internally pipped 19-day (filled symbols) and externally pipped 21-day (open symbols) chicken embryos exposed to 15% O₂ (squares) or 21% O₂ (circles) from day 6 until day 19 of the 21-day incubation. E: (in 19-day embryos) the maximal contraction induced by NE, U-46619, or ET-1 normalized for the 125 mM KCl response of each individual artery. Each point (or bar) represents the mean ± SE of 10 embryos. *P < 0.05, **P < 0.01, hypoxia vs. control. Only levels of statistical significance for the highest difference are shown.](http://ajpregu.physiology.org/
nary arteries were also reduced in the 19-day hypoxic embryos. However, as shown in Fig. 1E, when contractions were normalized for the 125 mM KCl response, no differences were observed between normoxic and hypoxic pulmonary arteries. In the pulmonary arteries of 21-day externally pipped embryos, the diameter at which maximal responses to depolarizing high-K+ solution were obtained (hypoxia 562 ± 21 μm, n = 8; control 581 ± 29 μm, n = 8) and the amplitude of the responses (Fig. 1A) were not significantly different. The responses to NE, U-46619, and endothelin-1 were also not significantly affected by hypoxia in the externally pipped embryo pulmonary arteries (not shown).

Relaxing responses were studied in femoral and pulmonary arteries from the same embryo after precontraction of the vessels with 62.5 mM KCl. As shown in Fig. 2B, ACh-induced relaxation was significantly impaired in the femoral arteries of hypoxic embryos (pD2: 6.51 ± 0.1) compared with the normoxic embryos (pD2: 7.05 ± 0.1; P < 0.01). In contrast, pulmonary arteries from the hypoxic and the control group showed similar ACh-induced relaxations (Fig. 2A). In the presence of the NO synthase inhibitor L-NAME, ACh-induced relaxation was markedly reduced in both types of artery from the two experimental groups (Fig. 2, A and B). In the presence of L-NAME, ACh-induced relaxation of femoral arteries was similar in hypoxic and normoxic embryos (Fig. 2B). Bubbling the organ chamber with 5% O2 (PO2 13.1 ± 0.1 kPa) instead of 95% O2 (PO2 74.3 ± 1 kPa) markedly reduced the relaxant effects of ACh, but this effect was similar in hypoxic and normoxic pulmonary arteries (Fig. 2C). The relaxing responses induced by the NO donor SNP and the adenylyl cyclase activator forskolin were unaffected by chronic hypoxia either in 19-day pulmonary or femoral arteries (Fig. 3). In addition, hypoxia did not produce any significant alteration in the response to ACh, SNP, or forskolin of externally pipped embryo pulmonary arteries (not shown).

Pulmonary vessel density (Fig. 4C) and percent wall area (Fig. 4B) were not different between control and hypoxic embryos. The density of catecholamine-containing perivascular nerves (stained with glyoxylic acid) was very low in pulmonary arteries when compared with previous data of our laboratory in femoral arteries (49). Chronic hypoxia did not significantly change sympathetic nerve density in pulmonary arteries (Fig. 4D).

The heart mass/body mass ratio, as a first-order index of cardiac hypertrophy, was higher (1.22 ± 0.19 vs. 1 ± 0.13%, P < 0.01) in the 19-day hypoxic embryos (n = 14) than in the 19-day normoxic embryos (n = 13). The LV wall area was also higher (4.51 ± 0.37 vs. 3.25 ± 0.38 mm2, P < 0.05) in the hypoxic group, but RV area and thickness of LV, RV, and septum were not significantly different. However, when normalized for body mass, LV and RV free wall thickness and area (Fig. 5A) were significantly higher in hypoxic compared with normoxic embryos, whereas septum thickness was similar (Fig. 5B). The RV area/LV area ratio (hypoxia 35 ± 2%; control 67 ± 7%; P < 0.05) and the RV area/LV area + septum area ratio (hypoxia 28 ± 1%; control 49 ± 2%; P < 0.01) were significantly decreased in the hypoxic animals, indicating a more marked left than right hypertrophy. Average number of nuclei per unit area was similar either in LV (control 331 ± 16 nuclei/area; hypoxia 349 ± 17 nuclei/area) or RV (control 269 ± 12 nuclei/area; hypoxia 266 ± 9 nuclei/area) from both experimental groups.

![Fig. 2. Concentration-dependent relaxant effects of ACh in endothelium-intact pulmonary (A and C) and femoral (B) arteries of 19-day chicken embryos exposed to 15% O2 (squares) or 21% O2 (circles) from day 6 until day 19 of the 21-day incubation. Arteries were precontracted with 62.5 mM KCl. Open symbols represent experiments in the presence of the NO synthase inhibitor N’-nitro-L-arginine methyl ester (L-NAME). C represents experiments performed, in pulmonary arteries, under lower oxygenation conditions (5% instead of 95% O2) in the organ chamber. Each point represents the mean ± SE of 8–10 embryos. **P < 0.01 for the difference in sensitivity between arteries of embryos exposed to hypoxia and arteries of normoxic controls.]

**DISCUSSION**

The present study investigated the pulmonary arterial and cardiac effects of chronic hypoxia during incubation in the chicken embryo. Our results indicate that chronic prenatal
hypoxia transiently reduced pulmonary arterial contractile reactivity but did not affect pulmonary arterial relaxations. Structural remodeling of the pulmonary vascular bed was not observed after chronic hypoxia. In contrast, hypoxia induced biventricular cardiac enlargement.

Effects of chronic hypoxia in the chicken embryo pulmonary artery. From the first descriptions of the PPHN syndrome, it has been speculated that chronic intrauterine hypoxemia might be responsible for the condition by increasing the muscularity of the pulmonary vasculature (31). In newborns who died soon after birth with PPHN precipitated by severe intrauterine or intrapartum hypoxia, postmortem examination showed thick-walled undilated pulmonary arteries (7, 21). Abnormal phenotypic modulation of both smooth muscle cells and fibroblasts may be induced by hypoxia, producing an extension of vascular muscularization, the newly muscularized vessels being densely innervated (15, 20). However, whether chronic hypoxia alone can cause PPHN is controversial and has not been clearly demonstrated by experimental models (1). Initial studies demonstrated that newborn rats exposed in utero to chronic hypoxia showed smooth muscle thickening in small pulmonary arteries (14). Other studies have failed to consistently find morphological evidences of hypertensive changes in fetal or neonatal pulmonary arteries after chronic intrauterine hypoxia (11, 39). In contrast, studies in fetal sheep have suggested that hypoxia caused by placental embolization (10), maternal hypotension (12), or partial cord compression (56) may induce neonatal pulmonary hypertension. However, these models were associated with several technical difficulties and a high fetal mortality of up to 60%, which probably explains why these models have not been further developed (57).

In our experimental model, exposure to chronic moderate hypoxia increased mortality during incubation and induced growth retardation. In this model, we have previously demonstrated increased hematocrit levels (49), indicating that the embryos were indeed exposed to hypoxemia in these conditions. Previous studies of our group demonstrated that this level of hypoxia produced, in the chicken embryo femoral artery, impairment of endothelium-dependent relaxation and increase of periarterial sympathetic innervation (47–49). In the present work, the effects of hypoxia in femoral arteries were confirmed but not reproduced in pulmonary arteries, indicating that there are fundamental differences in oxygen sensing and cell signaling between the two types of vessels in the prenatal period. Although the focus of intense research for nearly a century, the mechanisms underlying the differential vascular responses to hypoxia in systemic and pulmonary vessels remain unclear. It is well known that acute hypoxia elicits systemic vasodilation yet causes acute pulmonary vasoconstriction. The opposing hypoxic responses of the pulmonary vs. systemic arteries may result either from tissue-specific differences in the O2 sensor or in the response of the vessels to a common redox mediator (4). In addition, hypoxia-induced vascular remodeling and the molecular regulation of gene transcription during chronic postnatal hypoxia also differ between the systemic and pulmonary circulations (18, 64).

In the present study, 19-day pulmonary artery responses to KCl were significantly decreased by chronic hypoxia. Moreover, contractions induced by U-46619, endothelin-1, NE, and electrical-field stimulation were reduced in the hypoxic embryos to proportions similar to that obtained for KCl-induced contractions. Normalization of agonist responses to the magnitude of the maximal KCl response allows for determination of whether differences in the active responses to various agonists are specific to a particular agonist or are due to a general alteration in contractility. Thus, in the 19-day hypoxic pulmonary arteries, the decrease in the absolute ability of the smooth muscle to develop tension may account for the reduc-
tion in receptor-mediated contractions. A decrease in smooth muscle content or a smooth muscle phenotypic change would explain this pulmonary vascular hyporeactivity. However, morphometric analysis showed no differences in pulmonary artery medial thickness between normoxic and hypoxic embryos. Smooth muscle contractions in response to high K$^+$ are independent of receptor-ligand interactions and are brought about by membrane depolarization and Ca$^{2+}$ influx (7). Therefore, depressed responses to high K$^+$ would suggest either an alteration in resting membrane potential or in Ca$^{2+}$-channel proteins or impaired contractile or regulatory proteins. In several mammalian species, after prolonged postnatal hypoxic exposure, pulmonary vascular smooth muscle exhibits alterations in K$^+$ currents, membrane depolarization, elevation in resting intracellular Ca$^{2+}$, and changes in signal transduction pathways (43). Moreover, chronic hypoxia-induced alterations of mammalian pulmonary arterial reactivity have been related not only to an effect on Ca$^{2+}$ signaling but also on Ca$^{2+}$...
sensitivity of the contractile apparatus (6). In addition, Packer et al. (41) demonstrated an increase in the proportion of nonmuscle myosin to muscle myosin isoforms in the pulmonary arterial wall of chronic hypoxic rats. Further experimentation is, therefore, required to delineate the etiology of the functional changes induced by in ovo hypoxia in the chicken embryo pulmonary vascular smooth muscle.

Hypoxia-induced reductions of 19-day chicken embryo pulmonary vascular reactivity and body mass were not observed in 21-day externally pipped embryos. In these experiments, both normoxic and hypoxic embryos were incubated under 21% O₂ between days 19 and 21 to avoid the interference of hypoxia with the processes of internal and external pipping (i.e., to avoid alveolar hypoxia) and because hypoxia during the last 2 days of incubation produces a dramatic decrease in hatchability (48, 55), while embryos maintained under hypoxia from day 6 of incubation and transferred (after examination of viability) to normoxia on day 19 demonstrated hatchability similar to embryos incubated under normoxic conditions (48). Therefore, impaired reactivity of hypoxic pulmonary arteries returned toward control levels after exposure to normoxia. Several investigators have observed that hypoxia resulted in altered constrictor responsiveness to several stimuli in systemic and pulmonary vessels that is reverted after restoration of normoxia (17, 24). In contrast, we previously reported that hypoxia-induced alterations in femoral artery sympathetic and endothelial function persisted during normoxic postnatal life (48). However, these alterations did not lead to changes in blood pressure (48). Interestingly, a link between hypoxic exposure in the early period of life and susceptibility for pulmonary vascular dysfunction has been suggested by several experimental studies. Perinatal hypoxia increased the severity of pulmonary hypertension in rats when the animals were reexposed to hypoxia (27, 58). Moreover, transient hypoxic pulmonary hypertension during the first week of life predisposed to exaggerated pulmonary hypertension in young adults exposed to high altitude despite having normal pulmonary artery pressure at low altitude (51). Thus the pulmonary vascular effects of hypoxia seem to be related to the degree of lung maturation achieved at the onset of exposure (20), and hypoxic exposure during a critical period of lung growth may alter its development and leave structural and functional changes that cause an exaggerated response to adverse stimuli later in life (58). Whether the presently reported hypoxia-induced transient alterations of chicken embryo pulmonary artery reactivity could lead to a differential response to postnatal hypoxic exposure warrants further investigation.

As mentioned above, our results show that chronic hypoxia impaired endothelium-dependent relaxation in femoral but not in pulmonary arteries. In contrast, Shull and Wells (54) determined the acute effects of decreased oxygenation on NO production in ovine fetal pulmonary and systemic (mesenteric) endothelial cells, and they observed that in pulmonary, but not systemic, endothelium basal and stimulated endothelial NO production were reduced by low oxygenation. The role of NO production in chronic hypoxia-induced pulmonary hypertension in adults and newborns remains unclear. In the adult rat lung, gene expression for endothelial NOS (eNOS) is upregulated in pulmonary arteries after 3 wk of normobaric hypoxic exposure (53). In contrast, an impairment of ACh-induced relaxation has been reported after chronic hypoxic exposure in the adult and neonatal lung (3, 53). This observation is not incompatible with normal or even upregulated eNOS. Hislop et al. (22) showed an interesting pattern of up- and downregulation in neonatal piglets, depending on the time of exposure to hypoxia. In animals made hypoxic from birth, the lung vessel eNOS immunoreactivity was decreased, but in those animals made hypoxic after 3–6 days, it was increased. Moreover, Chicoine et al. (8) demonstrated that adult rats with hypoxia-induced pulmonary hypertension had higher levels of pulmonary eNOS compared with normoxic controls, whereas chronic hypoxic neonatal rats demonstrate the opposite profile. These data support a developmental difference in the response of
pulmonary eNOS expression and NO production to chronic hypoxia.

The autonomic nervous system plays a role in the control of pulmonary blood flow and may be involved in the pathophysiology of pulmonary vascular diseases (50). Thus perivascular sympathetic innervation has been implicated in the proliferation and differentiation of arterial smooth muscle cells, and its increase has been described as a possible etiological factor in PPHN (20, 42). Moreover, conditions such as hypoxia through an increase in α1-adrenoreceptor gene synthesis, density, or activity may lead to the development of pulmonary hypertension (50). Adrenergic and cholinergic nerve fibers are present within the adventitia of chicken pulmonary arteries and veins (5), but the absence of axons in the terminal arterioles contrasts with the profuse innervation of these vessels in mammals (28).

In the present work, we have observed that the density of sympathetic innervation of the chicken embryo main intrapulmonary artery was very low compared with the femoral artery (49) and was unaffected by chronic hypoxia during incubation. Accordingly, we have previously described a very weak contractile response of chicken embryo pulmonary arteries to adrenergic agents and perivascular sympathetic nerve stimulation (63). We have now observed that this weak sympathetic response remained unchanged after chronic hypoxia. Therefore, a role of adrenergic tone in the pulmonary vascular effects of chronic hypoxia in chicken embryos seems unlikely.

Cardiac effects of chronic hypoxia in the chicken embryo.

RV and LV areas and thickness, corrected for body weight, were increased in the hypoxic animals. This is consistent with previous observations reported in fetal sheep after chronic placental embolization (38) or chronic anemia (34) and with cardiac hypertrophy observed in small for gestational age infants (30, 61). Accordingly, the heart mass/body mass ratio, a first-order index of cardiac hypertrophy, has been previously reported to be augmented in chicken embryos after exposure to chronic hypoxia during incubation (36, 45). However, as the increase in the heart mass/body mass ratio is a consequence, in our experiments, of reduced body weight without increased heart mass, an alternative explanation could be the combination of a heart with preserved growth and a body with restricted growth. Nevertheless, we observed that LV wall areas of the hypoxic embryos were significantly larger than normoxic LV areas also when areas were not corrected for body mass. This indicates that hypoxic LV growth was not only preserved but actually increased.

In the fetal sheep, the standard mammalian model in the study of perinatal cardiovascular regulation, elevated arterial pressure (afterload), and/or increased workload are speculated to be the causative agents for prenatal hypoxia-induced ventricular hypertrophy rather than a direct hypoxic influence (32). However, on exposure to hypoxia, chicken embryos display both similar features and unique regulatory mechanisms compared with fetal sheep (9). Thus instead of the reflex hypertension displayed by fetal sheep in response to hypoxia (13), the hypoxic response in chicken embryos is characterized by hypotension (9, 59). Therefore, increased afterloads are unlikely. Another compensatory response also includes increased vasculogenesis to allow greater amounts of blood to flow to the tissues (2). Interestingly, Adair et al. (2) demonstrated that whole body flow rate increased and vascular resistance decreased in a dose-related manner in chicken embryos incubated under hypoxia. Finally, by altering the hematic viscosity, hypoxia-induced polycythemia might influence the modifications of the preload, which is also regulated by other circulating substances like the atrial natriuretic peptide (52).

Growth of the heart during embryonic and fetal life is characterized by hyperplasia of myocardial cells. Increased myocardial workloads due to chronic hypoxia exposure in fetal life lead to cardiac enlargement by causing an increased rate of hyperplasia of myocardial cells or continuation of hyperplasia beyond the normal period of hyperplastic growth (40). The switch from myocyte hyperplasia to hypertrophy occurs during the early postnatal period when cardiac myocytes lose their ability to proliferate, and the continued increase in heart mass is produced by the enlargement of individual preexisting cardiac myocytes (33). However, the chicken seems to be an exception because chicken myocytes undergo hypertrophy and continue to proliferate during posthatching maturation (33). In our experimental model, we counted the number of nuclei per unit area, including myocyte as well as nonmyocyte nuclei, in the hypoxic and control RV and LV. A smaller number of nuclei per unit area would reflect the presence of larger cells (i.e., hypertrophy). We have not observed significant differences in nuclei density between hypoxic and normoxic hearts. These results suggest that in our experimental model, myocyte hypertrophy was not responsible for LV and RV enlargement.

The decrease in pulmonary vascular resistance and pressure and increase in systemic blood pressure that occur after birth are later followed by a shift from RV to LV predominance. The difference in area and thickness between the walls of the LV and RV in the chicken embryo is an interesting point of difference from the mammalian condition (23). The LV and RV of the mammalian fetus are of equal thickness, and fetal RV pressure is normally at the systemic level (25). The difference between the anatomy of the mammalian fetal aortic arch and that of the chicken has been proposed to explain why a pressure difference in the ventricles of the chicken embryo heart is possible without left-to-right shunt. The extreme length of the ductus arteriosus and the acute angle at which the aorta and the ductus meet may hinder any backflow along the pulmonary vessels (23).

Perspectives

With impairments in blood gas exchange, mammalian fetuses, as well as avian embryos, are capable of a number of adaptive responses that will variously impact on fetal metabolism and oxygen transport and lead to a reduced rate of fetal growth and a reprogramming of tissue development (36, 37, 44). However, the extent to which these mechanisms are protective by decreasing substrate requirements for energy and growth, or give rise to pathological development of certain organ systems with time, is not known. Our findings support earlier studies in mammalian fetuses indicating that chronic moderate prenatal hypoxia does not produce structural alterations in the pulmonary vasculature (57). However, as the chicken embryo is a nonmammalian species, results may not directly be extrapolated to the human situation. Moreover, our experimental model was based on embryonic chickens of the White Leghorn strain that are resistant to postnatal pulmonary hypertension compared with meat-producing broiler strains (35). The differences in pulmonary vascular reactivity between
embryos of chicken strains with different susceptibility to pulmonary hypertension, the effects of chronic hypoxia on pulmonary vascular function and structure in the broiler chicken, and the consequences of chronic in ovo hypoxia for the postnatal pulmonary circulation are currently subjects of study in our laboratory (62). Finally, the chicken embryo model appears to be very interesting to study, at the molecular level, the cardiac effects of prenatal hypoxia and to provide important rationale for understanding whether hypoxia-induced cardiac alterations persist in the postnatal period.

REFERENCES


