Hypoxia Induces Aortic Hypertrophic Growth, Left Ventricular Dysfunction, and Sympathetic Hyperinnervation of Peripheral Arteries in the Chick Embryo

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Background—Low birth weight is associated with an increased incidence of cardiovascular diseases, including hypertension, later in life. This suggests that antenatal insults program for fetal adaptations of the circulatory system. In the present study, we evaluated the effects of mild hypoxia on cardiac function, blood pressure control, and arterial structure and function in near-term chick embryos.

Methods and Results—Chick embryos were incubated under normoxic (21% O₂) or hypoxic (15% O₂) conditions and evaluated at incubation day 19 by use of histological techniques, isolated heart preparations, and in vivo measurements of sympathetic arterial tone and systemic hemodynamics. Chronic hypoxia caused a 33% increase in mortality and an 11% reduction in body weight in surviving embryos. The lumen of the ascending aorta in hypoxic embryos was 23% smaller. Left ventricular systolic pressure was 22% lower, and heart weight/body weight ratio was 14% higher. In resistance arteries of hypoxic embryos, in vivo baseline tone was 23% higher, norepinephrine sensitivity was similar, and norepinephrine release from sympathetic nerves increased 2-fold, indicating sympathetic hyperinnervation. Mean arterial pressure and heart rate were similar under resting conditions, but chronically hypoxic embryos failed to maintain blood pressure during acute stress.

Conclusions—This study indicates that mild hypoxia during embryonic development induces alterations in cardiac and vascular function and structure and affects hemodynamic regulation. These findings reveal that antenatal insults have profound effects on the control and design of the circulatory system that are already established at birth and may program for hypertension and heart failure at a later age. (Circulation. 2002;105:2791-2796.)

Key Words: hypoxia ■ hypertension ■ nervous system, sympathetic ■ heart defects, congenital

Cardiovascular diseases account for the highest morbidity and mortality in Western society. Epidemiological studies have shown that low birth weight is associated with an increased propensity to coronary heart disease, hypertension, and non–insulin dependent diabetes in later life.1–4 On the basis of these observations, it has been proposed that antenatal events, reflected by low weight relative to gestational age at birth, predispose to cardiovascular diseases in adulthood. Furthermore, it was recently shown that low birth weight adversely influences endothelial function in childhood and early adult life independently of the classic risk factors.5,6 These studies suggest that insults at critical phases of development in utero induce permanent alterations in the circulatory system.

Despite accumulating epidemiological evidence in support of this fetal programming concept, the physiological mechanism responsible for the association between low birth weight and cardiovascular disease is largely unknown. In addition, one of the critical questions that remains unanswered is whether the circulatory system is already affected at the time of birth. Uteroplacental insufficiency, resulting in chronic impairment in fetal oxygen and nutrient supply, is a major cause of intrauterine growth retardation. The objective of this study was to investigate whether chronic hypoxia during embryonic development (1) induces aberrant formation of the arterial tree, (2) affects cardiac function, and (3) alters functional control of the cardiovascular system. The study was performed in near-term chick embryos that were exposed to a physiologically relevant level of hypoxia of 15% throughout incubation. The chick embryo is an established experimental model in developmental biology. Because the avian embryo develops independently of its mother animal, this allows a unique comprehensive approach to study the effects of isolated hypoxia, consisting of histological tech-
niques, isolated heart preparations, and in vivo measurements of arterial tone and systemic hemodynamics.

Methods
Experimental procedures were in accordance with the Dutch law on the use of laboratory animals. Fertile White Leghorn eggs were incubated at a temperature of 37°C and relative air humidity of 60% and were exposed either to 21% O2 in the normoxic (N) group or to 15% O2 in the chronically hypoxic (CH) group from the first day of incubation onward. Experiments were conducted at day 19 of the 21-day incubation period, corresponding to 0.9 of the total incubation time.

General Characteristics
To assess the effect of chronic hypoxia on general characteristics, we determined survival rate, body weight, and hematocrit. We also investigated whether chronic hypoxia prolonged the time to hatching.

Vascular Morphology
To determine the effect of chronic hypoxia on vascular morphology, embryos were killed, and the arterial tree was perfused at a standardized pressure of 30 mm Hg through a catheter introduced into the left ventricle (LV) with PBS and 1% phosphate-buffered paraformaldehyde (pH 7.4), both containing 0.1 mg/mL sodium nitroprusside.7 The ascending aorta was excised and fixed overnight in 1% phosphate-buffered paraformaldehyde. Segments were processed and embedded in paraffin, and 4-μm cross sections were stained with hematoxylin and eosin. Consecutive sections were stained with Lawson’s solution (a modified elastica van Gieson staining) to visualize elastic laminae and immunolabeled with smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker. Immunostaining was performed according to conventional methods with anti-FITC hrp (Chemicon Co) as a smooth muscle cell marker.
embryos. Statistical comparisons between groups were made with the Mann-Whitney U test. Statistical comparisons within groups were made with the Wilcoxon signed-rank test. Statistical significance was defined as $P < 0.05$.

## Results

### General Characteristics

Survival rate at 0.9 incubation time was lower in the CH (15% O$_2$, n=22) group than the N (21% O$_2$, n=21) group (50% versus 71.4%). Surviving chronically hypoxic embryos had a lower body weight (21.9±0.49 versus 24.7±0.40 g, $P < 0.001$) and higher hematocrit (0.34±0.014 versus 0.30±0.010, $P < 0.05$) than normoxic embryos. Hamilton stages (HH 43/44) at the time of experiments and the time to hatching (day 21 of incubation) were similar in CH and N embryos. This confirms that the experimental protocol induced small embryos without apparent developmental retardation.

### Vascular Morphology

The effect of chronic hypoxia on vascular morphology is shown in Figure 1. The luminal diameter of the ascending aorta was smaller in the CH (n=11) than the N (n=7) group (328±28 versus 430±28 μm, $P < 0.05$; Figure 2A), whereas the outer vessel diameters were not different between the 2 groups (896±40 versus 890±68 μm). As a result, the wall/lumen ratio was higher in the CH than the N group (2.0±0.32 versus 1.1±0.17, $P < 0.05$; Figure 2B). These data indicate that hypoxia is associated with reduced lumen size of the ascending aorta in the presence of hypertrophic growth.

### Cardiac Function and Geometry

LV function was determined under similar conditions (aortic pressure 30 mm Hg, heart rate 225 bpm) in the CH and N groups (both n=5). These values resembled the in vivo measured mean arterial pressure and heart rate at this stage of development (see below). There was no significant difference in end-diastolic pressure between the 2 groups (8±2.4 versus 8±2.3 mm Hg). LV systolic pressure was 22% lower in the CH than the N group (35±1.0 versus 45±3.5 mm Hg, $P < 0.05$; Figure 3). LV dP/dt$\text{\textsubscript{max}}$ and LV dP/dt$\text{\textsubscript{min}}$ were also lower in the CH group (836±104 versus 1109±101 mm Hg/s, $P = 0.076$, and 679±29 versus 957±68 mm Hg/s, $P < 0.01$).

The heart weight/body weight ratio, as a first-order index of cardiac hypertrophy, was higher (7.5±0.18×10$^{-3}$ versus 6.6±0.10×10$^{-3}$, $P < 0.05$) in the CH (n=14) than the N (n=8) group. LV free wall thickness and area were comparable between the 2 groups (0.73±0.06 versus 0.73±0.07 mm and 2.28±0.28 versus 2.23±0.21 mm$^2$, respectively), whereas septum thickness was reduced in the CH embryos (0.77±0.02 versus 1.18±0.07 mm$^2$, $P < 0.001$). These data indicate that hypoxia is associated with disturbed cardiac contractile function in the presence of mild hypertrophy.
Peripheral Arterial Control

Absolute arterial diameters under baseline conditions were smaller in the CH group than the N group (74±4.0 versus 91±3.4 μm). α-Adrenoceptor blockade with phentolamine induced a significantly stronger dilation of the mesenteric resistance arteries in the CH (n=5) group than the N (n=7) group (to 134±3.4% versus 111±2.4% of baseline, P<0.01; Figure 4), indicating a 23% higher baseline arterial tone in the CH group. Application of increasing concentrations of norepinephrine caused a decrease in arterial diameter in the CH (n=8) and N (n=9) groups. Maximal constrictor response at 10⁻³ mol/L norepinephrine was similar in the 2 groups (to 34±3.6% and 29±2.0% of baseline; Figure 5). Absolute maximal diameters in response to 10⁻² mol/L acetylcholine were similar in the 2 groups (104±5.0 and 104±4.9 μm).

Application of increasing concentrations of tyramine induced a gradual reduction in mesenteric arterial diameter in both the CH and N groups (both n=8, data not shown).

Systemic Hemodynamics

Under baseline conditions, mean arterial pressure and heart rate were similar in the CH (27±2.2 mm Hg and 205±6 bpm, n=8) and N (25±1.4 mm Hg and 199±9 bpm, n=9) groups. During acute hypoxic stress, however, mean arterial pressure decreased significantly in the CH group (to 22±1.8 mm Hg, P<0.05; Figure 7A), whereas it was maintained in the N group (37±3.6% versus 19±5.2% decrease of baseline, P<0.01; Figure 6). These data indicate that peripheral arterial tone was enhanced in CH embryos because of increased functional sympathetic innervation in the presence of similar α-adrenergic sensitivity of the peripheral vasculature.
We observed that as a consequence of medial hypertrophy, the lumen size of the ascending aorta was smaller in chronically hypoxic embryos. These changes in structural characteristics of the aortic wall are associated with an increase in resistance and a decrease in elastic properties, leading to reduced compliance. Similar alterations are implied in the pathogenesis of essential hypertension.11

Our in vivo measurements in the mesenteric vascular bed revealed an increase in arterial tone under baseline conditions. Furthermore, the contractile responsiveness to direct stimulation of perivascular sympathetic nerves by tyramine was 2-fold higher in chronically hypoxic embryos, whereas maximal responsiveness and sensitivity to norepinephrine were not altered. Together, these data indicate that chronic hypoxia induces sympathetic hyperinnervation of resistance arteries, leading to an increase in adrenergic arterial tone compatible with changes observed in adult hypertension.

Both reduced arterial lumen size and increased arterial tone play a fundamental role in the origin of essential hypertension. Therefore, our study shows, for the first time, that chronic embryonic hypoxia may establish the structural and functional basis for hypertension.

We found a 22% reduction in LV systolic pressure and lower positive and negative dP/dt in chronically hypoxic embryos, pointing to significantly diminished cardiac contractile function after chronic hypoxia. The heart weight/body weight ratio was 14% higher in hypoxic embryos, which is suggestive of a mild hypertrophic response. Together, these data indicate that chronic hypoxia induces alterations in function and mass of the embryonic heart compatible with moderate cardiac failure.

To extend this idea, we evaluated the regulation of systemic hemodynamics during acute severe hypoxic stress. This is a well-known stimulus for triggering a chemoreflex, which in the near-term embryo is normally characterized by bradycardia, elevated peripheral vascular resistance, and maintenance of blood pressure. This response is essential to maintain perfusion of the vital organs during an episode of acute hypoxia.12 Interestingly, we observed that arterial pressure dropped significantly in chronically hypoxic embryos during acute hypoxic stress, in contrast to the expected constant blood pressure in their normoxic counterparts. Because the degree of bradycardia and level of constriction in peripheral resistance arteries were similar in the 2 groups, it is highly likely that the decline in cardiac function in chronically hypoxic embryos accounts for this effect. These data provide the first evidence that hypoxia-associated alterations in the cardiovascular system lead to hemodynamic collapse under stress conditions in the near-term embryo.

Both molecular and hemodynamic mechanisms may account for the morphological and functional defects in the heart and vasculature of chronically hypoxic embryos. Endothelin-1, which, like vascular endothelial growth factor (VEGF), is a downstream target molecule of the hypoxia-sensitive transcription factor hypoxia-inducible factor (HIF)-1α,13 is a trophic factor for vascular smooth muscle cells and influences the development of neural crest–derived organs like the aortic outflow tract and the enteric nervous system.14 Because we find alterations at similar levels of the cardio-
vascular system, endothelin-1 may be a potential mediator and an interesting subject for future pharmacological interventions. We also observed a 2-fold increase in the constrictor response to tyramine after hypoxia, implying enhanced norepinephrine release from perivascular sympathetic nerves. This may be explained by hypoxia/HIF-2α–driven enhanced activity of the enzyme tyrosine hydroxylase, the rate-limiting enzyme in norepinephrine synthesis in sympathetic nerves.15,16 Finally, in line with our observations, mice with mild overexpression of VEGF-A show severe fetal cardiac and vascular abnormalities and die at midgestation.17 Genetic deletion of the HIF-1 response element or the VEGF coreceptor neuropilin-1 affects neuronal projection, implying a role for hypoxic signaling in neural regulation.18,19 Thus, on the basis of the fundamental role of these factors in the embryonic design of the circulatory system, we postulate that hypoxia may alter the orchestration of the expression of these factors during a critical period of embryonic development.

In addition, observed changes, such as enhanced sympathetic tone in the resistance vasculature, may reflect secondary hemodynamic adaptations. It is well known that patients with heart failure trigger similar compensatory neuroendocrine mechanisms aimed at restoring systemic arterial pressure. It may therefore be postulated that the decreased aortic lumen size imposes an increased workload on the heart, resulting in cardiac hypertrophy with subsequent LV dysfunction, which enhances sympathetic nervous activity.

By using an integrative physiological approach, the present study clearly showed that exposure of the embryo to mild hypoxia leads to abnormalities in cardiovascular function that may be related to the origin of hypertension. Our future investigations will address whether the changes observed in the chick embryo persist postnatally. Additional clinical research is needed to identify whether our postulates hold in human low-birth-weight neonates, for example, by noninvasive imaging of the heart and ascending aorta and naillfold microvascular microscopy to establish in vivo regulation of vascular tone.

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