Chronic Hypoxia Stimulates Periarterial Sympathetic Nerve Development in Chicken Embryo

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Background—Epidemiological findings suggest an association between low-for-age birth weight and the risk to develop coronary heart diseases in adulthood. During pregnancy, an imbalance between fetal demands and supply may result in permanent alterations of neuroendocrine development in the fetus. We evaluated whether chronic prenatal hypoxia increases arterial sympathetic innervation.

Methods and Results—Chicken embryos were maintained from 0.3 to 0.9 of the 21-day incubation period under normoxic (21% O\textsubscript{2}) or hypoxic conditions (15% O\textsubscript{2}). At 0.9 incubation, the degree of sympathetic innervation of the embryonic femoral artery was determined by biochemical, histological, and functional (in vitro contractile reactivity) techniques. Chronic hypoxia increased embryonic mortality (32% versus 13%), reduced body weight (21.9 ± 0.4 versus 25.4 ± 0.6 g), increased femoral artery norepinephrine (NE) content (78.4 ± 9.4 versus 57.5 ± 5.0 pg/mm vessel length), and increased the density of periarterial sympathetic nerve fibers (14.4 ± 0.7 versus 12.5 ± 0.6 counts/10\textsuperscript{4} mm\textsuperscript{2}). Arteries from hypoxic embryos were less sensitive to NE (pD\textsubscript{2}, 5.99 ± 0.04 versus 6.21 ± 0.10). In the presence of cocaine, however, differences in sensitivity were no longer present. In the embryonic heart, NE content (156.9 ± 11.0 versus 108.1 ± 14.7 pg/mg wet wt) was also increased after chronic hypoxia.

Conclusions—In the chicken embryo, chronic moderate hypoxia leads to sympathetic hyperinnervation of the arterial system. In humans, an analogous mechanism may increase the risk for cardiovascular disease in adult life. (Circulation. 2000;102:2892-2897.)

Key Words: hypoxia ■ cardiovascular diseases ■ nervous system, autonomic ■ nervous system, sympathetic

There is now substantial evidence that small size at birth is linked with a higher prevalence of cardiovascular and metabolic diseases in adult life.1,2 The mechanisms are unknown, but evidence from animal studies and preliminary evidence in humans suggest that an imbalance between fetal demands and supply leads to an adaptive series of stress responses that appear to permanently alter neuroendocrine development.3 Effects of maternal malnutrition on the fetal adrenocortical system have been addressed to some extent.4 We sought to determine whether prenatal hypoxemia interferes with the development of the cardiovascular sympathetic innervation.

In fetal mammals, acute hypoxia induces a redistribution of cardiac output from the periphery to vital organs such as adrenals, brain, and heart.6 Direct hypoxia-induced release of adrenal catecholamines (CA) participates in this acute fetal cardiovascular response. Prolonged periods of hypoxia may stimulate the expression of tyrosine hydroxylase (TH), the rate-limiting step in CA synthesis,7 as has also been shown in adult animals.8 This enzyme is essential for fetal development as knock-out mice die in utero at mid-gestation from cardiovascular failure.9 We hypothesize that chronic fetal hypoxia, besides stimulating adrenal CA release and synthesis, also promotes the growth and development of CA-producing autonomic nerves in the immediate vicinity of their cardiovascular effector tissues. Findings in spontaneously hypertensive rats (SHR) indicate that once established, sympathetic hyperinnervation of the cardiovascular system persists throughout life and leads after a substantial delay to the functional, structural, and hemodynamic characteristics of hypertension such as increased peripheral resistance, cardiovascular hypertrophy, and high blood pressure.10–12 In humans, high blood pressure involves the cardiovascular sympathetic nervous system and is a well-known risk factor for coronary heart disease,13 which was found to be associated with small for age birth weight.1,2

In avian embryos, unlike in mammalian species, direct effects of chronic hypoxia can be analyzed in the absence of restricted nutrient supply and maternal or placental hormones. In the chicken embryo, a widely used developmental biolog-
irical model, the cardiovascular effects of acute hypoxia mimic those seen in a broad variety of mammalian fetuses, and isolated organ techniques can be used that aim at perivascular autonomic nerves.

In the present study, we tested the hypothesis that prenatal chronic hypoxia stimulates the development of sympathetic nerves in the peripheral arterial system. Chicken embryos were maintained from 0.3 to 0.9 incubation in 21% or 15% O2, and the presence and function of sympathetic vasomotor nerves were evaluated in femoral arteries with the use of biochemical, histological, and pharmacological techniques.

Methods

Experiments were performed in accordance with Dutch law for animal experimentation. Fertile Lohman-selected White Leghorn eggs (‘t Anker), 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 oxygen level of 21% or 15% O2. On embryonic day 19 of the 21-day incubation, the animals were exposed to 15% O2 from 0.3 to 0.9 incubation and were sacrificed, while in the organ bath, in 2% glyoxylic acid and 10% sucrose bated, while in the organ bath, in 2% glyoxylic acid and 10% sucrose. After this, arterial segments were stretched until maximal responses. Endothelium was shown to be intact, as acetylcholine responses to nerve stimulation were tested with the use of electrical field stimulation (EFS, 0.25 to 32 Hz), which was previously shown to activate periarterial nerves of the chicken embryo femoral artery and to result in adrenergic nervous contractile responses. Endothelium was shown to be intact, as acetylcholine induced almost complete relaxation in femoral arteries of hypoxic and normoxic embryos (86.2±2.5% and 87.0±2.1% relaxation, respectively). Responses to acetylcholine have been shown by us to be completely endothelium dependent in chicken embryos.

At the end of the experiments, vessel segments were fixed in the organ chamber in phosphate-buffered formaldehyde (4%, pH 7.4, 37°C, 30 minutes) for subsequent morphometric analysis.

Morphometry

Cross sections (4 μm thick) of vessels were stained with Lawson solution and media cross-sectional area, media thickness, radius, and wall/lumen ratio were determined as previously described. Video images were generated by a Zeiss Axioscope and standard CCD camera (Stemmer) and were analyzed with commercially available software (JAVA 1.21, Jandel Scientific).

Staining of Perivascular Nerves

Whole mount preparations of femoral artery segments were incubated, while in the organ bath, in 2% glyoxylic acid and 10% sucrose in phosphate buffer for 10 minutes at room temperature. After this, the segments were air-dried (90 seconds), stretched at 100°C for 4 minutes, and enclosed with entellan and a coverslip. Glyoxylic acid–induced fluorescence was visualized with fluorescent microscopy (microscope objective Fluo 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.

NE and DNA Content

Drug and Solutions

Krebs-Ringer bicarbonate buffer contained (in mmol/L): NaCl 118.5, MgSO4 · 7H2O 1.2, KH2PO4 1.2, NaHCO3 25.0, CaCl2 2.5, and glucose 5.5. A 60-mmol/L K+ solution was prepared by replacing part of the NaCl by an equimolar amount of KCl. Phosphate-buffered solution consisted of 0.1 mol/L NaH2PO4 · H2O and 0.1 mol/L Na2HPO4 · 2H2O. Arterenal bitartrate (norepinephrine) and cocaine hydrochloride were obtained from Sigma Chemical Co, isopretene-noradrenaline and epinephrine were below detection limits in the embryonic arteries and heart, NE levels were expressed relative to arterial segment length or cardiac wet weight.

Data Analysis

Sensitivity to NE (expressed as pD2 [−log EC50]) and nerve stimulation were determined for each artery by fitting individual concentration-response data to a nonlinear sigmoid regression curve and interpolation (Graphpad Prism version 2.01, Graphpad Software Inc). pD2 values of arteries of normoxic embryos were compared with those of hypoxic embryos. Maximal responses (Emax) to NE and EFS for each artery were expressed in terms of active wall tension (N/m). The effect of cocaine was calculated as the difference between pD2 for NE in the presence and absence of cocaine (ΔpD2). Differences between findings in normoxic and hypoxic embryos were tested with Student’s t test or Mann-Whitney U test when normality test (Kolmogorov-Smirnov) failed. A value of P<0.05 was considered statistically significant. Data are presented as mean±SEM.

Results

Exposure of chicken embryos to 15% O2 from 0.3 to 0.9 incubation reduced embryonic survival (68% versus 87%). In the surviving embryos, the hematocrit was significantly increased (34.6±1.2% versus 30.0±1.8%) and body weight was significantly reduced (21.9±0.4 versus 25.4±0.6 g). Unlike kidney, liver, and heart weight, relative brain weight was not reduced after chronic hypoxia. Instead, the relative brain weight and brain-to-liver ratio were significantly increased in chicken embryos chronically exposed to low oxygen tension (Table 1).

A 36% increase in NE content was observed in femoral arteries of hypoxic chicken embryos (78.4±9.4 versus 57.5±5.0 pg NE/mg vessel length), though the difference failed to reach significance (P=0.08). In the heart, chronic exposure to hypoxia resulted in a significant increase of 45% (156.9±11.0 versus 108.1±14.7 pg NE/mg wet weight), but NE content was not altered in carotid arteries (Figure 1). Chronic hypoxia did not modify DNA content of embryonic femoral arteries (0.379±0.02 versus 0.350±0.03 μg/mm vessel length), carotid arteries (0.334±0.02 versus 0.314±0.03 μg/mm vessel length), and heart (3.11±0.07 versus 3.07±0.12 μg/mg wet wt).
nerve density by 13% (14.4 ± 0.6 versus 12.5 ± 0.6 μm²). The difference failed to reach statistical significance \((P=0.06)\). The \(\beta\)-adrenergic agonist isoproterenol (3 μmol/L) induced only marginal relaxation in \(K^+\)-precontracted femoral arteries. This effect did not differ between hypoxic (5.5 ± 1.0%) and control (9.8 ± 2.7%) embryos.

Maximal responses to EFS did not significantly differ between arteries of hypoxic embryos and control (1.45 ± 0.10 versus 1.21 ± 0.09 N/m). The frequency required to induce 50% of the maximal response to nerve stimulation was significantly higher in arteries of hypoxic embryos compared with controls (4.44 ± 0.85 versus 1.94 ± 0.58 Hz). This indicates that although NE content and nerve density in femoral arteries of hypoxic chicken embryos were increased, sensitivity to the constrictor effect of periarterial nerve stimulation was decreased. This is in line with the observed reduction of the arterial sensitivity to exogenously applied NE.

### Discussion

The present study shows that in chicken embryos, chronic moderate hypoxia increases arterial sympathetic innervation. Exposure of chicken embryos to 15% instead of 21% \(O_2\) increased embryonic mortality, suggesting an imbalance between embryonic oxygen demand and availability. In the surviving embryos, the hematocrit was significantly increased even at 0.9 incubation, indicating that moderate embryonic hypoxemia was chronically maintained. The observed reduction of total body weight after chronic hypoxia is in agreement with studies of babies born at high altitude\(^{21}\) and with experimental animal studies,\(^{22,23}\) which have pointed out the growth inhibiting effects of prolonged hypoxemia. The hypoxia-induced embryonic growth retardation was accompanied by an increase in relative brain weight and brain/liver ratio, suggesting disproportionate growth. The sparing effect on a vital organ such as the brain may be in line with the acute hypoxia-induced redistribution of cardiac output that we previously observed in the chicken embryo.\(^{14,15}\)

The findings in chicken embryo femoral artery indicate that chronic hypoxia increased periarterial sympathetic innervation. After exposure to 15% \(O_2\), NE content, sympathetic nerve fiber density, and cocaine-sensitive neuronal uptake of NE were increased in these arteries. The increase in sympathetic fiber density, which averaged 13%, represents a neurotrophic response. Though differences were not statistically significant, arterial NE content also appeared to be increased by chronic hypoxia. This is in line with reported effects of low oxygen tension on the expression of TH through binding

### Table 1. Effects of Chronic Hypoxia on General Characteristics of Chicken Embryos

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival, %</td>
<td>87</td>
<td>68†</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>25.40±0.56</td>
<td>21.86±0.43‡</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>30.00±1.81</td>
<td>34.63±1.23*</td>
</tr>
<tr>
<td>Heart, mg</td>
<td>200±5.5</td>
<td>149±3.0†</td>
</tr>
<tr>
<td>% body wt</td>
<td>0.79±0.02</td>
<td>0.69±0.01‡</td>
</tr>
<tr>
<td>Kidney, mg</td>
<td>100.0±2.80</td>
<td>68.0±1.69‡</td>
</tr>
<tr>
<td>% body wt</td>
<td>0.39±0.01</td>
<td>0.31±0.01‡</td>
</tr>
<tr>
<td>Liver, mg</td>
<td>596±14.9</td>
<td>472±8.4‡</td>
</tr>
<tr>
<td>% body wt</td>
<td>2.36±0.05</td>
<td>2.18±0.04‡</td>
</tr>
<tr>
<td>Brain, mg</td>
<td>853±12.6</td>
<td>798±11.5†</td>
</tr>
<tr>
<td>% body wt</td>
<td>3.39±0.07</td>
<td>3.69±0.08†</td>
</tr>
<tr>
<td>Brain/liver ratio</td>
<td>1.45±0.03</td>
<td>1.70±0.02‡</td>
</tr>
</tbody>
</table>

Values are mean±SEM; *P<0.05, †P<0.01, ‡P<0.001.

Cross-sectional area (16.2±1.3 versus 16.2±1.1 \(10^3 \) μm²), lumen radius (245±5 versus 250±4 μm), or medial thickness (10.3±0.7 versus 10.2±0.7 μm) of the femoral artery, changed by prolonged exposure to low oxygen tension.

In embryonic femoral arteries, chronic hypoxia not only increased NE content but also the density of catecholamine-containing perivascular nerves (Figure 2). Quantitative analysis of whole mounts, stained with glyoxylic acid, indicates that chronic hypoxia significantly increased sympathetic nerve density by 13% (14.4±0.7 versus 12.5±0.6 counts/\(10^2\) μm²).

Table 2 summarizes contractile reactivity of femoral artery segments isolated from chicken embryos exposed to 15% \(O_2\), and control embryos (21% \(O_2\)). The arterial preparations contracted in vitro in response to high potassium solution and to exogenous NE. Maximal responses to these vasoconstrictor stimuli were not significantly modified after chronic hypoxia. Sensitivity to NE was, however, significantly smaller in arteries of hypoxic embryos compared with controls (4.44 ± 0.85 versus 1.94 ± 0.58 Hz). This indicates that although NE content and nerve density in femoral arteries of hypoxic chicken embryos were increased, sensitivity to the constrictor effect of periarterial nerve stimulation was decreased. This is in line with the observed reduction of the arterial sensitivity to exogenously applied NE.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** NE content in femoral artery, carotid artery, and heart of normoxic (open bars) and hypoxic (filled bars) chicken embryos.

Values are mean±SEM \((n=17\) to 21\); **P<0.01.
of hypoxia-inducible factors to the promoter sequence of the TH gene.\textsuperscript{7,8} This mechanism, though not completely established in fetus, leads to increased NE synthesis. Increased NE levels may be accompanied by an increase in the number of adrenergic varicosities, as suggested by the increased neuronal reuptake of NE. We propose that hypoxia-induced redistribution of cardiac output from the periphery to vital organs in the embryo and fetus is ultimately maintained by stimulation of the peripheral sympathetic nerve development after hypoxia-induced release of adrenal NE and upregulation of tyrosine hydroxylase. The maintenance of cardiac output redistribution may also result in disproportionate growth.

Interestingly, in fetal llama, a species adapted to the chronic hypobaric hypoxemia of pregnancy at altitude, peripheral vasoconstriction in response to acute hypoxia is 4 to 5 times greater than in fetal sheep and is mediated by $\alpha$-adrenoceptors. Though the mechanism behind this enhanced $\alpha$-adrenergic response is not yet known, increased peripheral sympathetic innervation may play a role.\textsuperscript{6}

Despite biochemical and histological signs of sympathetic hyperinnervation, the adrenergic vasoconstrictor responsiveness of chicken embryo femoral arteries was not reduced after chronic hypoxia. Arterial sensitivity to exogenous NE was normalized when neuronal reuptake of NE was pharmacologically blocked with cocaine. Although we did not test this, the reduced sensitivity to sympathetic nerve stimulation may also be the consequence of enhanced neuronal reuptake of NE. In the adult, it is well established that chronic exposure to high concentrations of catecholamines results in desensitization and downregulation especially of $\beta$-adrenoceptors and their signal transduction pathways.\textsuperscript{24,25} Differences in the chronic control of adrenergic function have, however, been observed between adult and developing individuals. During developmental innervation and synaptogenesis, agonist-induced upregulation of adrenoceptors rather than downregulation has been observed.\textsuperscript{26} Furthermore, the chronic regulation of innervated arterial postjunctional $\alpha_{1}$-adrenoceptors may differ from that of $\beta$-adrenoceptors.\textsuperscript{27}

Whereas perivascular sympathetic nerves have been implicated in the proliferation and differentiation of arterial smooth muscle cells (for review see Reference 28), arterial structural consequences of hypoxia-induced sympathetic hyperinnervation were limited in chicken embryo femoral artery

**TABLE 2. Effects of Chronic Hypoxia on In Vitro Femoral Arterial Reactivity of Chicken Embryos**

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{max}$, N/m</td>
<td>1.66±0.12</td>
<td>1.89±0.07</td>
</tr>
<tr>
<td>$pD_{2}$, NE</td>
<td>6.21±0.10</td>
<td>5.99±0.04*</td>
</tr>
<tr>
<td>NE $E_{max}$, N/m</td>
<td>2.10±0.16</td>
<td>2.27±0.09</td>
</tr>
<tr>
<td>$pD_{2}$, NE i.p.o. cocaine</td>
<td>6.58±0.10</td>
<td>6.54±0.08</td>
</tr>
<tr>
<td>$\Delta pD_{2}$ (cocaine effect)</td>
<td>0.37±0.06</td>
<td>0.54±0.06</td>
</tr>
<tr>
<td>EFS (50% max), Hz</td>
<td>1.94±0.58</td>
<td>4.44±0.85*</td>
</tr>
<tr>
<td>EFS $E_{max}$, N/m</td>
<td>1.21±0.09</td>
<td>1.45±0.10</td>
</tr>
<tr>
<td>EFS $E_{max}$/NE $E_{max}$</td>
<td>0.59±0.04</td>
<td>0.62±0.03</td>
</tr>
</tbody>
</table>

$K_{max}$ indicates contractile response to 60 mmol/L K$^{+}$; NE $E_{max}$, maximal response to NE; EFS, electrical field stimulation; EFS $E_{max}$, maximal response to electrical field stimulation.

Values are mean±SEM; *$P<0.05$.  

**Figure 2.** Typical examples of perivascular sympathetic nerves stained with glyoxylic acid in femoral artery of chicken embryo exposed to normoxia (left) and hypoxia (right).  

**Figure 3.** Contractile responses (AWT, active wall tension) to exogenous NE in isolated femoral artery segments of normoxic and hypoxic chicken embryos. Before (top) and after (bottom) cocaine. Values are mean±SEM, *$P<0.05$ (for entire concentration-response curve).
important role of the sympathetic nervous system, but quantitative information on nerve density is lacking. Also, in insulin resistance, an important role of chronic sympathetic hyperactivity has been proposed. High blood pressure and insulin resistance markedly increase the risk for coronary heart disease, which occurs most frequently in individuals with a low-for-age birth weight. We propose that a prolonged moderate reduction of maternal oxygen supply interferes with fetal growth and stimulates peripheral cardiovascular sympathetic nerve development. The persisting elevated sympathetic nerve density leads in the long run to an increased risk for cardiovascular diseases.

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References

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