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% O₂) or hypoxic conditions (15% O₂). 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/10⁴ mm²). Arteries from hypoxic embryos were less sensitive to NE (pD₂, 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
ical 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.16

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, 75 μL of blood was obtained from a vessel of the chorioallantoic membrane to determine hematocrit values as an index of chronic hypoxemia. The embryo was removed from the egg, immediately decapitated, and weighed. Arteries and/or organs were isolated for determination of arterial reactivity, arterial structure, nerve density, norepinephrine (NE) content, and organ (wt) weights.

Arterial Reactivity
Two-millimeter segments of the right femoral artery were mounted (steel wires, diameter 40 μm) in a myograph organ bath (model 610, Danish Myotechnology by J.P. Trading, Denmark) for isometric force measurement. Organ baths were filled with a Krebs-Ringer buffer, maintained at 37°C, and aerated with 95% O2 and 5% CO2. The arterial segments were stretched until maximal contractile responses to 63 mmol/L potassium solution were achieved (optimal diameter). Concentration-response curves for NE (10⁻⁷ to 10⁻⁵ mol/L, half log units) were constructed in the presence and absence of cocaine (1 μmol/L), a blocker of neuronal uptake of NE. 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 perivascular nerves of the chicken embryo femoral artery and to result in adrenergic nervous contractile responses.16 Endothelium was shown to be intact, as acetylcholine induced almost complete relaxation in femoral arteries of hypoxic chicken 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.16

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.17 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.18

NE and DNA Content
Right femoral artery, right carotid artery segments, and heart were solubilized in 0.5 mol/L acetic acid (15 minutes, 100°C). NE content of the extract was measured by high-performance liquid chromatography and fluorescent detection,19 after which the tissues were solubilized in 1N KOH (24 hours, room temperature) and DNA content was determined.20 Unlike for NE, the levels of dopamine 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.

Drugs 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. Arterenol bitartrate (norepinephrine) and cocaine hydrochloride were obtained from Sigma Chemical Co, isoprotoreine nol hemisulfate hydrate from ICN Biomedicals Inc, Lawson solution from Boom b.v., and acetylcholine from Janssen Chimica. Agents were dissolved in distilled water.

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/mm 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). Neither were media
cross-sectional area (16.2 ± 1.3 versus 16.2 ± 1.1 mm²), 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/10⁵ μm²).

Table 2 summarizes contractile reactivity of femoral artery segments isolated from chicken embryos exposed to 15% O₂ and control embryos (21% O₂). 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 vessels from hypoxic embryos (Table 2 and Figure 3). Cocaine, an inhibitor of the neuronal reuptake of NE, increased the sensitivity of the arterial preparations to exogenously supplied NE. In the presence of cocaine, the sensitivity to NE no longer differed between arteries of hypoxic and control embryos (Table 2 and Figure 3), suggesting that the sensitizing effect of cocaine was larger in femoral arteries of hypoxic embryos compared with controls (ΔpD2, 0.54 ± 0.06 versus 0.37 ± 0.06). The difference failed to reach statistical significance (P=0.06). The β-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/m²). 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.

### 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 (n=39)</td>
<td>68† (n=65)</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>25.40 ± 0.56</td>
<td>21.86 ± 0.43‡ (n=29)</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>30.00 ± 1.81</td>
<td>34.63 ± 1.23* (n=10)</td>
</tr>
<tr>
<td>Heart, mg</td>
<td>200 ± 5.5</td>
<td>149 ± 3.0† (n=29)</td>
</tr>
<tr>
<td>% body wt</td>
<td>0.79 ± 0.02</td>
<td>0.69 ± 0.01‡ (n=29)</td>
</tr>
<tr>
<td>Kidney, mg</td>
<td>100.0 ± 2.80</td>
<td>68.0 ± 1.69‡ (n=29)</td>
</tr>
<tr>
<td>% body wt</td>
<td>0.39 ± 0.01</td>
<td>0.31 ± 0.01‡ (n=29)</td>
</tr>
<tr>
<td>Liver, mg</td>
<td>596 ± 14.9</td>
<td>472 ± 8.4‡ (n=29)</td>
</tr>
<tr>
<td>% body wt</td>
<td>2.36 ± 0.05</td>
<td>2.18 ± 0.04§ (n=29)</td>
</tr>
<tr>
<td>Brain, mg</td>
<td>853 ± 12.6</td>
<td>798 ± 11.5† (n=29)</td>
</tr>
<tr>
<td>% body wt</td>
<td>3.39 ± 0.07</td>
<td>3.69 ± 0.08† (n=29)</td>
</tr>
<tr>
<td>Brain/liver ratio</td>
<td>1.45 ± 0.03</td>
<td>1.70 ± 0.02‡ (n=20)</td>
</tr>
</tbody>
</table>

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

The present study shows that in chicken embryos, chronic moderate hypoxia increases arterial sympathetic innervation. Exposure of chicken embryos to 15% instead of 21% O₂ 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 and with experimental animal studies, 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 embryos.

The findings in chicken embryo femoral artery indicate that chronic hypoxia increased periarterial sympathetic innervation. After exposure to 15% O₂, 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

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**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. 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 α1-adrenoceptors. Though the mechanism behind this enhanced α1-adrenergic response is not yet known, increased peripheral sympathetic innervation may play a role.

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 β-adrenoceptors and their signal transduction pathways. 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. Furthermore, the chronic regulation of innervated arterial postjunctional α1-adrenoceptors may differ from that of β-adrenoceptors.

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 |
|-----------------------------|-----------------------------|
|                          | Normoxia | Hypoxia |
| Kmax, N/m                  | 1.66±0.12  | 1.89±0.07  |
| pD2 NE                     | 6.21±0.10  | 5.99±0.04* |
| NE Emax, N/m               | 2.10±0.16  | 2.27±0.09  |
| pD2 NE l.p.o. cocaine      | 6.58±0.10  | 6.54±0.08  |
| ΔpD2 (cocaine effect)      | 0.37±0.06  | 0.54±0.06  |
| EFS (50% max), Hz          | 1.94±0.58  | 4.44±0.85* |
| EFS Emax, N/m              | 1.21±0.09  | 1.45±0.10  |
| EFS Emax/NE Emax           | 0.59±0.04  | 0.62±0.03  |

Kmax indicates contractile response to 60 mmol/L K+. NE Emax, maximal response to NE; EFS, electrical field stimulation; EFS Emax, maximal response to electrical field stimulation.

Values are mean±SEM; *P<0.05.
investigated at 0.9 incubation. Media cross-sectional area and arterial lumen diameter were not modified; neither was arterial DNA content. Arterial structural consequences of increased periarterial sympathetic nerve density may require more time to develop.

Signs of hypoxia-induced sympathetic hyperinnervation were not restricted to the chicken embryo femoral artery. Also, the heart exhibited a 45% increase in NE content. In the chicken embryo, carotid artery hypoxia-induced changes were not observed, possibly because of low innervation density of this vessel.16

Several questions remain to be addressed. Is sympathetic nerve development accelerated or increased by hypoxia? Does the effect persist during postnatal development under normoxic conditions? What are the ultimate structural and functional cardiovascular effects? As regards the underlying molecular mechanism, several candidates may be considered. Growth and differentiation of peripheral sympathetic nerves and the production of target-derived nerve growth factors can be stimulated by low oxygen tension, catecholamines,29 and glucocorticoids.30 The direct oxygen sensitivity of the immature adrenal gland31 may link adrenal function to sympathetic nerve development through circulating catecholamines and glucocorticoids. We chose the chicken embryo to address the effects of prenatal hypoxia on sympathetic nerve development to avoid influences of fetal malnutrition and of maternal and placental hormones. In mammals, an imbalance between fetal demands and supply will of course not be restricted to oxygen. It will therefore be of interest to verify in the chicken embryo whether malnutrition, for instance, by removal of part of the ovalbumin,32 interferes with the observed hypoxia-induced hyperinnervation. Verification of the concept in mammalian situations of uteroplacental insufficiency will also be of interest.

During human pregnancy, important alterations in maternal renal and cardiovascular function develop. Blood flow changes markedly, especially in the uterine circulation. To accommodate an increasing fraction of the increasing cardiac output, the uterine arterial bed dilates and remodels extensively during pregnancy.33 Flow-induced vasodilation and outward arterial remodeling are endothelium-dependent processes.34–36 Preexisting and pregnancy-induced hyperlipidemia, hypertension, diabetes, and preeclampsia are accompanied by “endothelial dysfunction,”37–40 which may blunt dilation and remodeling, thereby altering the balance between maternal supply and fetal demands of oxygen and adversely affecting intrauterine fetal growth and possibly stimulating fetal peripheral cardiovascular sympathetic nerve development. In the pathogenesis of cardiovascular diseases, increased density and hyperactivity of the sympathetic nervous system play an important role. In SHR, most evidence indicates a pivotal role of early sympathetic hyperinnervation in the later development of high blood pressure.10–12 In SHR pups, the expression of nerve growth factor is increased,41 hyperinnervation precedes the development of hypertension,10 and neonatal sympathectomy prevents hypertension.42 Also, in essentially hypertensive humans, increased spillover of NE into plasma and electrophysiological evidence of increased sympathetic nerve firing rates are in line with an important role of the sympathetic nervous system, but quantitative information on nerve density is lacking.13 Also, in insulin resistance, an important role of chronic sympathetic hyperactivity has been proposed.43,44 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.1,2 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.

In summary, we observed that in the chicken embryo, chronic moderate hypoxia not only results in disproportionate growth but also leads to increased sympathetic innervation of peripheral arteries. This sympathetic hyperinnervation may increase the risk for cardiovascular disease.

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