

Chronic Moderate Hypoxia and Protein Malnutrition Both Induce Growth Retardation, But Have Distinct Effects on Arterial Endothelium-Dependent Reactivity in the Chicken Embryo

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ABSTRACT

Deviations in the rate of intrauterine growth may change organ system development, resulting in cardiovascular disease in adult life. Arterial endothelial dysfunction often plays an important role in these diseases. The effects of two interventions that reduce fetal growth, chronic hypoxia and protein malnutrition, on arterial endothelial function were investigated. Eggs of White Leghorn chickens were incubated either in room air or in 15% O₂ from d 6 until d 19 of the 21-d incubation. Protein malnutrition was induced by removal of 10% of the total albumen content at d 0. *In vitro* reactivity of the femoral artery in response to vasodilators was measured at d 19. Both chronic hypoxia and protein malnutrition reduced embryonic body weight at d 19 by 14% without affecting relative brain weight. Chronic hypoxia or protein malnutrition did not change sensitivity to the exogenous nitric oxide donor, sodium nitroprusside (5.74 ± 0.15 versus 5.85 ± 0.23 and 6.05 ± 0.18 versus 6.01 ± 0.34 , respectively). Whereas protein malnutrition did not modify arterial sensitivity to acetylcholine (7.00 ± 0.10 versus 7.12 ± 0.05), chronic hypoxia reduced sensitivity to this endothelium-dependent vasodilator (6.57 ± 0.07 versus 7.02 ± 0.06). In the presence of *N* ω -nitro-L-arginine methyl ester, this difference in sensitivity to acetylcholine was no longer apparent (6.31 ± 0.13 versus 6.27 ± 0.06), indicating that chronic exposure to hypoxia reduced sen-

sitivity to acetylcholine by lowering nitric oxide release. In additional experiments, a decrease in basal nitric oxide release in arteries of 3- to 4-wk-old chickens that had been exposed to *in ovo* chronic hypoxia was observed (increase in K⁺ contraction: -0.16 ± 0.33 N/m versus 0.68 ± 0.20 N/m). Protein malnutrition and chronic hypoxia both induce disproportionate growth retardation, but only the latter impairs arterial endothelial function. Intrauterine exposure to chronic hypoxia induces changes in arterial endothelial properties that may play a role in the development of cardiovascular disease in adult life. (*Pediatr Res* 53: 573–579, 2003)

Abbreviations

ACH, acetylcholine
AWT, active wall tension
EDHF, endothelium-derived hyperpolarizing factor
L-NAME, *N* ω -nitro-L-arginine methyl ester
NE, norepinephrine
NO, nitric oxide
eNOS, endothelial nitric oxide synthase
pD₂, sensitivity
SNP, sodium nitroprusside

Epidemiologic evidence links low birth weight to noninfectious diseases in the adult, including cardiovascular diseases. For instance, coronary heart disease, type 2 diabetes, atherosclerosis, and arterial hypertension have been associated with fetal growth retardation (1, 2). These observations have led to

the hypothesis that diseases in the adult, including cardiovascular disorders, may already be determined during intrauterine life.

An adverse intrauterine environment is most frequently the consequence of placental insufficiency resulting in decreased availability of nutrients and oxygen. Chronic exposure to hypoxia or undernutrition or a combination of both these stressors during prenatal life may interfere with fetal growth, which may result in changes in arterial function and structure. To investigate this relationship animal models have been es-

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tablished in which there is interference with fetal growth and development. Placental insufficiency can be induced by umbilicoplacental embolization, and reduction of placental blood flow, by ligation of the uterine artery (3, 4). Maternal food restriction or exposure to hypoxia can also create an adverse intrauterine environment (5–8). Although many animal studies have shown that growth retardation induced by intrauterine stress may lead to hypertension or other cardiovascular disorders [reviewed in Hoet and Hanson (9)], these disorders are not always accompanied by reduced body weight of the pups. This may indicate that adaptive responses to specific adverse intrauterine conditions rather than growth retardation *per se* may interfere with the physiologic programming of cardiovascular function and structure.

The studies that have provided evidence for the programming of cardiovascular disease have been performed in mammalian species in which the fetus is exposed to intrauterine stress via the mother. In these models it is difficult to distinguish between the effects of hypoxia and protein malnutrition. As the chicken embryo develops outside the hen, this model rules out these disadvantages. In previous work we have shown that the model is suitable to study cardiovascular response mechanisms (10–12) and that exposure to chronic moderate hypoxia changes peripheral artery function and structure at the level of sympathetic innervation (13).

Besides sympathetic hyperinnervation [reviewed in Julius and Valentini (14)], endothelial dysfunction plays an important role in cardiovascular disease (15). In children and young adults with low birth weight, endothelial function is reduced (16–18). Endothelium-dependent relaxation involves mediators such as NO and EDHFs (15), which have also been demonstrated to participate in the response to ACH in chicken embryo arteries (19). As acute and prolonged exposure to hypoxia can interfere with NO production and release (16, 20, 21), endothelial dysfunction resulting from chronic *in utero* exposure to hypoxia could be an interesting mechanism to link intrauterine stress and the programming of cardiovascular disease in the adult. On the other hand, decreased endothelial function after fetal protein malnutrition has also been described (5, 22).

In the present study, effects of chronic hypoxia and protein malnutrition on embryonic growth and arterial endothelial relaxing function were investigated. We hypothesize that ACH-induced relaxation of peripheral arteries of the chicken embryo may be modulated by a specific *in ovo* insult, such as chronic exposure to reduced oxygen or nutrients.

METHODS

Interventions. Experiments were performed in accordance with Dutch law for animal experimentation. Fertile Lohman-selected White Leghorn eggs (‘t Anker, Ochten, The Netherlands) were incubated at 38°C and 60% relative air humidity. To induce chronic hypoxia, eggs ($n = 65$) were transferred on embryonic d 6 to an incubator (Salvis Biocenter 2001) maintained at an oxygen level of 15%. Another set of eggs ($n = 39$) was transferred to a comparable incubator that was kept at 21% O₂ to serve as normoxic controls.

The albumen content of a separate set of eggs ($n = 18$) was determined using the methods described by Hill (23). In brief, eggs were weighed and subsequently hard-boiled. The components (albumen and yolk) were separated and weighed. Then, the relationship between the egg weight and total albumen weight was determined [albumen weight = $-a + b(\text{egg weight})$]. A new set of eggs ($n = 88$) was used for the *in vitro* studies, and protein malnutrition was induced at d 0. For each egg the albumen content was calculated (in grams). With a sterile needle (18-gauge), a hole was made in the eggshell (opposite the air cell) to carefully extract 10% of the total albumen (in milliliters) using a syringe. In another set of eggs ($n = 45$) a hole was made without extracting albumen to serve as sham controls. Holes were sealed with cyanoacrylate (RS Components, Corby, U.K.).

Survival was reduced by both chronic hypoxia (68% *versus* 87% in normoxic conditions) and protein malnutrition (56% *versus* 80% in sham control group). On embryonic d 19 of the 21-d incubation period surviving embryos were removed from the egg and immediately decapitated. Embryos were weighed, and brains were isolated to determine wet weight. Isometric force measurements were performed in femoral arteries of embryos that were exposed to chronic hypoxia or to protein malnutrition and in normoxic and sham controls.

Arterial reactivity. Segments of 2 mm of the right femoral artery were mounted (steel wires, diameter 40 μm) in a myograph organ bath (model 610M, Danish Myotechnology by J.P. Trading, Denmark) for isometric force measurement. Organ baths were filled with a Krebs-Ringer bicarbonate buffer, maintained at 37°C and aerated with 95% O₂ and 5% CO₂. The arterial segments were stretched until maximal contractile responses to 63 mM potassium solution (K⁺) were obtained; this length was the optimal diameter for the remainder of the study. Concentration-response curves for ACH (10⁻⁸ to 10⁻⁵ M, half-log units) were made during contraction with 63 mM K⁺. SNP (10⁻⁷–10⁻⁴ M) -induced relaxation was studied after arterial stimulation with 10 μM NE. The effect of ACH was also tested in arteries of normoxic and hypoxic embryos during NE-induced contraction.

The effect of NO synthase inhibition on ACH-induced relaxation was evaluated by constructing concentration-response curves in the presence of L-NAME (10⁻⁴ M). In additional experiments, the effect of L-NAME on K⁺-induced contraction was evaluated in side branches of the femoral artery of 3- to 4-wk-old chickens that had been exposed to chronic hypoxia (15% O₂) from embryonic d 6 to 19 and in control chickens. Responses to ACH (during K⁺ contraction) were also evaluated in these chickens.

Drugs and solutions. Krebs-Ringer bicarbonate buffer contained the following (in mM): NaCl, 118.5; MgSO₄·7H₂O, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; CaCl₂, 2.5; glucose, 5.5. Solutions containing different concentrations of K⁺ were prepared by replacing part of the NaCl by an equimolar amount of KCl. Arterenol bitartrate (NE) and L-NAME were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), ACH chloride from Janssen Chimica (Beersen, Belgium), and SNP from Acros (Geel, Belgium). All agents were dissolved in distilled water.

Data analysis. Body and organ weights of all embryos exposed to protein malnutrition and of sham controls were measured at d 19. A randomly selected group of embryos was included in the myograph studies. In previous work we described body and organ weights at embryonic d 19 of large numbers of hypoxic and normoxic embryos (13). A separate group of embryos was used in the myograph studies. AWT was calculated by dividing force by 2 times the length of the vessel segment (N/m). Responses to vasodilators were expressed as percentage change of the AWT induced by contraction. Sensitivity to ACH and SNP [expressed as pD_2 ($= -\log EC_{50}$, or effective dose to produce 50% of the maximal response)] was 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 Inc, San Diego, CA, U.S.A.). Maximal responses (E_{max}) for each artery were expressed in terms of AWT (N/m). The effect of L-NAME was calculated as the difference between pD_2 for ACH in the presence and absence of L-NAME (ΔpD_2).

Because the incubation procedures for the chronic hypoxia and protein malnutrition experiments were different, the intervention groups can only be compared with their own controls. Therefore, *t* tests (Sigma Stat 2.0, Jandel Scientific) were performed to compare differences in pD_2 , ΔpD_2 , and E_{max} values between arteries of hypoxic and normoxic embryos. Similar statistical procedures were followed to analyze differences in arterial responses of embryos exposed to protein malnutrition and sham controls. To test whether differences in pD_2 and E_{max} values within one artery segment in the presence and absence of L-NAME were statistically significant, paired *t* tests were performed. The nonparametric variants (Mann-Whitney *U* test, Wilcoxon signed rank test) were used when normality test failed (Sigma Stat 2.0, Jandel Scientific). Data are presented as mean \pm SEM of *n* embryos, and $p < 0.05$ was considered statistically significant.

RESULTS

Protein malnutrition from d 0 resulted in a 14% decrease of body weight measured at d 19 (19.4 ± 0.5 g versus 22.5 ± 0.5 g, $n = 49$, $n = 29$, $p < 0.001$). Relative brain weights of embryos exposed to protein malnutrition were larger compared with sham controls ($3.81 \pm 0.08\%$ versus $3.49 \pm 0.06\%$, $n =$

40, $n = 29$, $p < 0.01$). Previous work from our group demonstrated that exposure of chicken embryos to chronic hypoxia from d 6 of incubation also reduced total embryonic body weight measured at d 19 by 14% (21.9 ± 0.4 g versus 25.4 ± 0.6 g, $n = 32$, $n = 29$, $p < 0.001$). As observed after protein malnutrition, this was accompanied by relatively larger brain weights ($3.69 \pm 0.08\%$ versus $3.39 \pm 0.07\%$, $n = 39$, $n = 29$, $p < 0.01$) (13).

Optimal diameters of femoral arteries of embryos exposed to chronic hypoxia ($n = 14$ versus 9 normoxic control embryos) were not different from those of normoxic control embryos ($p = 1.0$). The same was observed when artery segments of embryos exposed to protein malnutrition ($n = 17$ versus 15 sham control embryos) were compared with sham controls ($p = 0.78$). Protein malnutrition, but not chronic hypoxia (2.02 ± 0.12 N/m versus 2.36 ± 0.20 N/m, $p = 0.13$), reduced contractile responses to 63 mM K^+ in comparison with sham controls (1.27 ± 0.11 N/m versus 1.72 ± 0.10 N/m, $p < 0.01$).

Table 1 summarizes sensitivity and maximal responses to the endothelium-dependent agonist ACH and the exogenous NO donor SNP in arteries of embryos exposed to chronic hypoxia or protein malnutrition and normoxic or sham control embryos.

Arterial sensitivity and maximal responses to ACH of embryos exposed to protein malnutrition were not different from their controls (pD_2 : $p = 0.42$, E_{max} : $p = 0.33$; Fig. 1B). Neither was the response to SNP changed by protein malnutrition (pD_2 : $p = 0.92$, E_{max} : $p = 0.59$; Fig. 1D). Although maximal relaxation was unchanged ($p = 0.51$, Fig. 1A), arterial segments of embryos chronically exposed to hypoxia were significantly less sensitive to ACH than arteries of normoxic control embryos ($p < 0.001$, Fig. 1A). This was not accompanied by changes in sensitivity to SNP ($p = 0.71$, Fig. 1C). Maximal responses to SNP were slightly decreased in the hypoxia group ($p = 0.03$, Fig. 1C). The decrease in sensitivity but not in maximal relaxation to ACH was also observed when arteries of hypoxic and normoxic control embryos were contracted with NE instead of 63 mM K^+ (pD_2 : 6.62 ± 0.09 versus 7.15 ± 0.09 , $p < 0.001$, E_{max} : $100.9 \pm 1.0\%$ versus $100.4 \pm 0.3\%$, $p = 0.87$).

Contractile responses to 63 mM K^+ of arteries of hypoxic and normoxic embryos were not changed in the presence of

Table 1. Sensitivity and maximal responses to ACH (in presence and absence of L-NAME) and SNP of femoral arteries of embryos exposed to chronic hypoxia or malnutrition and normoxic and sham control embryos

	Chronic hypoxia	Normoxic control	Malnutrition	Sham control
Sensitivity				
ACH	$6.57 \pm 0.07^*$	7.02 ± 0.05	7.00 ± 0.11	7.12 ± 0.05
ACH + L-NAME	6.31 ± 0.13	$6.27 \pm 0.06^\dagger$		
SNP	5.74 ± 0.15	5.85 ± 0.23	6.04 ± 0.18	6.01 ± 0.34
Maximal relaxation (%)				
ACH	85.3 ± 3.2	89.9 ± 2.2	92.3 ± 1.0	89.7 ± 2.2
ACH + L-NAME	$41.0 \pm 5.0^\dagger$	$48.6 \pm 5.3^\dagger$		
SNP	$101.0 \pm 0.8^*$	104.0 ± 1.7	103.0 ± 7.2	98.7 ± 3.9

Sensitivities to ACH and SNP in the absence and presence of L-NAME were presented as pD_2 values ($-\log EC_{50}$) and differences between pD_2 values in the absence and presence of L-NAME as ΔpD_2 . Maximal responses to ACH and SNP in the absence and presence of L-NAME are presented as percentages of the contraction to K^+ (in case of ACH) and to NE (in case of SNP). Mean \pm SEM; * $p < 0.05$ for the difference in response between arteries of embryos exposed to hypoxia and normoxic controls; $^\dagger p < 0.05$ for differences in responses in the absence and presence of L-NAME.

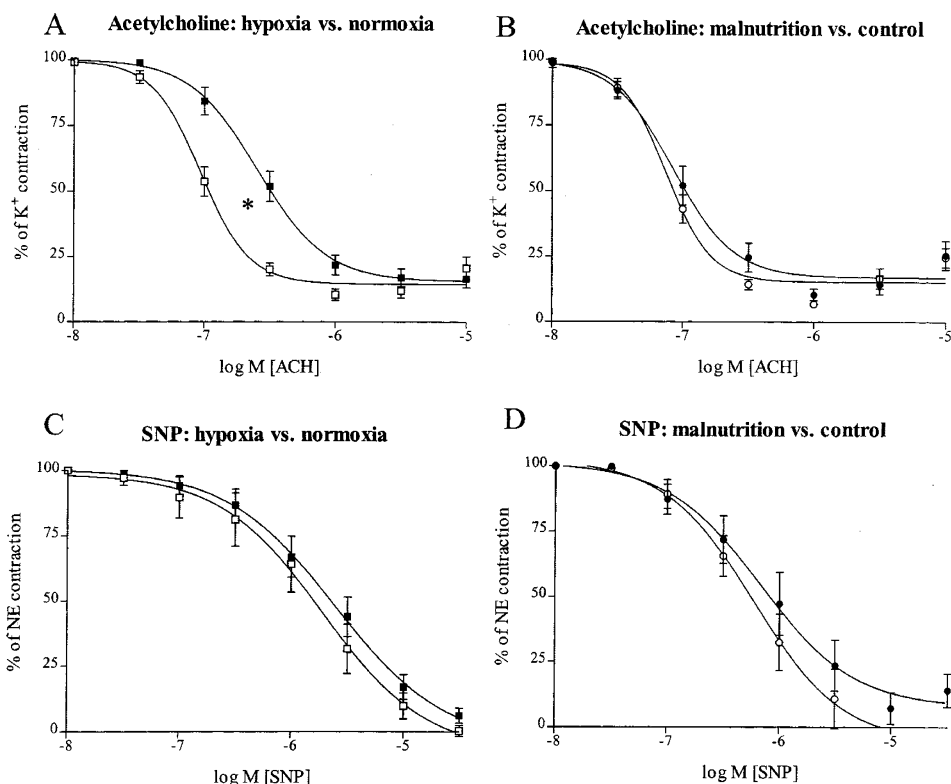


Figure 1. ACH- and SNP-induced relaxation in femoral arteries contracted with 63 mM K⁺ of embryos exposed to chronic hypoxia or protein malnutrition and normoxic and sham control embryos. Sensitivity to ACH was decreased in artery segments of hypoxic (A, filled symbols) compared with normoxic embryos (A, open symbols), whereas responses to SNP (C) were unchanged after chronic hypoxia. Arterial responses to ACH (B) and SNP (D) were not significantly different between embryos exposed to protein malnutrition (filled symbols) and sham control embryos (open symbols). Mean \pm SEM, * p < 0.05 (for the entire concentration-response curve).

L-NAME (hypoxia group: 2.00 ± 0.14 N/m versus 2.02 ± 0.12 N/m, $p = 0.66$; normoxia group: 2.29 ± 0.22 N/m versus 2.36 ± 0.20 N/m, $p = 0.42$). Inhibition of NO synthase with L-NAME significantly reduced sensitivity and maximal relaxing responses to ACH in arteries of normoxic embryos (pD_2 : $p < 0.01$, E_{max} : $p < 0.001$; Table 1 and Fig. 2) and maximal responses of embryos that had been exposed to hypoxia (pD_2 : $p = 0.07$, E_{max} : $p < 0.001$; Table 1 and Fig. 2). Under these conditions arterial sensitivity to ACH was no longer different between hypoxic and normoxic embryos ($p = 0.32$, Table 1 and Fig. 2), indicating that chronic exposure to hypoxia reduced sensitivity to ACH by lowering ACH-induced NO release.

At 3–4 wk of age total body weight of chickens that had been exposed to chronic hypoxia during *in ovo* development was smaller than weight of control chickens (172.82 ± 6.81 g versus 191.77 ± 6.72 g, $p < 0.05$). Responses to 63 mM K⁺ in side branches of the femoral artery of 3- to 4-wk-old chickens exposed to chronic hypoxia during *in ovo* development and in arteries of control chickens were comparable (2.42 ± 0.36 N/m versus 2.36 ± 0.25 N/m, $n = 14$, $n = 14$). L-NAME induced an increase of contraction stimulated with 63 mM K⁺ of 0.68 ± 0.20 N/m ($n = 10$, $p < 0.05$; Fig. 3). No significant effect (-0.16 ± 0.33 N/m, $n = 10$; Fig. 3) of L-NAME on K⁺ contraction was observed in chickens that had been exposed to chronic hypoxia during development, suggesting that chronic hypoxia before hatching affects basal NO

release after hatching. Responses to ACH were not altered by *in ovo* exposure to chronic hypoxia (pD_2 : 6.87 ± 0.10 versus 6.94 ± 0.07 , $n = 14$, $n = 14$; E_{max} : $81.88 \pm 2.90\%$ versus $79.65 \pm 3.49\%$). Effects of L-NAME on ACH-induced relaxation were comparable between groups (ΔpD_2 : 0.68 ± 0.28 versus 0.72 ± 0.10 , $n = 6$, $n = 9$; ΔE_{max} : $41.35 \pm 5.10\%$ versus $46.09 \pm 3.55\%$).

DISCUSSION

The present study demonstrates that exposure to chronic hypoxia and protein malnutrition both induce disproportionate growth retardation of the chicken embryo. However, only chronic hypoxia had consequences for arterial function. Endothelium-dependent relaxation was reduced in embryos that were exposed to chronic hypoxia from d 6 to 19.

Our group previously showed that the response to ACH in the femoral artery of the chicken embryo at the end of incubation is entirely endothelium-dependent (19). Precontraction with high K⁺, blockade of NO synthase with L-NAME, and inhibition of cyclooxygenase with indomethacin are not sufficient to entirely block ACH-induced relaxation in the chicken embryo [this study and (19)] and adult chicken (24, 25). This indicates that besides NO, prostaglandins, and EDHF, an additional unknown factor contributes to the endothelium-dependent response in this species.

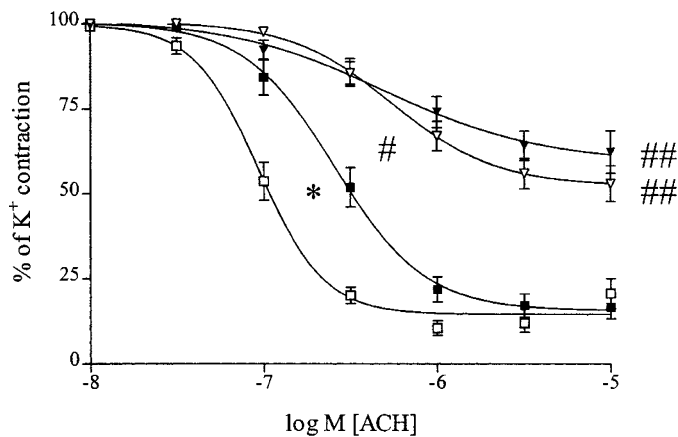


Figure 2. ACH-induced relaxation in the presence and absence of L-NAME in femoral arteries of embryos exposed to chronic hypoxia and normoxic embryos. NO synthase blockade by L-NAME decreases maximal responses to ACH in arteries of chronic hypoxic embryos (*filled squares* in absence of L-NAME, *filled triangles* in presence of L-NAME) and sensitivity and maximal responses in arteries of normoxic embryos (*open squares* in absence of L-NAME, *open triangles* in presence of L-NAME). Mean \pm SEM, * $p < 0.05$ for the difference in sensitivity between arteries of embryos exposed to hypoxia and arteries of normoxic controls in the absence of L-NAME; # $p < 0.05$ for the difference in sensitivity in the absence and presence of L-NAME in normoxic controls. ## $p < 0.05$ for the difference in maximal relaxation in absence and presence L-NAME in both arteries of the hypoxic embryos and arteries of normoxic controls. The rightward shift (ΔpD_2 , see Table 1) of the curves that is induced by L-NAME is significantly smaller after chronic hypoxia.

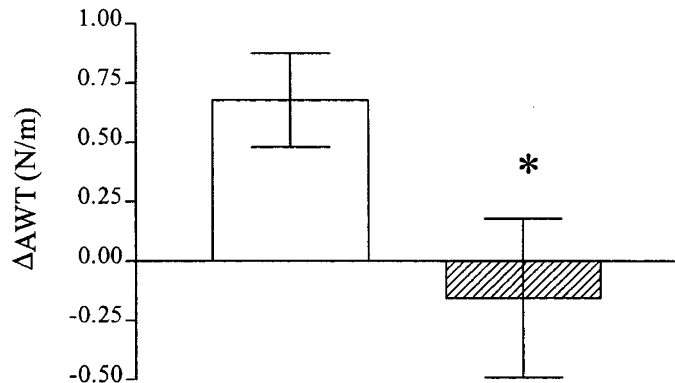


Figure 3. Effect of NO synthase blockade by L-NAME on contraction induced by 63 mM K^+ in side branches of the femoral artery of 3- to 4-wk-old chickens that had been exposed to chronic hypoxia during *in ovo* development (*hatched bars*) and chickens that developed in normoxic conditions (*open bars*). The increase in K^+ -induced AWT (Δ AWT) by L-NAME was significantly decreased by *in ovo* chronic hypoxia. Mean \pm SEM, * $p < 0.05$.

The effect of chronic hypoxia on ACH-induced relaxation seems to involve only the NO synthase-dependent component of the response, as during incubation with L-NAME responses to ACH in arteries of chronic hypoxic embryos were comparable to those in arterial segments of normoxic control embryos. Sensitivity to SNP, an exogenous NO donor, was not modified by chronic hypoxia, indicating that the capacity of the smooth muscle cells to relax in response to NO was not changed. Although the small change in maximal relaxing response to SNP was statistically significant, this is probably physiologically of little importance.

During acute fetal hypoxemia *in vivo*, activation of the chemoreflex results in a redistribution of the cardiac output so

that less blood is directed toward peripheral vascular beds (11, 26). The decrease in peripheral blood flow leads to reduced shear stress, which in turn may result in a decrease in arterial eNOS expression (27). Furthermore, inhibitory effects of hypoxia on endothelium-derived NO production during acute and long-term exposure in isolated fetal pulmonary (20) and adult systemic arteries (20, 21) have been demonstrated. Moreover, acute hypoxia completely inhibits ACH-induced relaxation in femoral arteries of the chicken embryo (28). This indicates that a decrease in oxygen availability might directly or indirectly (consequences of chemoreflex activation) modify both the presence and effects of NO. Similar effects would not be expected to occur as a result of protein malnutrition. However, to elucidate the mechanisms involved in the effects of chronic hypoxia on endothelial function, future studies are necessary. These should investigate a range of endothelium-dependent vasodilators, mechanical stimuli, NO synthase expression, and NO production.

A relationship between intrauterine growth retardation and impaired endothelial function has been described in humans. Flow-mediated dilation of the brachial artery (16, 17) and ACH-induced dilation (18) are reduced in healthy children and young adults who had low birth weights compared with normal birth weight subjects. Animal studies have focused on prenatal insults that may interfere with fetal growth to investigate effects on endothelial arterial function. Endothelial function was evaluated in offspring (100–120 d old) of food-restricted rats. Only when total food intake was reduced by 50% in the second half of gestation were reductions in endothelium-dependent relaxation observed (22); a reduction of the maternal food intake by 30% did not alter endothelial reactivity (5). In guinea pigs, effects of exposure of the mother to chronic hypoxia on endothelial function were observed in the offspring. Although the contribution of NO to the ACH-induced relaxing response and eNOS mRNA levels in fetal hearts were increased (29), chronic hypoxia reduced maximal responses to ACH in fetal carotid arteries (8).

We used two different models to induce growth retardation in the chicken embryo. We (13) and others (30, 31) previously showed that long-term exposure to moderate hypoxia reduces total embryonic body weight. In these experiments we also demonstrated increased hematocrit levels, indicating that the embryos were indeed exposed to hypoxemia in these conditions (13). In our model reduced total body weight was accompanied by a sparing effect on brain weight. Protein malnutrition was induced by removal of 10% of the albumen. The eggs of chickens contain yolk, which consists of water, fat, and proteins and primarily determines the degree of development of the chick at hatching. The other component of the egg, albumen, mainly (approximately 95%) contains water and amino acids, which are used by the developing embryo for whole-body protein synthesis and growth (23, 32, 33). As previously shown (23, 34), extraction of albumen from the egg was found to reduce total embryonic weight toward the end of incubation. Similar to exposure to chronic hypoxia, relative brain weight was spared, indicating that growth was reduced in a disproportionate way in both models. Disproportionate growth retardation has been described in other animal models in which

prenatal stress was induced by altering the intrauterine environment (4, 7, 9).

Comparisons between the two models should be made with caution because incubation procedures were different and making a small hole in the eggshell (as performed in the protein malnutrition studies) has been shown to induce changes in embryonic metabolism and body and organ weights (34). It is therefore possible that this procedure induced an additional small reduction in growth. However, whereas the growth retardation induced by the whole procedure of albumen removal was of comparable (or even greater) magnitude as the reduction in growth after chronic moderate hypoxia, both interventions had different effects on arterial reactivity.

Two critical points may be raised here. First, the period of exposure to the insult may be different between the two models. In the model of chronic moderate hypoxia, the embryo is constantly exposed to low oxygen levels from d 6 to 19. However, the effects of albumen removal may occur primarily during the second half of incubation (23). This may be of importance considering that insults may affect developing organ systems like the cardiovascular system at critical time points. Second, although chronic hypoxia reduced relative body weights of all organs (heart, liver, and kidney) apart from the brain (13), protein malnutrition did not significantly modify relative heart, liver, and kidney weights (data not shown), suggesting that the reduction in total body weight is accounted for by other tissues, like skeletal muscle or the skeleton (34). The authors like to stress that although there may be differences in exposure period and the exact features of the reduction in embryonic growth, growth retardation *per se* does not seem to be the cause of the changes in arterial functional properties. We clearly showed that the disproportionate reduction in growth caused by chronic hypoxia is accompanied by changes in ACH-induced relaxation whereas the reduction in *in ovo* growth induced by protein malnutrition is not.

Although evidence is limited others have reported effects of maternal food restriction on endothelial function (22). Important in this respect is that maternal undernutrition in mammals may modify maternal hormone balances and metabolism, which may indirectly influence fetal vascular function. Furthermore, the effects of maternal food restriction were demonstrated in adult offspring, whereas we evaluated endothelial function at the end of incubation time (0.9 gestation). Therefore, changes in endothelium-dependent relaxation as a result of chronic hypoxia in the chicken embryo may point to a more early and causal role of impaired endothelial function in the fetal programming of cardiovascular disease, whereas indirect effects of protein malnutrition may become evident postnatally. Future studies in posthatch chickens will address these points.

There is now substantial evidence from epidemiologic studies in humans and from animal studies associating an adverse intrauterine environment with cardiovascular diseases in adult life (1, 2). Endothelial dysfunction plays an important role in diseases such as atherosclerosis, heart failure, and hypertension (15). Changes in endothelial function may be causally involved in the development of these diseases. The role of endothelial NO as mediator may be considerable, as it may affect both smooth muscle tone and smooth muscle cell proliferation (35,

36). We show that prolonged exposure to chronic moderate hypoxia does, and protein malnutrition does not, reduce endothelium-dependent relaxation as a result of decreased NO release in peripheral arteries of the chicken embryo. Furthermore, our findings in 3- to 4-wk-old chickens indicate that effects on the NO system induced by chronic hypoxia during development are also present after hatching. These results warrant further investigation in chickens of various ages.

As the chicken embryo is a nonmammalian species, results may not directly be extrapolated to the human situation. However, inasmuch as studies in human neonates have suggested that the role of chronic hypoxia in creating intrauterine conditions that may lead to human growth retardation is substantial (37), the investigation of effects of a prolonged decrease in oxygen availability on vascular reactivity may be of great importance. We suggest that in addition to the previously demonstrated effect of embryonic hypoxemia on sympathetic nervous development (13), changes in endothelial function, especially the NO-dependent component, induced by chronic exposure to hypoxia during fetal life may contribute to the development of cardiovascular disease in later stages of life.

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