INTRODUCTION

Intrauterine growth retardation (IUGR) is most frequently the consequence of placental insufficiency resulting in decreased availability of nutrients and oxygen (1). Infants with IUGR are prone to intestinal dysfunction which is manifested by feeding intolerance, poor growth, malabsorption, and, in the most severe cases, necrotizing enterocolitis (NEC) (1, 2). Moreover, it has been suggested that some of the intestinal alterations induced by IUGR may persist in later life (3). The morphological and molecular mechanisms that lead to these complications are not completely understood and suitable experimental models are necessary. The aim of this study was to characterize mesenteric artery (MA) reactivity, small intestine morphometry and intestinal expression of vascular endothelial growth factor (VEGF) in a chicken model of hypoxia-induced fetal growth restriction. Chicken embryos (15 and 19 incubation days) and hatchlings (<3-h-old and 1-d-old) were incubated under hypoxic (15% O₂ from day 0 to day 19 of incubation) or normoxic conditions. Vascular reactivity was studied using wire myography. Intestinal morphometry was assessed in hematoxyline-eosine-stained sections. VEGF mRNA expression was determined by RT-PCR analysis. Hypoxia increased the responsiveness of chicken embryo MAs to the adrenergic agonist norepinephrine, the polypeptide endothelin (ET)-1, and the nitric oxide donor sodium nitroprusside and decreased the responsiveness to the endothelium-dependent relaxant agonist acetylcholine. However, the majority of these alterations, with the exception of the hyperresponsiveness to ET-1, were not present in the hypoxic hatchlings. When intestinal histology was analyzed, subtle hypoxia-induced changes were noted in the villi and the muscularis propria from the hatchlings. Hypoxic incubation also diminished the expression of VEGF mRNA in the terminal ileum of the hatchlings. In conclusion, chronic moderate hypoxia during incubation results in subtle but significant alterations in chicken MA reactivity, small intestine morphology and VEGF expression. Whether these alterations may have a direct effect on the functional status of the intestine remains to be investigated.

Key words: vascular endothelial growth factor, endothelin-1, chicken embryo, hypoxia, intrauterine growth retardation, mesenteric, necrotizing enterocolitis

MESENTERIC ARTERY REACTIVITY AND SMALL INTESTINE MORPHOLOGY IN A CHICKEN MODEL OF HYPOXIA-INDUCED FETAL GROWTH RESTRICTION

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Infants with intrauterine growth retardation are prone to intestinal disorders. The morphological and molecular mechanisms that lead to these complications are not completely understood and suitable experimental models are necessary. The aim of this study was to characterize mesenteric artery (MA) reactivity, small intestine morphometry and intestinal expression of vascular endothelial growth factor (VEGF) in a chicken model of hypoxia-induced fetal growth restriction. Chicken embryos (15 and 19 incubation days) and hatchlings (<3-h-old and 1-d-old) were incubated under hypoxic (15% O₂ from day 0 to day 19 of incubation) or normoxic conditions. Vascular reactivity was studied using wire myography. Intestinal morphometry was assessed in hematoxyline-eosine-stained sections. VEGF mRNA expression was determined by RT-PCR analysis. Hypoxia increased the responsiveness of chicken embryo MAs to the adrenergic agonist norepinephrine, the polypeptide endothelin (ET)-1, and the nitric oxide donor sodium nitroprusside and decreased the responsiveness to the endothelium-dependent relaxant agonist acetylcholine. However, the majority of these alterations, with the exception of the hyperresponsiveness to ET-1, were not present in the hypoxic hatchlings. When intestinal histology was analyzed, subtle hypoxia-induced changes were noted in the villi and the muscularis propria from the hatchlings. Hypoxic incubation also diminished the expression of VEGF mRNA in the terminal ileum of the hatchlings. In conclusion, chronic moderate hypoxia during incubation results in subtle but significant alterations in chicken MA reactivity, small intestine morphology and VEGF expression. Whether these alterations may have a direct effect on the functional status of the intestine remains to be investigated.

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INTRODUCTION

Intrauterine growth retardation (IUGR) is most frequently the consequence of placental insufficiency resulting in decreased availability of nutrients and oxygen (1). Infants with IUGR are prone to intestinal dysfunction which is manifested by feeding intolerance, poor growth, malabsorption, and, in the most severe cases, necrotizing enterocolitis (NEC) (1, 2). Moreover, it has been suggested that some of the intestinal alterations induced by IUGR may persist in later life (3). The morphological and molecular mechanisms that lead to these complications are not completely understood and suitable experimental models are necessary. In addition, it is difficult to demonstrate or isolate the effects of hypoxia because all human conditions or experimental animal models that induce fetal hypoxia are also accompanied by changes in nutrient delivery (4). In the last years, the chicken embryo has emerged as a valuable model for the study of the developmental consequences of hypoxia and other prenatal insults (5-11). Hypoxia is easily induced by incubating the egg in a low oxygen environment and its effects can be studied without interferences of maternal hormonal, metabolic, or hemodynamic alterations.

Hypoxia has profound effects on endothelial and vascular smooth muscle cellular physiology affecting the transcriptionally regulated expression of vasoactive substances, the modulation of receptor populations, the density and activities of ion channels and the signal transduction pathways involved in modulating vascular tone (7, 8, 12-14). Numerous studies have shown that chronic hypoxia decreases the growth of developing chicken embryos (4, 7, 8, 10, 15-18) and induces a broad spectrum of structural and functional vascular alterations (7, 8, 14, 15, 18-20). However, the effects of chronic hypoxia on intestinal development, and more particularly on the mesenteric circulation, have been only scarcely investigated (18).

One key growth factor involved in the pathophysiological effects of hypoxia is vascular endothelial growth factor (VEGF). VEGF is a peptide cytokine that couples hypoxia sensing to angiogenesis in developing and neoplastic tissue (21). Evidence from human and experimental studies indicates involvement of VEGF in the pathogenesis of several perinatal complications such as neonatal pulmonary vascular disease, chronic lung disease, retinopathy of prematurity, intraventricular hemorrhage and acute renal failure (22). In humans, the carrier state for the VEGF-2578 mutant allele, which predisposes to low VEGF
production, is associated with the development of NEC in preterm infants (22).

In a recent study, we analyzed the maturational differences in the reactivity of mesenteric artery (MA) rings isolated from normoxic incubated chicken embryos and hatchlings (23). We observed that, as early as 15 of the 21 days of incubation, MAs responded to K+-evoked depolarization and to a wide variety of contractile and relaxant agonists, indicating the presence at this stage of development of electro- and pharmaco-mechanical coupling as well as cGMP- and cAMP-mediated relaxation. In vivo development and transition to ex-ovo life was accompanied by alterations in the response of the MAs but a different developmental trajectory was observed for each reactivity pathway tested (23). In the present study we hypothesized that these developmental trajectories would be altered by incubation under hypoxia. We also hypothesized that chronic hypoxia would lead to changes in intestinal morphology and VEGF expression. To test these hypotheses, we analyzed the response to contractile and relaxant agonists of mesenteric arteries from chicken embryos (15 and 19 days) and hatchings (<3 hours-old and 1-day-old) incubated under hypoxic (15% O2 from day 0 to day 19 of incubation) or normoxic conditions. We also examined the effects of chronic hypoxia on small intestine histology and on the intestinal expression of VEGF.

MATERIAL AND METHODS

Incubation of chicken embryos

All experimental procedures were carried out in accordance with the Dutch Law on Animal Experimentation and the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU) and approved by the Committee on Animal Experimentation of the University of Maastricht. Fertilized eggs from White Leghorn chickens (Anker, Ochten, The Netherlands) were incubated at 37.8°C, 45% humidity and rotated once per hour over an angle of 90° (incubator model 25HS, Masalles Comercial, Spain). Control embryos were incubated under normoxic conditions (21% O2, 0.03% CO2). Experimental embryos were incubated in a second 25HS incubator where hypoxic conditions (15.0±0.3% O2 and 0.03% CO2) were maintained by providing a constant flow of N2 and compressed air with a flow meter (AGA Gas BV, The Netherlands) (10). The O2 and CO2 concentrations in the incubator were monitored with a DrDAQ O2 sensor (Pico Technology, United Kingdom) and an infrared CO2 analyzer (Beckman Instruments, Inc., Fullerton, USA). At day 15 (E15) or 19 (E19) of the 21 days of incubation some embryos were used for experiments and others allowed to hatch. The embryos allowed to hatch were transferred to the normoxic 25HS incubator at day 19. Hatchlings were used within the first 3 hours (NH3h) or transferred to a brooder unit under constant light, and temperature of 35°C and provided ad libitum access to water and a standard starter diet (10). This last group was studied within the second day posthatch (NH1d).

Recording of mesenteric artery reactivity

On the experimental day, the animals were killed by decapitation, placed on the dorsal side on a Petri-dish coated with silicon and a midline laparotomy and sternotomy were performed. With the aid of a dissecting microscope, the cranial MA was carefully dissected free from surrounding tissue. The cranial MA is the vessel which supplies most of chicken intestine and also the yolk sac during the embryonic period (24). Only the intestinal part of the artery was used in our study.

Two stainless steel wires (diameter 40 µm) were inserted into the lumen of the MA, which was mounted as a 1.7-2 mm length ring segment between an isometric force transducer and a displacement device in a myograph (Danish Myo Technology A/S model 610M, Aarhus, Denmark). The myograph organ bath (5 mL vol) was filled with Krebs-Ringer bicarbonate (KRB, composition in mmol L−1: NaCl, 118.5; KCl, 4.75; MgSO4•7H2O, 1.2; KH2PO4, 1.2; NaHCO3, 25.0; CaCl2, 2.5; glucose, 5.5) buffer maintained at 39°C. After an equilibration period of 30 min the vessels were distended to a resting tension corresponding to a transmural pressure of 10 mmHg (E15) or 20 mmHg (E19 and hatchlings) (9). These pressures correspond to the mean arterial blood pressure reported in chicken at the corresponding age (25) and elicited the highest contractile response to KCl, as determined in pilot experiments. After 30 min. of incubation under basal tone, a control contraction was elicited by raising the K+ concentration of the buffer (62.5 mM) in exchange for Na+. During mounting, stabilization, and the experiments, MA rings were maintained in KRB buffer aerated with 95% O2:5% CO2.

Contractile agonists were evaluated under basal tone. Concentration-response curves to KCl (31.25–125 mM), norepinephrine (NE; 10 nM–0.1 mM), the thromboxane A2 mimetic U-46619 (10 nM–3 µM), and endothelin (ET)-1 (0.1 nM–0.1 µM) were constructed by increasing the organ chamber concentration of the drug incrementally after a steady-state response had been reached. When two or more agonists were studied in the same arterial preparation, the vessels were repeatedly washed and allowed to equilibrate for at least 30 min. If the tone did not recover to resting level, the vessels were discarded for further experiments.

To study relaxant responses MA rings were contracted with NE (10 µM). When contraction reached a plateau, concentration-response curves to acetylcholine (ACh, 10 nM–0.1 mM), the NO donor sodium nitroprusside (SNP, 10 nM–0.1 mM) and the adenylate cyclase activator forskolin (10 nM–10 µM) were constructed.

Morphometric measurements

After sacrifice, full-thickness segments of terminal ileum were immediately removed, fixed overnight in phosphate-buffered 4% formaldehyde solution, stored in 70% ethanol, and embedded in paraffin. Paraffin sections (4 µm thickness) were cut at 0.1 mm intervals, deparaffinized, and stained with hematoxylin and eosin (H&E). Images were captured using a 5× objective on a standard upright microscope (Leica DM2000) with an attached digital camera (Leica DFC280). Typically, 30 images were captured per tissue section. Using image analysis software (Quantimet 570, Leica), villus height, villus area, number of crypts/villus, and muscularis propria thickness were determined (Fig. 1A) Villi developmental stage was classified using the system of Uni et al. (26). Briefly, villi were divided into three stages (V1, V2, and V3), differing in length and shape (Fig. IB), with the larger V1 villi often being pear-shaped and the intermediate V2 villi (approximately 50% of the size of the V1 villi) being narrower and with a rocket-like shape. The smallest V3 villi were arrow-shaped. Only V1 villi were used for the measurement of length and area.

RNA isolation and measurement of vascular endothelial growth factor mRNA expression

The chicken VEGF-A gene gives rise to four isoforms generated by alternative splicing of exons (VEGF122, VEGF146, VEGF166, and VEGF190, homologous to human VEGF121, VEGF145, VEGF165, and VEGF189, respectively).
We examined the expression of the VEGF-A isoforms by RT-PCR of total RNA isolated from the terminal ileum of normoxic and hypoxic chickens. The ileum section was cut into pieces and tissue RNAs were stabilized in RNA later™ (Qiagen, Venlo, The Netherlands) for 1 day at 4°C, after which the samples were stored at −80°C until use. After thawing, total RNA was isolated using the RNeasy® minikit (Qiagen) according to the manufacturer's protocol, including a DNase treatment step (RNase-free DNase set for use with RNeasy columns; Qiagen). Total RNA was quantified using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer. Part of the RNA was denatured at 65°C for 10 min and immediately placed on ice. Then, 500 ng of RNA was translated to cDNA using Ready-To-Go™ You-Prime First-Strand beads (GE Healthcare, Eindhoven, The Netherlands) and 200 pmol pd(N) 6 random hexamer primers (GE Healthcare) in a total volume of 25 µl. RT-PCR was performed at 37°C for 1 hour, after which the samples were heated to 95°C for 3 min to stop the reaction. Subsequently, samples were cooled to 4°C and stored at 4°C until use. PCR was performed with VEGF primers (FW 5’-caggccatcctgtgtgcctct-3’, RV 5’-ttccgctgctcaccgtctccgg-3’) designed to yield the four isoforms of avian VEGF (29).

PureTaq™ Ready-To-Go™ PCR beads (GE Healthcare) were used with 10 pmol of each primer and 1 µl of cDNA per reaction with a total volume of 25 µl. PCR steps were as follows: denaturation 3 min at 94°C (denaturation at 94°C - 30 s, annealing at 60°C - 1 min, elongation at 72°C - 1 min). Ribosomal 18S was used as an internal standard and analyzed by the same procedure (primer sequences: FW 5’-ccatccaatcggtagtagcg-3’, RV 5’-cgataacgaacgagactctgg-3’). PCR products in the exponential phase of the PCR reaction were yielded after 18 cycles. PCR products were visualized at a 2% agarose gel stained with ethidium bromide and band densities were measured using ImageQuant™ software (GE Healthcare).

Fig. 1. (A) Trace method used to determine villus height, villus area, number of crypts/villus, submucosal thickness and muscularis propria thickness in the chick terminal ileum. (B) Representative light microscopy of intestinal villi from the terminal ileum of a 3-h-old newly hatched chick exposed to normoxia during incubation. V1 to V3 = villi in different stages of development. Staining was with hematoxylin-eosin.

Solutions containing different concentrations of K+ were prepared by replacing part of the NaCl of the KRB buffer by an equimolar amount of KCl. Arterenol bitartrate (NE), ACh, and SNP were obtained from Sigma (St. Louis, MO); U-46619 was from Cayman Chemical (Ann Arbor, MI). All drugs were dissolved initially in distilled deionized water (except U46619 in DMSO) to prepare adequate stock solutions and further dilutions.
were also made in deionized water. The final bath concentration of DMSO did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity (23).

Data analysis

Results are shown as mean (S.D.) of measurements in n animals. For clarity, results are shown in the figures as mean ±S.E.M. Contractions are expressed in terms of active wall tension (mN/mm), calculated as the force divided by twice the length of the segment, while the relaxant responses are expressed as the percentage of reduction of the contraction induced by NE. Sensitivity (expressed as pEC$_{50}$ = log EC$_{50}$) and maximal contraction or relaxation (E$_{max}$) to agonists was determined by fitting individual concentration-response data to a non-linear sigmoidal regression curve. Differences in

Table 1. Effects of hypoxia during incubation in the body mass of 15-day (E15) and 19-day (E19) chicken embryos and 3-h-old (NH3 h) and 1-day-old (NH1d) newly hatched chicks.

*P 0.001 vs. control (normoxia).

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<td>hypoxia (n=30)</td>
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*P 0.001 vs. control (normoxia).

Fig. 2. Concentration-dependent contractile effects of KCl and the thromboxane A2 mimetic U46619 in mesenteric artery rings from 15-day (E15) and 19-day (E19) chicken embryos and 3-h-old (NH3 h) and 1-day-old (NH1d) newly hatched chicks exposed to hypoxia (●) or normoxia (○) during incubation. Each point represents the mean ±S.E.M. of 5-8 embryos/chicks.
RESULTS

Effects of hypoxia on chicken embryo growth

Exposure of chicken embryos, from the first day of incubation, to 15% instead of 21% O₂ induced a significant decrease of body mass at E15 and E19 (Table 1). When the hypoxic embryos were incubated under normoxia (from day 19 onward), no significant differences in body mass were observed at NH3h or NH1d (Table 1).

Mesenteric artery reactivity

The developmental changes in MA reactivity have been described in a recent publication of our laboratory (23). Therefore, in this work we focused on the effects of hypoxic incubation in MA reactivity. The contractions evoked by KCl (Fig. 2A-2D), and U-46619 (Fig. 2E-2H) were not affected by hypoxic incubation. In contrast, hypoxic incubation induced a significant increase in the effectivity (Eₘₐₓ) of NE at E19 (Eₘₐₓ normoxia: 1.23 mN/mm, S.D. 0.61, n=6; Eₘₐₓ hypoxia: 2.08 S.D. 0.49 mN/mm, n=8; P<0.01; Fig. 3B). No effects of hypoxia on NE responsiveness were observed at the other ages studied (Fig. 3). Hypoxia also induced a significant increase in the responsiveness to ET-1 at E15 (Fig. 4A) and NH1d (Eₘₐₓ normoxia: 0.40 mN/mm, S.D. 0.16, n=7; Eₘₐₓ hypoxia: 0.73 mN/mm, S.D. 0.23, n=6; P<0.01; Fig. 4D). Moreover, high concentrations (≥0.1 M) of ET-1 induced relaxation in the MA from NH1d chicks (Fig. 4E) but this relaxant effect of ET-1 is not observed after hypoxic incubation (Fig. 4F).

In the above-mentioned study, we demonstrated that ACh evoked endothelium-dependent relaxation in MA from embryonic and NH chickens (23). Herein, we observed that the sensitivity to ACh was significantly diminished in the hypoxic E15 animals (pEC₅₀ normoxia: 6.44, S.D. 0.24, n=11, pEC₅₀ hypoxia: 5.73, S.D. 0.24, n=6, P<0.001, Fig. 5A). Hypoxia did not affect the sensitivity to ACh at the other ages examined (Fig. 5B-5D) but induced a slight decrease in the Eₘₐₓ at NH1d (Fig. 5D). As shown in Fig. 5F the sensitivity to the NO-donor SNP was significantly greater in the hypoxic E19 (pEC₅₀ 7.31, S.D. 0.34, n=7) embryos when compared with their respective age-matched controls (pEC₅₀ 6.05, S.D. 0.32, n=10, P<0.001). Hypoxia did not affect the responsiveness to SNP at E15, NH3h or NH1d (Fig. 5E, 5G-5H).

Morphological examination

Histological analysis indicated that the chicken embryo terminal ileum was growing rapidly. Villi at E15 were rudimentary and crypts were not present (Fig. 6A). In the E19 intestines, only a few crypts were observed. These crypts were small and contained few cells (Fig. 6B). In the E15 and the E19 intestines only V1 and V2 villi were present and the distribution of these two villus stages did not change significantly between the two ages (Fig. 7F) The terminal ileum of the hatchlings (NH3h and NH1d) showed a marked increase in size and complexity with longer fingerlike villi and, generally, more than one crypt per villus in the intervillous epithelium (Fig. 6C, 6D, Fig. 7). Villi at the three stages of development (V1, V2 and V3) were present in a similar proportion at the two hatchling ages (Fig. 7F). Numerous goblet cells were observed on the villi surface of the hatchlings (Fig. 6C-6D). Ileal morphology of the hypoxic animals was, in general, comparable to that of controls (Fig. 7). The only exceptions to this were a small, but significant, decrease in villus height and muscularis propria thickness that was observed in the NH3h hypoxic animals (Fig. 7A-7E). The distribution of the three villus stages did not change between hypoxic and normoxic animals (Fig. 7F).

Fig. 3. Concentration-dependent contractile effects of norepinephrine (NE) in mesenteric artery rings from 15-day (E15) and 19-day (E19) chicken embryos and 3-h-old (NH3h) and 1-day-old (NH1d) newly hatched chicks exposed to hypoxia (●) or normoxia (○) during incubation. Each point represents the mean ±S.E.M. of 5-8 embryos/chicks. *P<0.05 for difference (assessed by t-test) in Eₘₐₓ compared with normoxia.
**Ileal vascular endothelial growth factor mRNA expression**

VEGF mRNA expression was investigated in the ileum of normoxic E15 (n=8), E19 (n=9) and NH3d (n=10) chicks and hypoxic E15 (n=7), E19 (n=8) and NH3d (n=12) chicks. When the mRNA expression of the VEGF-A isoforms was examined by RT-PCR, we detected two bands corresponding to VEGF122 and VEGF166 isoforms (Fig. 8A). No signal was detected for the VEGF146 and VEGF190 isoforms. As shown in Fig. 8B, there was a trend toward higher levels of VEGF122 expression in the ileum of the NH compared with embryonic chicks, but this did not reach statistical significance. The expression of VEGF166 was significantly higher in the NH chicks (Fig. 8C). This developmental increase in VEGF was impaired by hypoxic incubation. Thus VEGF expression was significantly lower in the NH hypoxic chicks compared with normoxic controls (Fig. 8).

**DISCUSSION**

Structural and functional changes occur in the prenatal intestine with development in preparation for enteral feeding following birth. Afterwards, the postnatal development of the gastrointestinal system is a very dynamic process in which an intense growth and remodeling of the tissues take place (30). The present study focused on how intestinal development of chicken embryos and hatchlings is affected by chronic moderate hypoxia (15% O2) during incubation. Hypoxia altered the responsiveness of chicken embryo mesenteric arteries to the adrenergic agonist NE, the endothelium-dependent relaxant agonist acetylcholine, the NO donor SNP and the constrictor polypeptide ET-1. However, the majority of these alterations, with the exception of the hyperresponsiveness to ET-1, were not present in the hypoxic hatchlings. When intestinal histology was analyzed, subtle hypoxia-induced changes were noted in the villi and the muscularis from the hatchlings. Hypoxic incubation also diminished the expression of VEGF mRNA in the terminal ileum of the hatchlings.

In our study, we deliberately chose for a model in which hypoxia was not applied from day 19 onward. Unlike the rapid transition from an intrauterine to an extrauterine environment displayed in most mammals, bird hatching from eggs is an event that may take place over several days (31, 32). O2 demand increases exponentially during development, and it

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**Fig. 4.** Concentration-dependent contractile effects of endothelin-1 (ET-1) in mesenteric artery rings from 15-day (E15) and 19-day (E19) chicken embryos and 3-h-old (NH3 h) and 1-day-old (NH1d) newly hatched chicks exposed to hypoxia (●) or normoxia (○) during incubation. Each point represents the mean ±S.E.M. of 6-12 embryos/chicks. * P<0.05 for difference (assessed by t-test) in E_max compared with normoxia. $P<0.05$ for difference (assessed by two-way ANOVA) in the overall contractile response compared with normoxia. In panels (E) and (F) (representative tracings), numbers above the arrows indicate log M [ET-1] and arrows without numbers indicate half-log increments in concentration.
exceeds the capacity of the chorioallantoic membrane gaseous diffusion by the end of the avian incubation period (32). At this point, around day 19 of incubation in the chicken, the beak of the embryo penetrates the air cell, air enters the lung, and breathing is initiated. This process is termed internal pipping and is followed by external pipping when an opening of the shell is achieved and ambient air is breathed for the first time (31, 32). In our 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 (8, 10, 14). Nevertheless, it is also known that these two days of normoxic incubation are sufficient to revert some of the effects of hypoxia on growth and vascular responsiveness (8, 10). A possible explanation for this reversion of the effects of hypoxic incubation might be a delayed hatching in the hypoxic group which allowed a longer exposure to normoxia between day 19 and hatching. We do not know if this was the case in our study because our experimental design did not include assessment of hatching time. Molenaar et al. analyzed this issue in chicken embryos incubated between day 7 and day 19 under 21 or 17% O₂ and found no significant differences in hatching time (33). In the present study, we observed that the hypoxia-induced reduction in body mass and the alterations in the responsiveness to NE, ACh and SNP were only present in the prenatal period but not in the NH chicks. In contrast, the responsiveness to ET-1 was altered in the embryonic and the NH chicks and the changes in intestinal morphology and VEGF expression were only observed in the NH chicks. Altogether, our data suggest that chronic hypoxia during incubation altered the developmental trajectory of chicken intestine and that this altered development leaded to changes in intestinal homeostasis that are present in the post-hatch period.

Fig. 5. Concentration-dependent relaxant effects of acetylcholine (ACh) and the nitric oxide donor sodium nitroprusside (SNP) in mesenteric artery rings from 15-day (E15) and 19-day (E19) chicken embryos and 3-h-old (NH3 h) and 1-day-old (NH1d) newly hatched chicks exposed to hypoxia (●) or normoxia (○) during incubation. Each point represents the mean ±S.E.M. of 4-10 embryos/chicks. *P<0.05 for difference (assessed by t-test) in E₅₀ compared with normoxia. #P<0.05 for difference (assessed by two-way ANOVA) in the overall relaxant response compared with normoxia.
Effects of hypoxia on contractile responses of mesenteric arteries

The redistribution of fetal cardiac output during hypoxia may be partially dependent on increased circulating levels of several vasoactive factors, including NE and ET-1, but may also involve changes in the local vascular responses to these circulating factors (34). In the present work, we observed that the contractile response evoked by K⁺-induced depolarization was similar in the normoxic and the hypoxic MAs. K⁺-induced contraction is often used to examine the smooth muscle contraction function and to standardize the receptor-mediated contraction (23). Our data suggest, therefore, that the intrinsic contractile properties of the MAs are not affected by hypoxic incubation. In contrast, chronic hypoxia increased the responsiveness to NE in the E19 and to ET-1 in the E15, E19 and NH1d chicks.

Exposure to mild chronic hypoxia during incubation resulted in increased NE levels in E19 chicks (35) suggesting that catecholamines are involved in the prenatal adaptation to chronic hypoxia in this species. However, the alterations in vascular adrenergic responsiveness that accompany the increase in catecholamines are markedly tissue-dependent. Thus, hypoxia increased adrenergic contraction in the MA (present work) and the DA of the E19, but did no affect the adrenergic responsiveness of the femoral and the pulmonary arteries (8, 14). Tintu et al., (18) analyzed the reactivity of second-order mesenteric resistance arteries from chicken embryos incubated under normoxia or hypoxia. They observed that the hypoxic E19 MAs showed an enhanced vasoconstriction in response to the sympathetic nervous stimulator tyramine and an enhanced vasodilation in response to the α-adrenoceptor blocker phentolamine. These data suggest an increased activity of perivascular sympathetic nerves in chronically hypoxic embryos at this stage of development. Our present results suggest that in larger arteries an increased sensitivity to catecholamines is also part of the spectrum of alterations induced by chronic prenatal hypoxia in the mesenteric circulation.

ET-1 is a vasoactive and mitogenic polypeptide produced mainly by the vascular endothelium. Its binding to ETₐ and ETₐ receptors on vascular smooth muscle induces contraction, whereas its binding to endothelial ETₐ receptors causes vasodilatation (36). ET-1 is constitutively produced, but its production can also be stimulated by a wide range of stimuli, including hypoxia. ET-1 levels in amniotic fluid in the human near-term fetus have been found to inversely correlate with PO₂ in serum as measured by cordocentesis (37). In hypoxic pulmonary hypertension in newborn mice, rats, and piglets, alternations in ET-1 level and ET₁ receptor binding indicate that they may be responsible for the increased pulmonary vasoconstriction and vascular remodeling (38). In some mammalian species, ET-1 is considered as the primary vasoconstrictor stimulus in the perinatal intestinal circulation (36). In the chicken embryo, ET-1 evoked contraction in the mesenteric (23), the pulmonary (39), the femoral, and the chorioallantoic arteries (9), as well as in the ductus arteriosus (40).
In the MA from normoxic chicks, ET-1-induced contraction reached a peak at E19 and decreased in the hatchlings. Moreover, high concentrations of ET-1 induced relaxation in the MA from NH1d chicks (23) but this relaxant effect of ET-1 is not observed after hypoxic incubation (Fig. 4F). Although, we have not characterized the nature of the response to ET-1, our previous results suggested the presence of ETα-mediated contraction and ETβ-mediated relaxation in the MA from hypoxic chickens (23). Our present results suggest that ETβ-mediated relaxation might be impaired by chronic prenatal hypoxia. Accordingly, prolonged exposure to hypoxia increased the contractile effect of ET-1 and abolished relaxant effect mediated by the ETα receptor in murine and porcine pulmonary arteries (41, 42). Our present findings in the MA from hypoxic chicks warrant further investigation into the constitutive production of ET-1 and the hypoxia-induced changes in ETα and ETβ receptor expression.

**Effects of hypoxia on relaxant responses of mesenteric arteries**

In the chicken embryo ACh induced an endothelium-dependent and, at least partially, NO-mediated relaxation of the mesentric (23), the pulmonary (39), the femoral (39), the caudal arteries (43) and the ductus arteriosus (11). As reported elsewhere, chronic moderate hypoxia in the chicken embryo led in the femoral artery (7, 8) and the ductus arteriosus (44) to impairment of ACh-induced relaxation. In contrast, ACh-induced relaxation of chicken embryo pulmonary arteries was not affected by chronic hypoxic incubation (8). Herein, we observed diminished sensitivity to ACh in the E15 hypoxic animals. Interestingly, the sensitivity to the endothelium-independent soluble guanylate cyclase (sGC) stimulator SNP was significantly increased in the MAs from E19 hypoxic embryos. This effect can be attributed to an enhanced sensitivity or expression, of sGC and could have partially masked the impairment of ACh-induced relaxation in the hypoxic MAs. The work from numerous laboratories shows that the activation of endothelium-independent pathways may compensate for the loss of endothelium-dependent relaxation (45-47). In opposition with our present results, chronic hypoxia did not affect SNP-mediated relaxation in chicken embryo femoral and pulmonary arteries and impaired it in the ductus arteriosus (7, 8, 19, 20). Altogether, this suggests that the effects of hypoxia in endothelium-dependent and -independent relaxation are strongly vascular bed-dependent.

**Effects of hypoxia on intestinal morphology**

The development of the gastrointestinal tract in pre- and posthatch chicks has been described by several authors (26, 48-51). Between days 17 and 19 of incubation, a progressive but slow increase in villus development is observed (26). Additional rapid morphological changes occur during later embryonic development and, specially, after hatch (26, 48-51) The accelerated rate of development posthatch is reflected in the several-fold elevation in numbers of enterocytes during first few days posthatch, resulting from the dramatic increase in villus length and surface (50). Our present results confirm this pattern of pre- and posthatch development.

Tibboel et al. reported that from day 14 of incubation onward an acute (3 hours) exposure to 12–13% O2 produced serious
necrotic changes in the intestine of the chicken embryo (52). In contrast, when we investigated the effects of chronic exposure to 15% O₂ on the morphological development of chick terminal ileum, we only observed subtle alterations. In fact, prehatch intestinal development was similar in normoxic and hypoxic embryos. After hatching, the hypoxic chicks showed decreased villus length and muscularis propria thickness (only observed at NH3h). We can only speculate on the functional significance of these subtle hypoxia-induced alterations. An important consideration is that, in parallel with the morphological changes, the ability of the intestinal tissue to digest and absorb nutrients increases steadily during the first days posthatch (50, 51). Interestingly, Molenaar et al. demonstrated that the efficiencies of nutrient utilization for growth improved in the posthatch period in chicken embryos incubated between day 7 and 19 at 17% O₂ when compared with normoxic-incubated chicks (33). This suggests that embryos incubated under suboptimal environmental conditions may develop adaptive mechanisms that still continue in the posthatch period (33).

**Effects of hypoxia on intestinal vascular endothelial growth factor expression**

We found that intestinal VEGF expression increased toward hatching and that this increase was impaired by hypoxic incubation. Similarly, developmental increases in VEGF have been demonstrated in lamb intestine (53), suggesting a role for VEGF in the development of the intestinal vascular network during fetal and early postnatal life. Although hypoxia is regarded as the most potent regulator of VEGF (21), we did not find a higher VEGF expression in the intestine from the hypoxic embryos. In addition, when the hypoxic embryos were exposed to normoxia for the two last days of incubation, they did not undergo the posthatch increase in VEGF expression observed in the normoxic embryos. It can be speculated that the rapid intestinal growth that takes place between 19 and 21 days of incubation induces relative tissular hypoxia leading to increased VEGF expression. Embryos incubated in low O₂ increase carrying capacity through increases in red blood cells, hemoglobin mass, or blood volume (33). Such adaptations to low O₂ may have reduced the physiological hypoxia induced by rapid intestinal growth and, consequently, the intestinal expression of VEGF.

**Future perspectives**

Acute or chronic hypoxic injury to the gastrointestinal tract is believed to be a major contributing and potentially inciting factor in several neonatal conditions, including NEC. The series of experiments reported here present evidence that chronic moderate hypoxia during incubation results in subtle but significant alterations in chicken MA reactivity, small intestine morphology.
and VEGF expression. Whether these slight alterations may have a direct effect on the functional status of chick intestine remains uncertain. It can be speculated that the modest degree of the alterations induced by hypoxia is the result of the adaption of the embryo to the prolonged exposure to a relatively moderate insult. Critical time windows exist during development, and if environmental changes are experienced in the window of vulnerability, then the trajectory of development of the responding organ may be changed in ways that result in transient or persistent alterations (52). Therefore, new experiments will have to be conducted where embryos are exposed to different degrees of hypoxia across different temporal windows.

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