Fetal programming of pulmonary vascular dysfunction in mice: role of epigenetic mechanisms

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EPIDEMIOLOGICAL STUDIES INDICATE that pathological events during the fetal period predispose to systemic cardiovascular disease later in life, but little is known about the pulmonary circulation and the underlying mechanisms. Maternal undernutrition during pregnancy may represent a model to investigate underlying mechanisms, because it is associated with systemic vascular dysfunction in the offspring in animals and humans. In rats, restrictive diet during pregnancy (RDP) increases oxidative stress in the placenta. Oxygen species are known to induce epigenetic alterations and may cross the placental barrier. We hypothesized that RDP in mice induces pulmonary vascular dysfunction in the offspring that is related to an epigenetic mechanism. To test this hypothesis, we assessed pulmonary vascular function and lung DNA methylation in offspring of RDP and in control mice at the end of a 2-wk exposure to hypoxia. We found that endothelium-dependent pulmonary artery vasodilation in vitro was impaired and hypoxia-induced pulmonary hypertension and right ventricular hypertrophy in vivo were exaggerated in offspring of RDP. This pulmonary vascular dysfunction was associated with altered lung DNA methylation. Administration of the histone deacetylase inhibitors butyrate and trichostatin A to offspring of RDP normalized pulmonary DNA methylation and vascular function. Finally, administration of the nitroxide Tempol to the mother during RDP prevented vascular dysfunction and dysmethylation in the offspring. These findings demonstrate that in mice undernutrition during gestation induces pulmonary vascular dysfunction in the offspring by an epigenetic mechanism. A similar mechanism may be involved in the fetal programming of vascular dysfunction in humans.

METHODS

All animal protocols were approved by the Centre Hospitalier Universitaire Vaudois Institutional Animal Care Committee.

Calorie Restriction Diet During Gestation

Timed mating was performed in female C57Bl/6 mice (Charles River, L’Arbresle, France; age 8–10 wk). Two females were mated to one male. Following confirmation that mating had occurred (presence of a vaginal smear plug), the females were housed individually in standard cages and randomly divided into two groups. The control group was fed standard chow (Safe, Epinay sur Orge, France) ad

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libitum. The nutritionally restricted group was fed 65% of the ad libitum intake (determined by the amount of food consumed by the control group the previous day) from day 7 of pregnancy until parturition. All offspring received food ad libitum.

To avoid variability of the results related to the hormonal cycle in females, only male offspring were studied. All measurements were performed by investigators who were blinded to the study group.

**Pulmonary Endothelial Function In Vitro**

Twelve- to fourteen-week-old male offspring of restrictive diet pregnancy and control mice were housed under normoxic conditions or housed for 2 wk in Plexiglas cages in which the fraction of the inspired oxygen (FIO₂) was kept at 16%, a level of hypoxia known to induce exaggerated hypoxic pulmonary hypertension and right ventricular hypertrophy in mice with pulmonary vascular dysfunction (7). At the end of the 2-wk hypoxic exposure, the mice were killed with an intraperitoneal injection of pentobarbital sodium (200 mg/kg). The lungs were immediately removed, and the pulmonary arteries were dissected free of parenchyma and cut into a ring. Pulmonary artery rings were then suspended in organ chambers filled with 10 ml of modified Krebs-Ringer bicarbonate solution (composition in mmol/l: 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose), maintained at 37 ± 0.5°C and aerated with 95% O₂-5% CO₂ (pH 7.4). Two stirrups were passed through the lumen to suspend the ring. One stirrup was anchored to the bottom of the organ chamber, and the other was connected to a strain gauge (PowerLab/8SP; AD Instruments, Colorado Springs, CO) to measure the isometric force. At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension (0.5 g) and allowed to equilibrate for 1 h. Next, the effects of acetylcholine (10⁻⁸ to 10⁻⁴ mol/l) or sodium nitroprusside (10⁻⁹ to 10⁻⁵ mol/l) were determined in pulmonary artery rings preconstricted with phenylephrine (10⁻⁵ mol/l) at a level corresponding at least to the maximal response to potassium (100 mmol/KCl) (22). The drug-induced change in tension was expressed as the percentage of the initial contraction induced by phenylephrine.

**Measurement of Pulmonary Artery Pressure**

Twelve- to fourteen-week-old male offspring of restrictive diet pregnancy and control mice were housed for 2 wk in Plexiglas cages in which the FIO₂ was kept at 16% or at 21%. At the end of the 2-wk exposure, the mice were anesthetized with ketamine/xylazine (100/10 μg/kg body wt ip) and placed on a heating table to keep the body temperature between 37 and 38°C. Via a PE 50 catheter inserted into the right jugular vein, a microtip pressure transducer (Millar, Houston, TX; 1.4 F) was advanced through the superior vena cava into the right ventricle. The right ventricular pressure was recorded with a computer data acquisition system (DI-400, Windaq; Dataq Instruments, Akron, OH). The systolic pulmonary artery pressure was assumed to be equal to the systolic right ventricular pressure (6). After 15 min of room air breathing, the mice were breathing a hypoxic gas (FIO₂, 16%) through a specially designed face mask.

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Fig. 1. Pulmonary vascular function, pulmonary artery pressure, and right ventricular weight in offspring of restrictive diet pregnancy and control mice at the end of a 2-wk exposure to hypoxia. Acetylcholine (A) and sodium nitroprusside-induced (B) pulmonary artery vasodilation in vitro and pulmonary artery pressure (PAP; C) and right ventricle-to-left ventricle + septum (RV to LV + S) weight ratio (D) in vivo in offspring of restrictive diet pregnancy and control mice. Error bars represent SE; n ≥ 10 animals in each group. *P = 0.002 vs. control mice; #P = 0.013 vs. control mice.
Right Ventricular Hypertrophy

Fourteen-week-old male mice were exposed to hypoxia (FiO2, 16%) for 2 wk. At the end of this hypoxic exposure, mice were killed with an intraperitoneal injection of pentobarbital. The heart was removed and the right ventricle (RV) was carefully dissected from the left ventricle and septum (LV + S) after removal of the atria. The tissue was weighed, and the RV-to-LV + S ratio was calculated (5).

Epigenetic Mechanisms

DNA methylation of lung tissue. DNA methylation studies were performed in lung tissue harvested from 12- to 14-wk-old mice after exposure to hypoxia. The SssI methyltransferase assay of Balaghi and Wagner was used (19). This assay uses the universal methyl donor S-adenosyl methionine to transfer a tritium-labeled methyl group to nonmethylated cytosines in CpG sites. As a result, higher counts demonstrate less genomic DNA methylation. A 30-μl reaction volume was used to incubate 0.5 μg of genomic DNA with 3 μmol/l (74 kBq) [3H-methyl]methionine (NEN, Boston, MA), 3 μl of 10 × reaction buffer, and 3 U of SssI methyltransferase (New England Biolabs, Beverly, MA). The reaction was incubated at 37°C for 60 min, and then 15 μl of the reaction mixture were spotted onto DE81 paper circles (Whatman, Ann Arbor, MI). The paper circles were washed five times in 0.5 mol/l acidic phosphate buffer (pH 6.8) and dried in air. Radioactivity was determined by liquid scintillation counting. Blank values were determined from reactions without SssI methyltransferase enzyme.

Effects of histone deacetylase inhibitor administration. Sodium butyrate (Sigma-Aldrich Chemie, Steinheim, Germany; 2 mg·kg body wt⁻¹·day⁻¹ in 200 μl of PBS), trichostatin A (Sigma-Aldrich Chemie; 1 mg·kg body wt⁻¹·day⁻¹ in 200 μl of PBS), or vehicle was administered intraperitoneally for 2 wk to 10-wk-old male offspring of restrictive diet pregnancy and control mice kept under hypoxia for 2 wk.

Transmission of vascular dysfunction to the next generation. To test for this possibility, male offspring of restrictive diet pregnancy who had been treated with vehicle or sodium butyrate (Sigma-Aldrich Chemie; 2 mg·kg body wt⁻¹·day⁻¹ in 200 μl of PBS) were mated to female control mice and pulmonary vascular function in vitro was examined in the progeny having been exposed to 2 wk of hypoxia.

Role of oxidative stress. To test for the potential pathogenic role of oxidative stress during pregnancy on vascular function in the offspring, Tempol (Sigma-Aldrich Chemie; 10⁻² mmol/l in the drinking...
water) was administered to pregnant mice during the entire period of the restrictive diet.

Statistical Analysis

Statistical analyses were made using JMP v. 7.0 software (SAS Institute, Cary, NC). Bivariate analyses were made using the unpaired two-tailed Student’s t-test or two-factor ANOVA. Multivariate analysis was performed with a three way ANOVA taking into account group (diet/control), treatment (vehicle/butyrate/trichostatin A), and acetylcholine concentration (as log10, with linear and square factors). Two models were tested; one without interaction and one including a group × treatment interaction. Post hoc comparisons were made using the Tukey’s honestly significant difference test. A P value <0.05 was considered to indicate statistical significance. Unless otherwise indicated, data are given as means ± SD.

RESULTS

The litter size (5.2 ± 1.7 vs. 5.3 ± 1.5) and the body weight (4.3 ± 0.7 vs. 4.0 ± 0.6 g at the age of 5 days, P = 0.10, and 23.3 ± 1.7 vs. 24.0 ± 2.3 g at the age of 3 mo, P = 0.4) were not different in restrictive diet pregnancy and control mice.

In mice kept under normoxia, acetylcholine-induced vasodilation in vitro (P = 0.8) and pulmonary artery pressure responses to hypoxia were similar in offspring of restrictive diet pregnancy and control mice. After 2 wk of hypoxic exposure, acetylcholine-induced vasodilation of pulmonary artery rings in vitro was significantly smaller (P < 0.0001) in offspring of restrictive diet pregnancy than in offspring of normal pregnancies (Fig. 1A), whereas sodium nitroprusside-induced vasodilation was similar in the two groups (Fig. 1B). In offspring of restrictive diet pregnancy, pulmonary endothelial dysfunction in vitro translated into exaggerated hypoxic pulmonary hypertension (35.8 ± 3.5 vs. 30.8 ± 4.9 mmHg, P = 0.002, restrictive diet vs. controls; Fig. 1C), and right ventricular hypertrophy (RV-to-LV + S ratio; 0.329 ± 0.028 vs. 0.289 ± 0.049, P = 0.013, restrictive diet vs. controls; Fig. 1D) in vivo.

Epigenetic mechanisms

The uptake of radioactive methyl groups in lung tissue was significantly higher in offspring of restrictive diet pregnancy than in control mice (85 ± 18 vs. 58 ± 16 CPM/ng genomic DNA, P = 0.02; Fig. 2A), where CPM is counts/min. Administration of butyrate (57 ± 14 CPM/ng genomic DNA, P = 0.046, vs. diet plus vehicle; Fig. 2A) or trichostatin A (58 ± 15 CPM/ng genomic DNA, P = 0.02, vs. diet plus vehicle; Fig. 2A) to offspring of restrictive diet pregnancy normalized pulmonary DNA methylation. Restoration of pulmonary DNA methylation by butyrate and trichostatin A was associated with normalization of the hypoxic pulmonary artery pressure response in vivo (32.9 ± 2.3 vs. 35.8 ± 3.5 mmHg, P = 0.01, diet plus butyrate vs. diet plus vehicle; 29.4 ± 2.8 vs. 35.8 ± 3.5 mmHg, P = 0.03, diet plus trichostatin vs. diet plus vehicle; Fig. 2B) and pulmonary endothelium-dependent vasodilation in vitro (P < 0.0001; Fig. 2C). Trichostatin A and butyrate had no detectable effect on pulmonary DNA methylation and pulmonary vascular responses in control mice (Fig. 2, A–C).

Vascular Function in the Progeny of Offspring of Restrictive Diet Pregnancy

The progeny of male offspring of restrictive diet pregnancy displayed impaired acetylcholine-induced pulmonary vasodilation in vitro (P < 0.0001; Fig. 3) that was comparable to the one observed in their fathers. Butyrate administration to male offspring of restrictive diet pregnancy before mating prevented the transmission of pulmonary vascular dysfunction to their progeny (Fig. 3).

Effects of Tempol Administration to the Mother During Restrictive Diet Pregnancy on Pulmonary DNA Methylation and Vascular Responsiveness in the Offspring

Tempol administration during restrictive diet pregnancy normalized the pulmonary uptake of methyl groups in the offspring (71 ± 11 CPM/ng genomic DNA, P = 0.14, diet plus Tempol vs. controls; Fig. 4A). Restoration of pulmonary DNA methylation was associated with the prevention of pulmonary endothelial dysfunction in vitro (P < 0.0001; Fig. 4B), exaggerated hypoxic pulmonary hypertension (32.4 ± 1.8, P = 0.009, vs. vehicle; Fig. 4C) and right ventricular hypertrophy (RV-to-LV + S ratio; 0.295 ± 0.037, P = 0.037, vs. vehicle; Fig. 4D) in vivo of offspring of restrictive diet pregnancy.

DISCUSSION

There is increasing evidence in humans and experimental animals that pathologic events during the fetal period predispose the offspring to cardiovascular disease, but the underlying mechanism is poorly understood (8, 16, 23). Here, we show that restrictive diet pregnancy in conjunction with hypoxic stress later in life causes pulmonary endothelial dysfunction in vitro and exaggerated hypoxia-induced pulmonary hypertension and right ventricular hypertrophy in vivo in the offspring that appear to be related to an epigenetic mechanism.

Several lines of evidence suggest that pulmonary vascular dysfunction in offspring of restrictive diet pregnancy was related to an epigenetic mechanism. First, we found that DNA

Fig. 3. Vascular function in the progeny of offspring of restrictive diet pregnancy. Acetylcholine-induced vasodilation in the progeny of male offspring of restrictive diet pregnancy treated with butyrate (2 mg·kg body wt–1·day–1; ip, during 2 wk) or vehicle before mating. For comparison, the responses in control mice and offspring of restrictive diet pregnancy are also shown. Error bars represent SE; n > 10 animals in each group.
methylation in lung tissue was altered in offspring of restrictive diet pregnancy. Second, epigenetic changes can be reversed by histone deacetylase inhibitors (28). We, therefore, examined the effects of butyrate administration to adult male offspring of restrictive diet pregnancy on pulmonary methylation and vascular function. Butyrate normalized pulmonary methylation and pulmonary vascular function in vitro and in vivo in these animals. The finding that administration of the more specific (20) histone deacetylase inhibitor trichostatin A to offspring of restrictive diet pregnancy had similar favorable effects on pulmonary methylation and vascular function further strengthens the concept of an epigenetic mechanism. Finally, epigenetic changes may be transmitted to the next generation (11, 30). To test for this possibility, we assessed pulmonary vascular function in the progeny of male offspring of restrictive diet pregnancy mated to control females. We found that pulmonary vascular dysfunction in the progeny was comparable to the one observed in their fathers. Most importantly, butyrate administration to male offspring of restrictive diet pregnancy before mating prevented the transmission of the vascular dysfunction to the progeny.

The present study not only provides evidence for an epigenetic mechanism underpinning vascular dysfunction in offspring of restrictive diet pregnancy but also suggests that this mechanism may be induced by oxidative stress during pregnancy. In rats, restrictive diet during pregnancy increases oxidative stress in the placenta (26). Moreover, oxidative stress is known to alter DNA cytosine methylation (29) that may result in changes in gene expression that are maintained throughout the life span (12, 15, 17). In investigating the functional importance of this problem, we found that administration of the nitroxide Tempol (13, 31) to the mother during restrictive diet pregnancy prevented pulmonary DNA dysmethylation as well as pulmonary vascular dysfunction in the offspring. Collectively, these findings suggest that in mice restrictive diet pregnancy induces pulmonary vascular dysfunction in the offspring by an epigenetic mechanism that appears to be triggered by exaggerated oxidative stress.

Low birth weight has been found to be associated with vascular dysfunction in the systemic circulation in experimental animal models (8, 9, 21, 25) and humans (3) and has been suggested to play a pathogenic role in the systemic vascular dysfunction induced by restrictive diet pregnancy in rats (8, 9). This factor does not appear to have played an important role in the present studies, since birth weight was not different between offspring of restrictive diet pregnancy and control mice. Finally, we found that in mice pulmonary vascular dysfunction in offspring of restrictive diet pregnancy became only manifest
with the addition of hypoxic stress. In line with this observation, in humans, offspring of mothers with preeclampsia display exaggerated hypoxic pulmonary hypertension only when living at high altitude (16).

The present data in mice are consistent with findings in humans indicating that famine during pregnancy, a condition known to predispose the offspring to premature cardiovascular disease, is associated with dysmethylation of the insulin-like growth factor-2 gene in the offspring (14). Along the same lines, preeclampsia, another condition known to predispose the offspring to systemic and pulmonary vascular dysfunction (16, 18), is associated with dysmethylation in the placenta (27, 32). Taken together, these findings could suggest that epigenetic mechanisms may contribute to vascular dysfunction in offspring of famine and offspring of mothers with preeclampsia in humans. The findings in mice also suggest that pharmacologic interventions during pregnancy may help prevent epigenetic alterations and vascular dysfunction in the offspring.

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DISCLOSURES

All authors have read and approved the manuscript. This material has not been reported previously and is not under consideration for publication elsewhere. No conflicts of interest, financial or otherwise, are declared by the author(s).

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