Mice generated by in vitro fertilization exhibit vascular dysfunction and shortened life span

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Children conceived by assisted reproductive technologies (ART) display a level of vascular dysfunction similar to that seen in children of mothers with preeclampsia. The long-term consequences of ART-associated vascular disorders are unknown and difficult to investigate in healthy children. Here, we found that vascular tone from mice generated by ART display endothelial dysfunction and increased stiffness, which translated into arterial hypertension in vivo. Progeny of male ART mice also exhibited vascular dysfunction, suggesting underlying epigenetic modifications. ART mice had altered methylation at the promoter of the gene encoding eNOS in the aorta, which correlated with decreased vascular eNOS expression and NO synthesis. Administration of a deacetylase inhibitor to ART mouse normalized vascular gene methylation and function and resulted in progeny without vascular dysfunction. The induction of ART-associated vascular and epigenetic alterations appeared to be related to the embryo environment; these alterations were possibly facilitated by the hormonally stimulated ovulation accompanying ART. Finally, ART mice challenged with a high-fat diet had roughly a 25% shorter life span compared with control animals. This study highlights the potential of ART to induce vascular dysfunction and shorten life span and suggests that epigenetic alterations contribute to these problems.

Introduction

Epidemiological studies showing an association between pathologic events during early life and the development of cardiovascular and metabolic disease in adulthood have led to the “fetal programming of adult disease hypothesis” (1, 2). In line with this hypothesis, experimental studies show that short-term hypoxemia after birth predisposes to exaggerated hypoxic pulmonary vasoconstriction later in life in rats (3) and humans (4) and preeclampsia predisposes the offspring to pulmonary and systemic endothelial dysfunction (5). Recent data suggest that assisted reproductive technologies (ART) represent another example for this hypothesis, since arterial pressure is increased in rats and children generated by ART (6, 7) and young children conceived by ART display generalized vascular dysfunction and signs of early arteriosclerosis (8).

While these findings demonstrate vascular dysfunction in ART children that is of magnitude similar to that observed in offspring of mothers with preeclampsia (5) or preadolescents with type I diabetes (9), conditions known to be associated with an increased risk for premature cardiovascular morbidity (10–12), several important issues remain unclear. First, in humans, it is difficult to determine whether this problem is related to the ART procedure itself or to parental factors. Second, the long-term cardiovascular consequences of ART are unknown because this population has not yet reached the age at which premature cardiovascular diseases typically start to occur. Finally, the underlying mechanisms of ART-induced vascular dysfunction are not known and are difficult to study in healthy children.

We, therefore, developed a mouse model of ART to first test the hypothesis that ART induces vascular dysfunction in the offspring of normal mice. We found that ART mice display endothelial dysfunction, increased arterial stiffness, and arterial hypertension. To study whether ART predisposes to premature mortality, we compared the life span of ART and control mice fed with normal chow or challenged with a high-fat diet (HFD). We then started to test for underlying mechanisms. Hormonal stimulation of ovulation is inherently needed for ART and may have effects on vascular function in the offspring. We, therefore, compared vascular function between mice born after hormonal stimulation in the mother and control mice. The time passed in culture medium may be another factor involved in ART-induced vascular dysfunction. To test this hypothesis, we compared vascular function between mice born after implantation of 2 cell embryos and blastocysts. Finally, in mice, epigenetic mechanism are involved in the fetal programming of vascular dysfunction (13), and in humans, the prevalence of very rare diseases caused by epigenetic mechanisms is increased in ART (14, 15). Epigenetic alterations may be transmitted to the next generation (13, 16, 17). Therefore, we examined whether male ART mice transmitted the vascular dysfunction to their progeny. In a next step, we then...
studied the methylation of imprinted genes and genes involved in cardiovascular regulation in ART mice, offspring of ART mice, and control mice. Finally, we examined the effects of administration of a deacetylase inhibitor on the methylation of genes and vascular function in these 3 groups of animals.

Results

Endothelial dysfunction, increased arterial stiffness, and arterial hypertension in ART mice
To assess the putative effects of ART on vascular function, we assessed acetylcholine-mediated and nitroprusside-induced mesenteric artery vasodilation in vitro in ART and control mice. We found that acetylcholine-induced vasodilation was impaired in ART mice compared with control mice (Figure 1A). In contrast, nitroprusside-induced vasodilation was normal in ART mice (Figure 1B), demonstrating mesenteric artery endothelial dysfunction. To assess for premature vascular senescence, we examined carotid artery vascular stiffness. We found that vascular stiffness was increased in ART mice (Figure 1, C and D). To examine whether endothelial dysfunction in vitro had functional consequences in vivo, we measured arterial blood pressure and found that it was higher in ART mice than in control mice, both during short-term measurements and during chronic measurements by telemetry over 48 hours (Figure 1, E and F).

Decreased life span in ART mice
To evaluate whether ART had consequences on the life span in mice, we compared the survival of ART and control mice. To facilitate the detection of potential differences, in addition to groups of mice fed with normal chow, groups of ART and control mice were challenged with a HFD. We found that in mice fed with normal chow, life span was similar in the 2 groups. In contrast, a HFD, while not having a detectable effect on life span in control mice, significantly reduced survival in ART mice ($P < 0.0001$, Figure 2).

Underlying mechanisms
Effects of stimulation of the ovulation in the mother on vascular function in the offspring
To examine whether hormonal stimulation of ovulation in the mother alters vascular function in the offspring, we

Figure 1
Mesenteric artery responsiveness, carotid artery pressure diameter relationship, and arterial blood pressure in mice generated by ART and control mice. (A) Acetylcholine-induced and (B) sodium nitroprusside–induced mesenteric artery vasodilation in vitro (data represent mean ± SEM). (C and D) Carotid artery pressure diameter relationship in vitro. C shows outer diameter (OD) and D shows inner diameter (ID). (E) Mean arterial blood pressure in vivo obtained by short-term measurements and (F) 48-hour long-term measurements by telemetry. Horizontal lines represent the median; boxes, 25th to 75th percentiles; and T bars, 5th and 95th percentiles. Data are for at least 10 mice per group except for C and D ($n$ = 5 per group) and F ($n$ = 4 per group).

Figure 2
Kaplan-Meier survival curves of ART and control mice fed with normal chow (NC) or challenged with a HFD. Median survival (days): Control NC = 853; control HFD = 787; ART normal chow = 736; ART HFD = 582.
compared mesenteric artery responses to acetylcholine in offspring of superovulated and control mice. Overall, acetylcholine-induced mesenteric vasodilation was comparable in the 2 groups (Figure 3), but at lower doses of acetylcholine, curves tended to diverge, and the responses to the 1 × 10⁻⁸ dose were statistically different between the 2 groups (P = 0.04).

Vascular dysfunction is similar in ART mice generated by implantation of 2 cell embryos and blastocysts. To examine whether a shorter duration of embryo culture attenuates/prevents ART-induced vascular dysfunction in the offspring, we compared mesenteric artery responses to acetylcholine and arterial blood pressure between mice generated by implantation of 2 cell embryos (30 hours of incubation) and blastocysts (96 hours of incubation). ART-induced vascular dysfunction and arterial hypertension were comparable in the 2 groups of mice (Figure 4).

Male ART mice transmit the vascular dysfunction to their progeny. There is evidence that ART may alter epigenetic mechanisms (14, 15). These alterations may be transmitted to the next generation (13, 16, 17). To test for this possibility, we examined vascular function and arterial blood pressure in male offspring generated by mating male ART mice with control females. Endothelial dysfunction and arterial hypertension in offspring of male ART mice were comparable to those observed in their fathers (Figure 5, A and B).

Methylation of imprinted genes in the aorta is altered in ART mice and their progeny

Normalization by administration of the deacetylase inhibitor butyrate. To evaluate whether ART altered the methylation of genes in the vasculature, we studied the methylation of imprinted genes in the aorta. We found that the methylation of the imprinted genes H19, Gtl2, and Peg3, but not of Peg1 and Snrpn, was altered in the aorta of ART mice (Figure 6). To determine whether these alterations were specific for vascular tissue or generalized, we examined the methylation of these genes in the liver and found that it was similar in the 2 groups (data not shown). Finally, to study whether altered methylation was transmitted to the next generation, we examined the methylation of imprinted genes in the aorta of the progeny of male ART mice. The alterations of the methylation of imprinted genes in the aorta of the offspring were comparable to those observed in their fathers (Figure 6).

Deacetylase inhibitors are capable of normalizing altered methylation (13, 18). We therefore examined the effects of butyrate administration to male ART mice on the methylation of imprinted genes. We found that butyrate normalized the methylation of the imprinted genes H19, Gtl2, and Peg3 (but had no detectable effect on the methylation of the normally methylated imprinted genes Peg1 and Snrpn) in the aorta of ART mice, whereas it had no detectable effect on the methylation of these genes in control mice (all P > 0.22, control + vehicle vs. control + butyrate; Figure 6). Finally, butyrate administration to male ART mice before mating prevented the transmission of altered methylation to the progeny (Figure 6).

Altered DNA methylation of the promoter of the eNOS gene, altered eNOS and eNOS mRNA expression in vascular tissue, and decreased nitric oxide plasma concentration in ART mice

Normalization by administration of the deacetylase inhibitor butyrate. While the above findings show altered methylation of imprinted genes in ART mice, the consequences of these alterations are not known, since the physiologic function of these genes is unknown. We, therefore examined DNA methylation of genes known to be important in cardiovascular regulation. DNA methylation of the promoter of the eNOS gene was increased in the aorta of ART mice (Figure 7A), whereas DNA methylation of the promoter of the endothelin-1 and the angiotensin converting enzyme (ACE) gene was not different between ART and control mice (endothelin-1: 15.9% ± 1.0% vs. 16.4% ± 1.0%, control vs. ART, P = 0.27; ACE: 4.0% ± 0.5% vs. 3.9% ± 0.3%,
Increased promoter DNA methylation downregulates eNOS expression in endothelial cells in vitro (19). In line with this concept, eNOS expression (Figure 7B) and eNOS mRNA expression (0.56% ± 0.08% vs. 1.0% ± 1.21%, \( P = 0.047 \), ART vs. control) in the carotid artery were decreased in ART mice compared with control animals. Decreased eNOS expression appeared to be of functional importance, because nitric oxide (NOx) plasma concentration was lower in ART than in control mice (Figure 7C).

Butyrate administration to ART mice normalized DNA methylation of the promoter of the eNOS gene (Figure 7A) and eNOS expression (Figure 7B) and restored NOx plasma concentration (Figure 7C).

**Butyrate administration to ART mice normalizes endothelial function and prevents transmission of vascular dysfunction to the offspring.** To evaluate whether normalization of the DNA methylation and vascular NO synthesis by butyrate had favorable effects on vascular function in ART mice, we examined vascular responsiveness to acetylcholine. We found that butyrate administration to ART mice normalized mesenteric artery function, whereas it had no detectable effect on this function in control mice (Figure 8A).

Moreover, butyrate administration to male ART mice before mating prevented the transmission of vascular dysfunction to the next generation (Figure 8B).

**Discussion**

We recently showed that children conceived by ART display vascular dysfunction (8). The long-term consequences of this problem are unknown, and its underlying mechanisms are difficult to investigate in healthy children. Here, we show for what we believe is the first time that in mice generated by ART, endothelial function of the systemic circulation in vitro is defective and translates into arterial hypertension in vivo. Moreover, ART also had consequences on the life span; when mice were challenged with a HFD, life span was roughly 25% shorter in ART than in control mice.

Consistent with findings in children conceived by ART showing endothelial dysfunction (8), in ART mice, acetylcholine-induced mesenteric artery dilation was impaired while endothelium-independent dilation was preserved. Moreover, as in ART...
children, arterial stiffness was increased in ART mice. Whereas in young ART children, endothelial dysfunction was not associated with arterial hypertension, in adult ART mice, endothelial dysfunction in the systemic circulation was associated with arterial hypertension. In humans, it has been speculated that parental factors (e.g., older age of mothers necessitating to resort to ART, sterility-associated vascular dysfunction that is transmitted to the offspring) contribute to vascular dysfunction in offspring of ART. The present findings showing that in normal mice ART induces vascular dysfunction in the offspring indicate that ART per se is the main culprit. Data in humans showing no relationship between the age of the mother and vascular function in the offspring and demonstrating normal vascular function in sterile parents are in line with this concept (8).

Due to the young age of the ART population in humans (the first ART child was born in 1978), it is not known yet whether ART will affect life expectancy in humans. Here we found that when fed normal chow, life span was not different in ART and control mice. However, when mice were challenged with a Western style HFD, life span in ART mice was roughly 25% shorter than in control mice. Premature vascular senescence and exaggerated HFD-induced glucose intolerance and insulin resistance (20) may contribute to this problem. These effects of ART were observed in FVB mice, a mouse strain less susceptible to atherosclerosis than, for example, the C57BL/6 strain (21, 22), suggesting that the effects of IVF may have a greater impact in genetically susceptible individuals. These findings suggest that it may be worthwhile to provide dietary advice to persons born with the help of ART.

Figure 7
Butyrate administration to ART mice normalizes DNA promoter methylation and expression of the eNOS gene in vascular tissue and NO\textsubscript{x} plasma concentration. DNA methylation of the promoter of the eNOS gene in the aorta (A), eNOS expression in the carotid artery (B), and NO\textsubscript{x} plasma concentration (C), in control + vehicle, ART + vehicle, ART + butyrate, and control + butyrate mice. Data represent mean ± SEM for at least 13 mice per group. *P = 0.004, †P < 0.0001, ‡P = 0.03 vs. control + vehicle by 1-way ANOVA and Dunnett’s post hoc test. In B, the lanes were run on the same gel but were noncontiguous.

Figure 8
Butyrate administration to ART mice normalizes vascular function and prevents transmission of dysfunction to offspring. (A) Effects of butyrate administration to ART and to control mice on acetylcholine-induced mesenteric artery dilation. (B) Effects of butyrate or vehicle administration to ART mice before mating on acetylcholine-induced mesenteric artery dilation in the progeny. Data represent mean ± SEM for at least 10 mice per group.
In mice, fetal programming of pulmonary vascular dysfunction is associated with altered lung DNA methylation (13) and there is evidence for transmission of epigenetic alterations to the next generation. Here, we found that vascular dysfunction and arterial hypertension of the progeny of male ART mice mated with normal females was similar to what was found in their fathers. In line with this observation, the methylation of imprinted genes was altered similarly in the aorta of ART mice and their progeny. While these findings provide proof of principle for epigenetic alterations in the vasculature of ART mice, the consequences of these alterations for vascular regulation, if any, are unknown. In a next step, we, therefore, examined the methylation of genes known to be important for vascular regulation. We found that DNA methylation of the promoter of the eNOS gene was increased in arterial tissue of ART mice and associated with decreased eNOS expression, decreased eNOS mRNA expression, and lower NO, plasma concentration in ART than in control mice. Taken together, these findings indicate that in mice, ART alters the entire chain of events starting from altered eNOS methylation in the vasculature over endothelial dysfunction and premature vascular senescence to arterial hypertension and possibly perinatal G1 and G2 medium preequilibrated at 37°C under the CO2-enriched atmosphere. Eggs were then transferred to 25 μl drops of G1 medium covered with paraffin oil. The embryo culture was conducted up to the blastocyst stage in sequence media. For example, culture media may lack or contain at different concentration key metabolites and/or growth factors present in oviductal fluid and are not capable of reproducing the dynamic changes of oviductal fluid naturally occurring along the female reproductive tract. Suboptimal in vitro culture conditions result in compromised ability to maintain genomic imprinting of in vivo compared with in vivo embryos (28) and may contribute to the observed epigenetic and cardiovascular alterations in ART mice in the present study. In line with this speculation, modification of the culture medium may allow ART-induced cardiovascular dysfunction in mice to be attenuated (29). Taken together, the present findings suggest that embryo culture per se is the main culprit underpinning ART-induced epigenetic and vascular alterations in mice. These alterations may be facilitated by hormonal stimulation of ovulation in the mother. We speculate that similar mechanisms may play a role in the rapidly growing population of humans conceived by ART, who now make up 2% to 4% of births in developed countries.

**Methods**

**Chemicals and reagents.** Pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), and ketamin were from Intervet. Human tubal fluid medium (HTF), human serum albumin (HSA), and paraffin oil were from Irvine Scientific. G1 and G2 media were from VitroLife. Sodium butyrate, acetylcholine and sodium nitroprusside were from Sigma-Aldrich. Xylazine was from Bayer HealthCare. Temgesic (buprenorphine) was from Essex Chemie. Bactrim was from Roche. Biokema Flunixin was from Biokema SA.

**Animals.** Wild-type (FVB and NMRI) mice were from the Charles-River Laboratory. Animals were fed a standard chow diet (Safe). Throughout the study, the mice were housed with lights on from 7:00 am to 7:00 pm, and access to food and water was ad libitum.

**In vitro fertilization and embryo culture.** Eight- to twelve-week-old female FVB mice were superovulated by i.p. injection of 5 IU (0.1 ml) PMSG, followed 50 hours later by an i.p. injection of 5 IU (0.1 ml) of hCG. Fourteen hours after hCG injection, cumulus-oocyte complexes were recovered from oviducts in HTF supplemented with 5 mg/ml of HSA. Spermatozoa were collected from the cauda epididymis of 10- to 14-week-old FVB mice and capacitated for 60 minutes in HTF/HSA medium at 37°C under a humidified atmosphere of 6% CO2 in air. Oocytes were inseminated 14 hours after hCG injection with 10⁶ spermatozoa in HTF/HSA medium for 4 hours at 37°C in a CO2-enriched (6%) atmosphere. Eggs were then transferred to 25 μl drops of G1 medium covered with paraffin oil. The embryo culture was conducted up to the blastocyst stage in sequential G1 and G2 medium sequentially at 37°C under the CO2-enriched atmosphere. Embryos were kept in the G2 medium for 48 hours before the transfer to pseudopregnant females. The culture media used in these studies are largely used during ART in humans. For these studies, the ART procedure was repeated about 50 times, each time in a different animal.
Embryo transfer. NMRI females of at least 6 weeks of age were placed with vasectomized males to mate 2.5 days prior to embryo transfer. The morning after mating, females were checked for the presence of a vaginal plug. On the transfer day, pseudopregnant females were anesthetized by i.p. injection of xylazine (15 mg/kg) and ketamine (100 mg/kg). From 14 to 20 embryos were transferred to the uterus of each female.

Unless stated otherwise, 12- to 14-week-old male ART and control mice were used for all the in vitro and in vivo studies described below.

Mesenteric artery function in vivo. ART and control mice were killed with an overdose of pentobarbital sodium (200 mg/kg, i.p.). The mesenteric artery was dissected free of peritoneum and cut into a ring. Each ring was positioned between 2 stainless steel wires (diameter 25 μm) in a 5-ml organ bath of a small vessel myograph (DMT 610M; Danish Myo Technology) (30). The organ bath was filled with modified Krebs-Ringer bicarbonate solution (composition: 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM Mg₂SO₄, 1.2 mM KH₂PO₄, 25.0 mM NaHCO₃, and 11.1 mM glucose), maintained at 37 ± 0.5°C and aerated with 95% O₂ plus 5% CO₂ (pH = 7.4). At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension corresponding to a transmural pressure of 100 mmHg and allowed to equilibrate for 1 hour. MyoDaq software (MyoDaq 2.01 M610; Danish Myo Technology) was used for data acquisition and display. Then, acetylcholine-induced (ACH, 10⁻⁶ to 10⁻³ M) or sodium nitroprusside-induced (10⁻⁶ to 10⁻³ M) vasodilation was assessed in mesenteric arteries preconstricted with phenylephrine (10⁻⁴ mol/l) at a level corresponding at least to the maximal response to potassium (100 mmol/l I KCl). The ACH-induced change in tension was expressed as the percentage of the initial contraction induced by phenylephrine.

To assess arterial stiffness, the left carotid artery was quickly excised, placed in modified Krebs-Ringer solution (composition, see above) cleaned of adhering tissue and fat. The artery was then mounted onto a pressure myograph (Danish Myo Technology) in an organ bath filled with physiological buffer, as described previously (31). After a 30-minute equilibration period, the carotid artery was transilluminated under an inverted microscope (Zeiss Axio Vert A1) connected to a camera and a computerized system (MyoVIEW II Software; Danish Myotechnology) allowing the continuous recording of the artery diameter. Measurements of the inner and outer artery diameter were obtained during stepwise increases (10 mmHg steps for 5 minutes from 0 to 180 mmHg) of the intravascular pressure and expressed as a percentage of the baseline value measured at 10 mmHg of intravascular pressure.

Arterial blood pressure. Arterial blood pressure was recorded continuously in awake mice, as described previously (32). Briefly, a fluid-filled PE-10 tubing connected to a pressure transducer was inserted into the carotid artery under isoflurane anesthesia and tunneled subcutaneously to exit at the back of the neck. Mice were allowed to recover for 4–5 hours before the blood pressure measurement.

In additional ART and control mice, we measured arterial blood pressure by telemetry, as described previously (33). Briefly, blood pressure transmitters (TA11PA-C10 transmitter; DataSciences International) were implanted under inhalational anesthesia with isoflurane on a heating surface set at 36°C. Preoperatively, mice received 1 ml of prewarmed 0.9% saline i.p. to support fluid homeostasis and trimethoprim (6 mg/kg)/sulfadoxin (30 mg/kg) (Bactrim) s.c. for anti-infective prophylaxis. The left common carotid artery was exposed, and the tip of the telemetry catheter was inserted, advanced into the aortic arch, and secured by silk sutures. The body of the transmitter was positioned in an s.c. pocket on the right flank. Postoperatively, mice received s.c. injections twice per day for 7 days of flunixin (5 mg/kg body weight) for pain relief and 300 μl of glucose (5%) and 300 μl of normal saline (0.9%) to prevent exsiccosis.

From 10–12 days after implantation, transmitters were activated magnetically and offset-corrected blood pressure was recorded at 4-minute intervals for 48 consecutive hours. The recording room was maintained at 22–24°C with a 12-hour light/12-hour dark cycle, and mice had free access to food and water. The telemeter signal was processed using RPC-1 receivers, an APR-1 ambient pressure monitor, and a Dataquest A.R.T. 4.31 Gold acquisition system (DataSciences International).

Histone deacetylase inhibitor administration. To test for the potential pathogenic role of epigenetic mechanisms, sodium butyrate (2 mg/kg body weight/d in 200 μl of PBS, i.p.) was administered to 10-week-old male ART and control mice for 14 days.

Methylation of imprinted genes in the aorta. The maternally expressed paternally methylated H19 (34) and G6l (35) genes and the paternally expressed maternally methylated Peg3, (36) PegI (37), and Sirpu genes (38) were studied in the aorta. DNA was extracted using the QIAampDNA Microkit (Qiagen). Using the EZ Methylation Gold-Kit (Zymo Research), the extracted DNA was treated with sodium bisulfite in order to convert unmethylated cytosine residues to uracil. The converted DNA was eluted in 10 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5). 2 μl of the bisulfite-treated DNA was used for subsequent PCR amplification.

The PCR amplifications were performed starting from 80–140 ng of bisulfite-treated DNA. All reactions were performed with PCR reaction mixtures (total volume 25 μl) containing oligonucleotides at 0.5 mM concentration and 12.5 μl of HotStarTag Master Mix (Qiagen). The PCR conditions were the same for all 5 genes tested, i.e., 94°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and by a 72°C 10-minute final extension step. The characteristics of the amplicons and the oligonucleotides chosen have been described earlier (39).

The biotinylated PCR products were purified using streptavidin-sepharose beads (Amersham) and sequenced using the PSQ 96 Gold Reagent Kit (Biotage AB) with the following primers: H19: 5′-GTGTA-AAAGTACGGGTGTG-3′; G6l: 5′-GTTGTAGGATTGGTATAGAG-3′; Peg3: 5′-TCAATATCACAATAAAATC-3′; Snrpn: 5′-GAATTTGAGTT- GTGTTG-3′; Peg3: 5′-AATGTACAGGTTGTTAGAG-3′. The degree of methylation at each CpG site was determined using Pyro Q-CpG Software (Biotage AB). All samples were analyzed in duplicate. Diagrams showing the location of the DMDs and the DNA fragments of the 5 genes amplified by PCR in the present study have been published previously (39).

Methylation of the eNOS gene promoter in the aorta. Using the same methods, methylation analysis was performed in the eNOS (GenBank AF091262) core promoter, where DNA methylation was shown to control gene expression (19, 40). The following oligonucleotides were designed with the PyroMark Assay Design 2.0 program (Qiagen): eNOS-F: 5′-TGAAGATTTCATTCGAGTAC-3′; eNOS-R: 5′-CTTGATACGCTACCTACGAAGTGT-3′; eNOS-S: 5′-CGTACACCATCTTTTCTGAGT-3′. The biotinylated PCR products were purified using streptavidin-sepharose beads (Amersham) and sequenced using the PSQ 96 Gold Reagent Kit (Biotage AB) with the following oligonucleotide: eNOS-S: 5′-TTGGTTTATATATCAGTTTATA-3′. This sequence analyzed by pyrosequencing was 151-bp long, encompassing 8 CpGs.

Protein extraction and eNOS Western blot. Four carotid arteries of the same condition were pooled and ground in liquid nitrogen. Each pool of carotid tissue was suspended in SDS lysis buffer (62.5 mM Tris, pH 6.8, 5% SDS, 10 mM EDTA). Following sonication and 5 minutes of full-speed centrifugation, supernatant proteins were quantified by the Lowry method. Samples were heated at 95°C for 3 minutes, loaded in duplicate (8 μg per well) in an 8% acrylamid gel, and transferred to PVDF membrane (Millipore). The membrane was blocked with 5% milk-TBS, 0.1% Tween (TBST) for 1 hour. Blots were probed with anti-eNOS (1:1000; BD Transduction Laboratories) and incubated for 1 hour at room temperature, washed with TBST, and then incubated with HRP-conjugated secondary antibody (BD Transduction Laboratories) for 30 minutes at room temperature. Blots were then washed and the protein bands were visualized using the ECL Plus Western Blotting Detection System (Amersham).
Laboratories) in 5% milk/TBST overnight and were detected using peroxidase-conjugated secondary antibody (1:10000) in 5% milk/TBST for 1 hour. Products were visualized by chemiluminescence (GE Healthcare), and band intensity was measured using ImageQuant 5.0 software (Molecular Dynamics). Equal protein loading was confirmed by β-actin (1:10000; Thermo Scientific Pierce Products) hybridization on the same membrane.

RNA preparation and gene expression analysis. Total RNA from aorta was extracted using the TRIzol RNA isolation Reagents according to the manufacturer’s protocol (Life Technologies Europe B.V.). From 500 to 1000 ng total RNA were reverse transcribed using 400 units of Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) in the presence of 1 U/μl RNasin (Promega Corp.), 0.2 μg of random primers (oligo(dN)6) (Promega Corp.), 2 nM dNTP, and 20 μM of dTT (Invitrogen). The expression of the cDNAs for mouse eNOS was determined by quantitative real-time PCR using an ABI StepOne Plus Sequence Detection System (Applera Europe) and normalized using the housekeeping gene R292. PCR products were quantified using Master SYBR Green Mix (Applera Europe), and results are expressed in AU relative to the control group mean value. Primers were designed using Primer Express software (Applera Europe) and chosen when possible on both sides of an intron to avoid amplification of eventual contaminating genomic DNA. Oligos were used at 217 nM each (Microsynth). The sequence of the primers used were as follows: eNOS, forward: 5′-AAGGACGCGGTGGAATTAA-3′; eNOS, reverse: 5′-TCACCTTTGGCCACGTGTAAC-3′.

Methylation of the endothelin-1 gene promoter in the aorta. Methylation analysis was performed in the Edn-1 (GenBank NC_000079) promoter in a region where DNA methylation was shown to control gene expression (41, 42). The following oligonucleotides were designed with the PyroMark Assay Design 2.0 program (Qiagen): Edn-F: 5′-GTGATTTTTTAGGATTGTTAAGG-3′; Edn-R: 5′-biotin-7GCGGTGGTTGGTATTATA-3′. The biotinylated PCR products were purified using streptavidin-sepharose beads (Amersham) and sequenced using the PSQ 96 Gold Reagent Kit (Biotage AB) with the following oligonucleotide: ACE-S: CCTCCACACTTAACTTTTCC-3′. The sequence analyzed by pyrosequencing was 85-bp long, encompassing 10 CpGs.

Methylation of the ACE gene promoter in the aorta. Methylation analysis was performed in the ACE promoter, in a CpG island region where methylation was reported to be modified in the mouse fetus after maternal dietary changes during pregnancy (49, 40, 43). The following oligonucleotides were designed with the PyroMark Assay Design 2.0 program (Qiagen): ACE-F: 5′-biotin-7CAGGGTTGCTGTTGGTATTATA-3′; ACE-R: 5′-CCCAACTACCCACCATACTTAAACAT-3′. The biotinylated PCR products were purified using streptavidin-sepharose beads (Amersham) and sequenced using the PSQ 96 Gold Reagent Kit (Biotage AB) with the following oligonucleotide: ACE-S: CCTCCACACTTAACTTTTCC-3′. The sequence analyzed by pyrosequencing was 239-bp long, encompassing 9 CpGs.

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