Maternal undernutrition inhibits angiogenesis in the offspring: a potential mechanism of programmed hypertension

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Khorram O, Khorram N, Momeni M, Han G, Halem J, Desai M, Ross M. Maternal undernutrition inhibits angiogenesis in the offspring: a potential mechanism of programmed hypertension. Am J Physiol Regul Integr Comp Physiol 293: R745–R753, 2007. First published May 16, 2007; doi:10.1152/ajpregu.00131.2007.—The underlying etiology of many chronic diseases such as hypertension and diabetes has been traced to the intrauterine environment. Our interest has focused on determining the mechanism of programmed hypertension. In our rodent model of 50% maternal food restriction (MFR) from day 10 of gestation to term, the offspring develop hypertension as adults. We hypothesized that maternal undernutrition inhibits angiogenesis such that the neonate is endowed with fewer microvessels, increasing their susceptibility to develop hypertension as adults. We found significantly reduced number of mesenteric branching and renal medullary microvessels in the 1-day-old MFR newborns. Endothelial cells from MFR offspring generated shorter neovessels in culture compared with controls. The inhibition of angiogenesis was associated with a significant decrease in VEGF protein expression in mesenteric microvessels and aortas in 1-day-old offspring. However, in adulthood there was a marked increase in VEGF expression in both vessel types. The expression of endothelial nitric oxide synthase protein was also found to be increased in both renal and mesenteric microvessels and in aortas in the 1-day-old MFR offspring. These results suggest that MFR results in inhibition of VEGF expression in microvascular and aortic endothelial cells early in life, resulting in decreased angiogenesis and increased peripheral vascular resistance, both of which may contribute to offspring hypertension.

Recent evidence has shown that metabolic and cardiovascular disorders that manifest in adult life have their origin before birth (2). Programmed hypertension has been produced in a number of animal species through prenatal interventions, such as maternal undernutrition, or maternal administration of glucocorticoids (GC) (for review, see Ref. 23). Although the phenotype of the in utero food-restricted offspring has been addressed in many different models, little is known about the underlying mechanism(s) that leads to hypertension later on in life. Most of the studies that have focused on blood vessels have examined the offspring as adults when hypertension has already developed; thus it is unknown whether the reported changes such as endothelial dysfunction are a cause or a result of hypertension.

In an animal model of intrauterine growth retardation developed in our laboratory (4) in which dams are food restricted from day 10 of gestation, our group demonstrated that the offspring have low birth weight and, by 2 mo of age, develop hypertension (17, 18). Furthermore, we characterized the phenotype of the vasculature of the offspring in terms of the extracellular matrix (17) and the vascular smooth muscle (18). We reported that maternal rat food restriction (MFR) induced marked structural changes in the offspring blood vessels, some of which were manifested as early as day 1 of life. We found marked remodeling of the extracellular matrix with collagen deposition in the offspring micro- and conduit vessels. This was associated with a significant increase in matrix metalloproteinase (MMP)-9 mRNA in 1-day-old MFR aortas and in the expression of MMP-2 and MMP-9 mRNAs in 4-mo-old MFR aortas (17), suggesting a role for these enzymes in the extensive vascular remodeling. In addition, both conduit and resistance vessels exhibited vascular smooth muscle hypertrophy, which led to changes in the caliber of the blood vessels (18).

Angiogenesis and vasculogenesis are complex processes regulated by a myriad of growth factors. Vasculogenesis is in situ differentiation and growth of blood vessels from mesoderm-derived hemangioblasts. It gives rise to the heart and the first primitive vascular plexus inside the embryo and its surrounding membranes (26). Angiogenesis is defined as formation of new vessels by capillary sprouting from preexisting vessels (7, 26). Many genes are involved in regulating these processes, but a key factor for regulating both angiogenesis and vasculogenesis is vascular endothelial growth factor (VEGF) and its receptors (26). Mice with knockout of VEGF receptor 2 (VEGF-R2) lack vasculogenesis and fail to develop blood islands throughout the embryo and the yolk sac, and they die at embryonic day 8.5 to 9.5 (30). In view of our recent data, we hypothesized that inhibition of VEGF expression in the offspring vessels results in reduced angiogenesis, thereby leading to increased peripheral resistance and hypertension.

Materials and Methods

Animals. First-time-pregnant Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) were housed in a facility with constant temperature and humidity and with a controlled 12:12-h light-dark cycle. After mating, pregnancy was detected by presence of vaginal plug the following day. At 10 days of gestation, rats received either an ad libitum diet of standard laboratory chow (LabDiet 5001: protein 23%, fat 4.5%, metabolizable energy 3,030 kcal/kg; Brentwood, MO) or a 50% food-restricted diet determined by quantification of normal intake in the rats fed ad libitum. The respective diets were given from day 10.220.32.246 on October 14, 2017

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ming of the placenta (6). Maternal body weights and the food intake were recorded daily. At day 1 after birth, all offspring from food-restricted and control rat dams were cross-fostered to rat dams fed ad libitum. At day 1 after birth, litter size was culled to four males and four females per litter. The characterization of this animal model has been previously published (4). Unless otherwise specified, throughout all studies six animals (each dietary group) representing equal genders derived from different litters were used.

Tissue harvest. After decapitation, the entire gastrointestinal tract was dissected and placed in 4% paraformaldehyde for analysis of mesenteric arterioles. The thoracic aorta was then dissected and some of the specimens were snap frozen in liquid nitrogen, whereas others were fixed in 4% paraformaldehyde for immunohistochemistry.

Endothelial cell culture. Aortic endothelial cells were isolated as described by Kurimoto et al. (19). Briefly, aortas from 1-day-old control and MFR animals were dissected and cleaned of fat and periadventitial tissue and then placed in PBS. The vessels were washed to remove any blood cells and were opened longitudinally and cut into 1-mm pieces. These pieces were then placed with their intimal surface down on Matrigel (1:2 dilution + 2 ml of serum-free DMEM/F-12)-coated plates in growth medium (DMEM/F-12, 10% fetal calf serum, 3% Nu-serum, 50 μg/ml endothelial cell growth supplement, 50 μg/ml heparin, 2% antibiotic-antimylocytic solution). After 4 days, endothelial cell outgrowths were visible. At this time aortic pieces were removed, and the endothelial cells were harvested. Cells (1.5 × 10^4 cells/well) were seeded onto Matrigel-coated six-well plates in

**Fig. 1.** A: representative mesentery from a 1-day-old control animal (left). Arrows demonstrate the bifurcations that were counted under high-power magnification. Bar plot shows summarized data based on n = 6 in each dietary group. FR, food restricted. ***P < 0.001. B: representative vas recta (×20) in 1-day-old control and maternal food-restricted (MFR) offspring. Brown staining represents endothelial nitric oxide synthase (eNOS) in the vasa recta. C: summarized data showing the number of vas recta (total number per ×20 field) and the intensity of eNOS staining (integrated optical density, IOD) within them in 1-day-old control and MFR offspring, obtained using Image-Pro Plus software. ***P < 0.001.
growth medium. After incubation for 48 h at 37°C, the cells generated tubelike structures that were examined by light microscopy. The rest of cells were immunostained for von Willebrand factor (vWF), a specific endothelial cell marker, demonstrating a pure endothelial cell culture.

Mesenteric microvessel branching. The entire gastrointestinal tract was removed and fixed in 4% paraformaldehyde. The mesentery was then fanned out under a magnifying glass, and the number of bifurcations in a square area of the mesentery within 2 mm of the proximal colon, starting at the junction of the small intestine [length of colon analyzed in control: average length of 32 ± 1.2 mm vs. MFR 33.3 ± 2.1 mm; P = nonsignificant (NS)] was counted. Primary, secondary, and tertiary branches were included.

Analysis of microvessel generated in vitro (see Fig. 2) was performed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) by an investigator blinded to the treatment groups. Three digital images (×20 magnifications) per culture well were taken for analysis. Each well represented cells isolated from six animals derived from different litters. A total of three wells per dietary group were used for the final analysis. From each image, three to six cells were analyzed in terms of length and the number of branches, and averages were obtained for both parameters and subjected to statistical analysis.

Immunohistochemistry. Tissues were fixed in 4% paraformaldehyde for 24 h and then stored in 70% ethanol. After paraffin embedding, 5-μm sections were cut and tissues were stained for PCNA (Cell Marque, Hot Springs, AK), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), and endothelial nitric oxide synthase (eNOS; Transduction Labs, Lexington, KY) using standard immunohistochemical techniques previously described in detail (17, 18).

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling assay. We used the ApopTag in situ apoptosis detection kit (Intergen, Purchase, NY) for terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay. The indirect fluorescent staining technique was used according to the manufacturer's instructions. Briefly, tissue sections were first deparaffinized with xylene and ethanol and then treated with proteinase K (20 μg/ml). The TdT enzyme (1:20) was applied, followed by a wash step and application of anti-digoxigenin conjugate (fluorescein or rhodamine) (65 μl/5 cm² of surface area).

Western blot analysis. Tissues were sonicated in protein lysis buffer, and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). For each sample, 70 μg of protein were separated on a 7.5% polyacrylamide gel. The separated proteins were transferred electrophoretically to Immobilon-P membranes (Millipore, New Bedford, MA). Membranes were blocked for 2 h in a 5% milk buffer before overnight incubation with the antibody against VEGF and VEGF-R2 (Santa Cruz Biotechnology) at a dilution recommended by the manufacturer. The blots were subjected to enhanced chemiluminescence (ECL Western blotting detection system; Amersham, Arlington Heights, IL), with enzyme conju-

![Image](http://ajpregu.physiology.org/pdf/293/10/220/32.246.4.png)
gate anti-mouse IgG horseradish peroxidase as a secondary antibody. Blots were then exposed to autoradiography film. The resulting bands were then compared by scanning densitometry. To ensure equal loading, protein blots were stripped and reprobed for GAPDH.

**Image analysis.** Equal numbers of control and food-restricted vessels were stained at the same time under identical conditions and analyzed under the same magnification and light intensity by an investigator blinded to the treatment groups. The area of staining and staining intensity were quantified by image analysis using the Image-Pro 4.01 software (Media Cybernetics) coupled to an Olympus BHS microscope/Spot RT digital camera. The images were calibrated for background intensity and converted in gray scale. The concentration of the immunoreactive antigen was determined using the integrated optical density (IOD). The IOD values were proportional to the unweighted average optical density per area. The linearity of the IOD was determined using an ISO transmission density tablet as standard (Kodak) (5). The IOD measurements from the tablet were stored in an intensity calibration curve in the software. All the samples were run simultaneously, and if a staining procedure could not be applied simultaneously to all specimens, adjacent sections of a single positive control and of a given specimen within the series were run to standardize the optical intensity comparisons.

There were no differences in the statistical analysis using either IOD or area of staining values. For aorta, at least three sections per

![A: immunofluorescent image demonstrating aortic endothelial cells (left, ×20; right, ×40) isolated from 1-day-old control and MFR offspring and stained for von Willebrand factor. B: representative images showing aortic endothelial cells (third passage) from control and food-restricted 1-day-old offspring (×20). C: bar plots demonstrating summarized analysis of neovessels generated from control and MFR aortic endothelial cells in terms of number of branches and average branch length.](image-url)
specimen were analyzed, with three fields per section and six animals per group. For mesenteric arterioles, three fields per section and six to nine animals per group. Each slide analyzed had its corresponding negative control.

For analysis of renal microvessels, kidneys were cross sectioned, and three males and two females in each dietary group were compared. Staining of medullary sections with eNOS antiserum identified the vasa recta selectively. The number of these microvessels and the eNOS staining within these vessels were then analyzed as described above using the Image-Pro Plus software under ×20 magnification.

Statistical analysis. Data were analyzed using SigmaStat software. Parametric data were analyzed using the Student’s t-test, and non-parametric data were analyzed using the Mann-Whitney U-test. Since no gender differences were found in any end points studied, the results for males and females were combined. Results are means ± SE. Significance was established at P < 0.05.

RESULTS

In utero undernutrition, as expected, resulted in low-birth-weight male and female offspring (control: 7.2 ± 0.09 g, n = 35; MFR: 5.7 ± 0.12 g, n = 36; P < 0.001). Blood pressure data were previously published (17). Maternal food restriction induced a highly significant (P < 0.001) decrease in the number of mesenteric microvascular branches in the 1-day-old offspring (Fig. 1A). This decrease was independent of the weight of the offspring. A similar decrease in microvessel density was found in the renal medulla of the 1-day-old MFR offspring (Fig. 1B). Microvessels were identified by the eNOS staining of vasa recta as shown in Fig. 1B. Both the number of vasa recta (control: 187 ± 6.6 vs. MFR: 151 ± 8.3; P < 0.001) and the density of eNOS staining within these microvessels (IOD) were highly significantly reduced (4.3 ± 0.36 vs. 2.7 ± 0.34; P < 0.001) in the 1-day-old MFR offspring. No gender differences were found for mesenteric branching and renal microvessel density.

When aortas and mesenteric arterioles were analyzed for eNOS expression, we found that in contrast to the renal medulla, the expression of eNOS protein was significantly higher in MFR aortas (P < 0.05) as determined by Western blot analysis (Fig. 2A) and in mesenteric arterioles (P < 0.001) as determined by quantitative immunohistochemistry (Fig. 2B). The majority of eNOS staining in both vessel types was confined to the endothelial cells. No differences in eNOS expression were found in mesenteric arterioles or aortas of adult animals (data not shown).

Endothelial cells were isolated from 1-day-old offspring aortas of control and MFR, and their ability to generate neovessels was then analyzed in vitro. As demonstrated in Fig. 3A, the cells isolated from a control blood vessel (shown at ×20 and ×40 magnification) were pure endothelial cells, as indicated by staining for vWF. These cells gave rise to neovessels, producing capillary-like structures (Fig. 3B). Analysis of
the neovessels generated in vitro (summary data shown in Fig. 3C) using Image-Pro Plus revealed that cells from the MFR animals were markedly shorter \((P < 0.001)\) than those from the controls. However, the number of branches was not significantly different between the two groups.

We then examined the expression of VEGF, the most significant regulator of angiogenesis, and its receptor VEGF-R2 by Western blot analysis in aortas. As shown in Fig. 4A, the expression of VEGF protein in aortas from 1-day-old MFR offspring was significantly lower \((P < 0.05)\) than in controls. However, in the aortas from 4-mo-old offspring, the expression of VEGF was 10 times higher \((P < 0.01)\) in MFR aortas compared with controls. The expression of VEGF-R2 in aortas (Fig. 4B) was opposite that of VEGF. VEGF-R2 expression was significantly higher \((P < 0.05)\) in MFR aortas compared with controls in the 1-day-old offspring, whereas in 4-mo-old offspring, the expression of aortic VEGF-R2 was significantly lower \((P < 0.05)\) than controls (Fig. 4B). The expression of VEGF in mesenteric arterioles (Fig. 5), determined by quantitative immunohistochemistry, showed the same pattern of expression as aortas. In mesenteric arterioles of 20-day-old fetuses and 1-day-old MFR offspring, VEGF expression was significantly lower \((P < 0.001)\) compared with controls. The expression of VEGF-R2 in arterioles was significantly higher \((P < 0.001)\) in MFR aortas compared with controls. The expression of VEGF-R2 in arterioles (Fig. 4B) was opposite that of VEGF. VEGF-R2 expression was significantly higher \((P < 0.05)\) in MFR arterioles compared with controls in the 1-day-old offspring, whereas in 4-mo-old offspring, the expression of mesenteric VEGF-R2 was significantly lower \((P < 0.05)\) than controls (Fig. 4B). The expression of VEGF in mesenteric arterioles (Fig. 5), determined by quantitative immunohistochemistry, showed the same pattern of expression as aortas.

We then determined whether the suppression of VEGF in neonatal vessels was associated with changes in cellular apoptosis and mitosis. As shown in Fig. 6, A and B, there was a significant increase \((P < 0.05)\) in the number of apoptotic cells in aortas from 1-day-old MFR vessels and mesenteric arterioles, respectively. These cells were distributed in all compartments of the vessel wall. The number of cells undergoing mitosis as determined by PCNA staining, shown in Fig. 6C, was significantly lower \((P < 0.001)\) in the MFR aortas compared with controls. There were no differences in mitotic rates in mesenteric arterioles of MFR offspring (data not shown).

**DISCUSSION**

This study demonstrates that in utero undernutrition inhibits angiogenesis in multiple vascular beds, including the mesenteric bed and renal microvessels. Furthermore, the endothelial cells of MFR animals are phenotypically altered such that neovessels generated from these cells are shorter in length, indicating an intrinsic defect in endothelial cells of MFR offspring. Both the aorta and mesenteric vessels of MFR offspring show increased rates of apoptosis and decreased mitosis. The inhibition of angiogenesis was associated with decreased expression of aortic and mesenteric VEGF in the pre- and neonatal periods. In adult MFR offspring, in contrast to the neonate, the expression of VEGF was markedly upregulated.

This study demonstrates that in utero undernutrition inhibits the major regulator of angiogenesis, namely, VEGF. We postulate that as a result of this inhibition of VEGF, a compensatory mechanism to upregulate VEGF-R2 occurs that enhances the sensitivity of the vessel to VEGF. At the time we published our group’s initial observation of inhibition of angiogenesis in MFR offspring (16), Pladys et al. (27) reported their findings showing decreased angiogenesis in the anterior tibialis muscle in a protein restriction rat model. Our findings differ from those of Pladys et al. (27) in that these investigators did not find changes in VEGF, VEGF-R2, or angiopoietin expression. This may be secondary to their use of the entire tissue for examination of angiogenic genes rather than the blood vessels, as was done in our study, and to the use of different animal models utilizing different nutritional insults in the two studies. Other investigators have also demonstrated an effect of maternal nutrition on VEGF expression. Overfeeding resulted in reduced expression of placental VEGF and its receptor (FLT1), which may cause placental hypovascularity compromising blood flow to the fetus (28). Ito et al. (15) demonstrated that uterine artery vasodilation response to VEGF is blunted by low dietary protein intake.

The kidney is another organ known to be affected by maternal undernutrition and a target of programming. Our results for the first time demonstrate an inhibition of angiogenesis and decreased eNOS expression in MFR kidneys. This would imply decreased blood flow and vasodilatory influence within the kidney, which would potentially compromise nephrogenesis, as has been reported by other groups (25). Of interest is that eNOS expression was reduced in the 1-day-old renal microvessels but increased in the 1-day-old MFR aorta and mesenteric arterioles. This suggests that in the mesenteric vascular bed, an adaptive mechanism to offset the effects of fewer microvessels occurs through an increase in the expres-
sion of eNOS, which would promote vasodilation. This compensatory mechanism does not appear to occur in the kidney. Previous studies have suggested that the effects of undernutrition on renal nephrogenesis may be mediated through the renal renin angiotensin system (RAS). A low-protein diet during pregnancy in rats induced a decrease in intrarenal RAS activity, and treatment of newborns with losartan during the first 12 days of life prevented the decrease in nephron number and the occurrence of adult hypertension (34). The potential connection between the RAS and our observed findings of eNOS expression remains to be determined.

Our data indicated that eNOS expression in both aortas and mesenteric vessels is increased in 2-mo-old MFR offspring. This suggests that the increase in eNOS expression is an adaptive mechanism to offset the lower blood flow secondarily to the reduced angiogenesis, and this adaptive mechanism is no longer evident in adulthood. Several but not all studies (33) have shown that endothelium-dependent and -independent vasodilation is impaired and flow-mediated dilation is decreased in low-birth-weight individuals at 3 mo of age, in later childhood, and in early adult life (11–12, 22). However, the reported effects on eNOS expression and activity in utero in undernourished offspring differ among published reports. Pladys et al. (27) did not find any changes in eNOS expression in their low-protein-diet model in 21-day-old fetal and 7-day-old offspring, whereas Franco et al. (8) reported decreased expression of eNOS in the aorta of adult male but not female offspring and a reduction in eNOS activity in both sexes. These differences could be related to different types of nutritional insults (i.e., global food reduction vs. protein restriction), the gestational period of undernutrition, and the age of offspring examined.

Although VEGF expression is suppressed in the neonatal MFR offspring, its expression is markedly higher in the adult offspring when the animals develop obesity. A number of human studies have demonstrated an increase in serum levels of VEGF and VEGF isoforms in obese individuals (24, 31). These studies have suggested that VEGF may have proinflammatory effects in high levels. Besides obesity, which is thought to be an inflammatory condition, the expression of VEGF is increased in inflammatory diseases such as asthma (13–14, 29). VEGF has been shown to induce Th2 cytokines, inducing an asthma-type phenotype with inflammation, edema, and vascular remodeling (1, 20). Previous studies by our group (17) have shown that in the adult MFR offspring, deposition of collagen occurs in the walls of both mesenteric arterioles and aortas, at

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**Fig. 6.** A: representative aortic section (left) from 1-day-old control and food-restricted offspring showing terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL)-positive cells (×40). Bar plot (right) shows summarized data. B: representative mesenteric arteriole (left) from 1-day-old control and MFR offspring showing TUNEL-positive cells (×40). Bar plot (right) shows summarized data. C: representative aortic sections (left) from 1-day-old control and food-restricted offspring showing cells staining for PCNA (×40). Bar plot (right) shows summarized data. ***P < 0.001.
which time hypertension has developed, raising the possibility that overexpression of VEGF in the adult MFR offspring could induce the expression of TGF-β1, thereby inducing collagen deposition. We have evidence that TGF-β1 expression is increased in adult MFR aortas, although it is not detectable in the newborn (unpublished data).

We also found that MFR induced an increase in cellular apoptosis in both mesenteric arterioles and aorta and decreased mitosis in the aortic cell wall. Apoptosis in endothelial cells could result in rarefaction (21), which may be a potential etiology of the reduced microvessel density reported presently. Pladys et al. (27) also found capillary rarefaction in the offspring in their protein restriction model. The inhibition of VEGF expression reported presently could be the cause of rarefaction, since VEGF regulates endothelial cell survival by inducing the expression of antiapoptotic proteins Bcl-2 and A1 (9) through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway (10).

The decreased number of microvessels in MFR offspring could have a number of consequences. With fewer vessels, blood flow to various target organs would be expected to decrease. Low perfusion could result in oxidative stress and reactive oxygen species production (3), which in turn contribute to vascular remodeling, apoptosis, and proliferation of vascular smooth muscle (32), all of which our group has previously reported occur as early as day 1 of life in the MFR offspring vessels (18). It is possible that hypertension occurs very early in life in MFR offspring because of increased peripheral vascular resistance, although these measurements have not yet been reported.

In summary, our data demonstrating an inhibition of angiogenesis in MFR offspring provide a basis for programmed hypertension. In utero nutritional stress through yet to be identified pathways suppresses VEGF expression in endothelial cells, resulting in decreased angiogenesis and increased peripheral vascular resistance. In postnatal life, other factors stimulate the expression of vascular VEGF, which in turn could induce an inflammatory state in the vessel wall, contributing to collagen deposition and stiffening of vessels. Potential rescue strategies may include augmentation of pre- or neonatal VEGF expression and/or inhibition of VEGF in adult offspring.

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REFERENCES


