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Abstract

The underlying etiology of many chronic diseases such as hypertension and diabetes has been traced to the \textit{in utero} 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 as 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 eNOS 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.

\textbf{Key Words:} Maternal food restriction, angiogenesis, VEGF, programmed hypertension, kidney, nitric oxide synthase.
Introduction

Recent evidence has shown that metabolic and cardiovascular disorders which manifest in adult life have their origin before birth\(^1\). 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 2). 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 which have focused on blood vessels have examined the offspring as adults when hypertension has already developed; thus it is unknown if the reported changes such as endothelial dysfunction are a cause or a result of hypertension.

In an animal model of intrauterine growth retardation (IUGR) developed in our laboratory\(^3\) in which dams are food restricted from day 10 of gestation we demonstrated that the offspring have low birth weight and by 2 months of age develop hypertension\(^4,5\). Furthermore, we characterized the phenotype of the vasculature of the offspring in terms of the extracellular matrix\(^4\) and the vascular smooth muscle\(^5\). 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 one 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 MMP-9 mRNA in one day old MFR aortas, and in the expression of MMP-2 and MMP-9 mRNA in 4
month old MFR aortas\textsuperscript{4} 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\textsuperscript{5}.

Angiogenesis and vasculogenesis are complex processes regulated by a myriad of growth factors. Vasculogenesis is in situ differentiation and growth of blood vessels from mesodermal derived hemangioblasts. It gives rise to the heart and the first primitive vascular plexus inside the embryo and its surrounding membranes\textsuperscript{6}. Angiogenesis is defined as formation of new vessels by capillary sprouting from re-existing vessels\textsuperscript{6,7}. 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\textsuperscript{6}. Mice with knockout of VEGFR-2 lack vasculogenesis and fail to develop blood islands throughout the embryo and the yolk sac, and die at e8.5 to e9.5\textsuperscript{8}. 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, Inc, Hollister, CA) were housed in a facility with constant temperature and humidity and on a controlled 12 h light-12 h 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, Brentwood, MO, USA: protein 23%, fat 4.5%, metabolizable energy 3030 kcal/kg) or 50% food restricted diet determined by quantification of normal intake in the ad libitum fed rats. The respective diets were given from 10 day of pregnancy to term. This period of undernutrition was chosen to eliminate the effects of the nutritional insult on programming of the placenta\textsuperscript{9}. 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 4 male and 4 female per litter. The characterization of this animal model has been previously published\textsuperscript{3}. Unless otherwise specified throughout all studies 6 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\textsuperscript{10}. Briefly, aortas from 1 day old control and MFR animals were dissected and cleaned off of fat and peri-adventitial tissue and placed in PBS. The vessels were washed to remove any blood cells, and opened longitudinally, and cut into 1 mm pieces. These pieces were then placed with their intimal surface down on Matrigel (1:2 dilution + 2ml of DMEM/F12 serum free) coated plates in growth media (DMEM/F12, 10% fetal calf serum, 3% Nu-serum, 50ug/ml endothelial cell growth supplement, 50 ug/ml heparin, 2% antibiotic-anti-mycotic solution). After 4 days endothelial cell outgrowths were visible. At this time aortic pieces were removed, and the endothelial cells were harvested $1.5 \times 10^4$ Cells per well were seeded on to Matrigel-coated 6-well plates in growth medium. After incubation for 48 hrs at 37°C the cells generated tube like structures which were examined by light microscopy. The rest of cells were immuno-stained for Von Willebrand factor (VWF), a specific endothelial cells 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 small intestine (length of
colon analyzed in control: average length of 32±1.2 mm vs MFR 33.3±2.1 mm (P=NS) was counted. Primary, secondary and tertiary branches were included.

Analysis of microvessel generated in vitro (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 (20x magnifications) per culture well were taken for analysis. Each well represented cells isolated from 6 animals derived from different litters. A total of 3 wells per dietary group were used for the final analysis. From each image 3-6 cells were analyzed in terms of length and number of branches and averages were obtained for both parameters and subjected to statistical analysis.

**Immunohistochemistry**

Tissues were fixed in 4% paraformaldehyde for 24 hours and then stored in 70% ethanol. After embedding in paraffin, 5 µm sections were cut and tissues were stained for PCNA (Cell Marque, Hot Springs, AK), and VEGF (Santa Cruz Labs, Santa Cruz, CA), and eNOS (Transduction Labs, Lexington, KY) using standard immunohistochemical techniques previously described in detail4,5.

**TUNEL Assay**

We used the ApopTag In Situ Apoptosis Detection kit (Intergen Co, Purchase, NY.). The indirect fluorescent staining technique was used according to the manufacturer. Briefly tissue sections were first deparaffinized with Xylene and ethanol, then treated with Proteinase K (20ug/ml). The TdT enzyme (1:20) was applied followed by a wash step and application of anti-Digoxigenin conjugate (fluorescein or rhodamine) (65ul/5 cm² of surface area).
Western Blot Analysis

Tissues were sonicated in protein lysis buffer and protein concentrations determined by the BCA Protein Assay kit (Pierce, Rockford, IL). For each sample, 70 ug of protein was separated on a 7.5% polyacrylamide gel. The separated proteins were transferred electrophoretically to Immobilon-P membranes (Millipore Corp., New Bedford, MA). Membranes were blocked for 2 h in a 5% milk buffer before overnight incubation with the antibody against VEGF and VEGFR2 (Santa Cruz Labs, Santa Cruz, CA) at a dilution recommended by the manufacturer. The blots were subjected to enhanced chemiluminescence (ECL Western Blotting Detection System, Amersham Corp, Arlington Heights, IL); with enzyme conjugate 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 re-probed for Glyceryl-aldehyde 3-phosphate dehydrogenase (GAPDH).

Image Analysis

Equal number of controls 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, Silver Spring, MD), 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
immunoreative antigen was determined using the IOD (integrated optical density). The IOD values were proportional to the un-weighted average optical density per area. The linearity of the IOD was determined using an ISO transmission density tablet as standard (Kodak)\textsuperscript{11}. 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 specimen were analyzed, with three fields per section and six animals per group. For mesenteric arterioles, three sections were analyzed with three fields per section and 6-9 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 was then analyzed as described above using the Image Pro Plus software under 20x magnification.

**Statistical Analysis**

Data was analyzed using the Sigmastat software. Parametric data was analyzed by the Student’s t-test and non-parametric data by the Mann-Whitney-U
test. Since no gender differences were found in any endpoints studied the results for males and females have been combined. Results are expressed as mean±SEM. Significance was established at P<0.05.
Results

In utero undernutrition as expected resulted in the birth of low-birth-weight male and female offspring (Control: 7.2±0.09 g, n=35; MFR: 5.7±0.12 g, n=36; P<.001). Blood pressure data have been previously published. 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<.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<.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 in contrast to the renal medulla the expression of eNOS protein was significantly (P<.05) higher in MFR aortas as determined by Western blot analysis (Fig. 2a) and in mesenteric arterioles (P<.001) 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 as 20x and 40x magnification) were pure endothelial cells as indicated by staining for Von Willebrand Factor. 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) by Image Pro Plus revealed that cells from the MFR animals were markedly shorter (P<.001) than 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 VEGFR2 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 (P<.05) lower than controls. However, in the 4 month old offspring aortas, the expression of VEGF was 10 fold higher (P<.01) in MFR aortas as compared to controls. The expression of VEGFR2 in aortas (Fig. 4b) was opposite to that of VEGF. VEGFR2 expression was significantly (P<.05) higher in MFR aortas as compared to controls in the 1 day old offspring, whereas in 4 month old offspring, the expression of aortic VEGFR2 was significantly (P<.05) lower than the 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 (P<.001) lower compared to controls, whereas in the 2 month old offspring
vessels VEGF expression was significantly (P<.001) higher in the controls compared with the MFR tissues.

We then determined if the suppression of VEGF in neonatal vessels was associated with changes in cellular apoptosis and mitosis. As shown in Figures 6a and 6b there was a significant increase (P<.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<.001) in the MFR aortas as compared to 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 up-regulated.

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 up-regulate VEGFR2 occurs which would enhance the sensitivity of the vessel to VEGF. At the time we published our initial observation of inhibition of angiogenesis in MFR offspring\textsuperscript{12}, Pladys et al\textsuperscript{13} reported their findings showing decreased angiogenesis in the anterior tibialis muscle in protein restriction rat model. Our findings differ from those of Pladys et al\textsuperscript{13} in that these investigators did not find changes in VEGF, VEGFR2 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 secondly 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\textsuperscript{14}. Ito et al\textsuperscript{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\textsuperscript{16}. Of interest is that eNOS expression was reduced in the one day old renal microvessels, but increased in the one 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 expression of eNOS which would promote vasodilation. This compensatory mechanism does not appear to occur in the kidney. Previous studies 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 intra-renal RAS activity, and treatment of newborn with losartan during the first 12 days of life prevented the decrease in nephron number and the occurrence of adult hypertension\textsuperscript{17}. 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 are increased in 2 month old MFR offspring. This suggests that the increase in eNOS expression is an adaptive mechanism to offset the lower blood flow secondary to the reduced angiogenesis and this adaptive mechanism is no longer evident in adulthood. Several but not all studies\textsuperscript{18} have shown that endothelial-dependent and independent vasodilation is impaired and flow-mediated dilation is decreased in low-birth weight individuals at 3 months of age, in later childhood and in early adult life\textsuperscript{19-21}. However, the reported effects on eNOS expression and activity \textit{in utero} in undernourished offspring differ among published reports. Pladys et al\textsuperscript{13} 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\textsuperscript{22} reported decreased expression of eNOS in the aorta of adult male but not female offspring and a reduction in eNOS activity in both genders. 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\textsuperscript{23,24}. These studies have suggested that VEGF may have pro-inflammatory 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\textsuperscript{25-27}. VEGF has
been shown to induce Th2 cytokines inducing an asthma type phenotype with inflammation, edema and vascular remodeling\textsuperscript{28,29}. Our previous studies showed that in the adult MFR offspring deposition of collagen occurs in the walls of both mesenteric arterioles and aortas at which time hypertension has developed\textsuperscript{4} raising the possibility that over expression of VEGF in the adult MFR offspring could induce the expression of TGF$\beta$-1 thereby inducing collagen deposition. We have evidence that TGF$\beta$-1 expression is increased in adult MFR aortas although it is non-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\textsuperscript{30} which may be a potential etiology of the reduced microvessel density reported here. Pladys et al.\textsuperscript{13} also found capillary rarefaction in the offspring in their protein restriction model. The inhibition of VEGF expression reported here could be the cause of rarefaction since VEGF regulates endothelial cell survival by inducing the expression of anti-apoptotic proteins Bcl-2 and A1\textsuperscript{31} through the phosphatidylinositol 3'-kinase (PI)/Akt signal transduction pathway\textsuperscript{32}.

The decreased number of microvessels in MFR offspring could have a number of consequences. Due to 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\textsuperscript{33}, which in turn contribute to vascular remodeling, apoptosis and proliferation of vascular smooth muscle,\textsuperscript{34} all of which we have previously reported occur as early as day one of life in the MFR
offspring vessels\textsuperscript{5}. It is possible that hypertension occurs very early in life in MFR offspring due to increased peripheral vascular resistance, though these measurements have not yet been reported.

In summary, our data demonstrating an inhibition of angiogenesis in MFR offspring provides a basis for programmed hypertension. \textit{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.
Acknowledgement

We appreciate the assistance of Ms. Hye Jin Park with manuscript preparation and Linda Day and Stacy Behare with specimen collection and animal care.
References


**Figure Legends**

**Figure 1A.** Representative mesentery from 1 day old control animal. Arrows demonstrate the bifurcations that were counted under high power magnification. Bar plot is summarized data representing data based on N=6 in each dietary group. ***P<.001.

**Figure 1B.** Demonstrates representative vas recta (20x) in 1 day old control and MFR offspring. Brown staining represents eNOS in the vasa recta.

**Figure 1C.** Summarized data showing the number of vas recta (total number per 20x field) and the intensity of eNOS staining (IOD) within them in 1 day old control and MFR offspring obtained from Image Prol Plus software. ***P<.001.

**Figure 2A.** Representative aortas from 1 day old control and MFR offspring (100x) demonstrating eNOS staining (brown). The western blot gel and the bar plot showing the summarized data of eNOS protein expression in control and food restricted (FR) offspring are shown to the right. *P<.05.

**Figure 2B.** Representative eNOS staining in mesenteric arterioles of 1 day old control and MFR offspring (40x). The bar plot shows the summary of immunohistochemical data. *P<.05.

**Figure 3A.** Immunofluorescent figure demonstrating aortic endothelial cells (20x, 40x) isolated from 1 day control and MFR offspring and stained for Von Willebrand factor.

**Figure 3B.** Representative images showing aortic endothelial cells (third passage) from control and food restricted 1 day old offspring (20x).

**Figure 3C.** 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 (um).

**Figure 4A.** Western blot gel demonstrating the expression of VEGF protein in lystes obtained from 1 day old and 4 month old control and MFR offspring. Lanes 1-4: control and 5-8 are from food restricted (FR) offspring. The summarized data (Mean band Density) is shown in bar plots. *P<.05; **P<.01.

**Figure 4B.** The expression of VEGF-R2 as determined by Western blot analysis in the same samples as fig. 4A.

**Figure 5.** Shows summary bar plot of quantitative immunohistochemical analysis of VEGF in mesenteric arterioles from 20 day old fetuses, 1 day old and 2 month old offspring as determined by Image Pro Plus software. ***P<.001.
**Figure 6A.** Representative aortic section from 1 day old control and food restricted offspring showing TUNEL positive cells (40x). Summarized data is shown as bar plot.

**Figure 6B.** Representative mesenteric arteriole from 1 day old control and MFR offspring showing TUNEL positive cells (40x). Summarized data is shown as a bar plot.

**Figure 6C.** Representative aortic sections from 1 day old control and food restricted offspring showing cells staining for PCNA (40x). Summarized bar plot is shown to the right. ***P<.001.
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