Excess Maternal Glucocorticoids in Response to In Utero Undernutrition Inhibit Offspring Angiogenesis

Omid Khorram, MD, PhD¹, Reza Ghazi, MD¹, Tsai-Der Chuang, PhD¹, Guang Han, MD¹, Joshua Naghi, MD¹, Youping Ni, MD, PhD¹, and William J. Pearce, PhD²

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Abstract

To test the hypothesis that inhibition of offspring angiogenesis by maternal undernutrition (MUN) is mediated by maternal glucocorticoids, 3 groups of dams were studied: controls received ad libitum food; MUN dams were food restricted by 50% from day 10 of gestation; and metyrapone (MET) dams were food restricted and treated with 0.5 mg/mL of MET, a glucocorticoid synthesis inhibitor. The MUN reduced birth weights, reduced vascular endothelial growth factor (VEGF) abundance in PI aortas, reduced VEGF and VEGF-R2 abundances in PI mesenteric arterioles, reduced arteriolar endothelial nitric oxide synthase abundance, reduced microvessel density in the anterior tibialis, reduced endothelial cell branching in culture, reduced arteriolar immunoreactivity for proliferating cell nuclear antigen (PCNA), increased active caspase 3 in PI mesenteric arterioles, and decreased matrix metalloproteinase (MMP)-2 and MMP-9 abundances in lysates of PI aortas. All of these effects were prevented by treatment with metyrapone. Collectively, these findings suggest that reduced angiogenesis in MUN offspring involves direct inhibitory effects of maternal glucocorticoid on fetal VEGF and its receptors.

Keywords

angiogenesis, fetal programming, glucocorticoids, maternal undernutrition, matrix metalloproteinases, VEGF

Introduction

Evidence accumulated over the past 3 decades strongly indicates that many metabolic and cardiovascular disorders that manifest in adult life have their origins before birth.¹ Fetal programming of adult onset hypertension has been demonstrated in multiple animal species and can be induced pharmacologically by maternal glucocorticoid (GC) administration, and uteroplacental insufficiency induced by uterine artery ligation² or physiologically by maternal undernutrition (MUN, for review see³). Maternal exposure to high circulating concentrations of GC also alters fetal development.^{4,5} Physiologically, excess GC can occur when the mother is stressed or when placental expression of 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD-2) is reduced. Interestingly, the timing of exposure to GC and the type of GC involved (cortisol or dexamethasone) influence the effects induced in the offspring.⁶ Based on these findings, maternal GC administration is a widely used model of in utero programming.⁷

The early work of Gardner et al demonstrated that in the protein restriction model of programming, GC of maternal origin is a pivotal programming agent for hypertension.⁸ In these studies, the administration of metyrapone (MET), which inhibits corticosterone synthesis in pregnant rats and their fetuses, prevented development of hypertension in the offspring of

mothers fed a low-protein diet.⁹ Given that infusion of GC directly into the fetus in utero or at birth can elevate blood pressure,¹⁰ the fetus appears to be responsive to GC early in development. However, maternal adrenalectomy prevented the development of hypertension in offspring of rats fed a low-protein diet,¹¹ suggesting that maternal, rather than fetal, GC is the main factor inducing offspring hypertension. Similarly, maternal administration of carbenoxolone, an inhibitor of placental 11 β -HSD-2, resulted in low-birth-weight and hypertensive offspring,⁷ and this effect was dependent on an intact maternal adrenal.¹² Recent studies have further shown that an intact adrenal in adult offspring is also essential for the development of hypertension following low-protein exposure in utero.⁸

In our previous studies, MUN has decreased vascular expression of vascular endothelial growth factor (VEGF) and

¹ Department of Obstetrics and Gynecology, La Biomedical Institute, Harbor-UCLA Medical Center, Torrance, CA, USA

² Center for Perinatal Biology, Loma Linda University, Loma Linda, CA, USA

Corresponding Author:

Omid Khorram, Department of Obstetrics and Gynecology, Harbor-UCLA Medical Center, 1000W. Carson St, Box 489, Torrance, CA 90502, USA.
Email: okhorram@obgyn.humc.edu

its receptors in the offspring and correspondingly has reduced angiogenic potential in microvascular endothelial cells.¹³ This finding, together with other published evidence that GCs inhibit angiogenesis, inspired our current hypothesis that excess GC in MUN dams contributes to the development of hypertension in adult offspring through inhibition of vascular VEGF and VEGF receptor expression and reduced angiogenic potential. The idea that angiogenesis and hypertension are intimately related is also supported by recent clinical studies for treatment of tumors with antiangiogenic drugs; hypertension is a common side effect of these agents.^{14,15} In preeclampsia, hypertension is associated with high circulating levels of soluble VEGF receptor 1, which forms inactive complexes with VEGF and placental growth factor.^{16,17} Other mechanisms associating hypertension with inhibition of angiogenesis have also been proposed, including microvascular rarefaction,^{18,19} reduction in nitric oxide (NO) availability,^{20,21} and increased endothelin 1 production²² in response to treatment with antiangiogenic factors.

To address our hypothesis, we utilized an animal model in which administration of MET in the drinking water effectively inhibited maternal GC synthesis.²³ This treatment blocked the MUN-induced increase in maternal GC secretion and strongly modulated the effect of MUN on vascular VEGF and VEGF receptor expression. Because VEGF plays a key role in control of vascular cellular proliferation and angiogenesis, we evaluated both the processes. Cellular proliferation and apoptosis were examined using the markers proliferating cell nuclear antigen (PCNA) and active caspase 3, respectively. Angiogenesis was assayed *in vitro* through quantitation of the ability of microvascular endothelial cells isolated from the lung to produce new branches and *in vivo* by determining microvessel density in the anterior tibialis. In addition, we determined the vascular expression of proteins that play an important role in the regulation of angiogenesis including matrix metalloproteinase MMP-2, MMP-9, and endothelial nitric oxide synthase (eNOS). For the protein expression studies, we focused primarily on the aorta as a representative conductance vessel and on the mesenteric arterioles as representative of the resistance vessels critical to blood pressure regulation. We chose the lung and the skeletal muscle as target sites for the effects of VEGF because both tissues are highly vascularized with abundant microvessels. The intent of this study was to elucidate the *in utero* effects of MET on responses to MUN, and therefore we examined the VEGF pathway in the P1 neonatal offspring.

Materials and Methods

Animals

The study was approved by the Animal Use and Care Committee at Los Angeles Biomedical Research Institute at the Harbor-UCLA Medical Center. We used a well-characterized animal model of fetal programming developed by our group²⁴ in which first-time pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, Massachusetts) were housed

under constant temperature and humidity with a 12-hour/12-hour light–dark cycle. On day 10 of gestation, rats were provided either an *ad libitum* diet (control group) of standard laboratory chow (Lab Diet 5001: protein 23%, fat 4.5%, metabolizable energy 3030 kcal/kg; Lab-Diet, St Louis, Missouri) or 50% food-restricted diet (MUN group) determined by quantification of normal intake in the controls. The third group (MET) was identical to the MUN group except that on day 10 of gestation these rats were also given water bottles with 0.5 mg/mL MET (Sigma-Aldrich, St Louis, Missouri). This concentration was chosen based on the study of Smith and Waddell.²³ Six dams per treatment group were studied. Maternal body weights and intake of food and water were recorded daily. Offspring on postnatal day 1 were sexed and sacrificed by decapitation after which tissues and trunk blood were collected. For blood pressure studies, offspring were nursed by their own mothers that were fed *ad libitum*, weaned on day 21 of postnatal life, and housed individually thereafter until sacrifice. After acclimatization to the procedure, blood pressure was determined by tail plethysmography. Blood samples from the dams were obtained from the tail vein after anesthesia.

Tissue Processing

Aortas were harvested and flash frozen in liquid nitrogen. Samples of small and large intestine with attached mesentery were fixed in 4% paraformaldehyde. For cell culture experiments, the peripheral lung edges were dissected under sterile conditions and collected into phosphate-buffered saline.

Immunohistochemistry

Using antibodies specific for VEGF and VEGF receptors, factor VIII, PCNA (Santa Cruz, California), and cleaved caspase 3 (Asp175; Cell Signaling Technology, Beverly, Massachusetts), and eNOS (Transduction labs, Lexington, Kentucky), immunohistochemical analysis was performed as described previously.^{13,25} The chromagen used was diaminobenzidine, and sections were counterstained with hematoxylin. For mesenteric arterioles, 3 to 5 vessels per animal were analyzed for cells expressing active caspase 3 and PCNA. Microvessel density was determined by counting the number of microvessels identified by factor VIII staining in 3 high-powered fields per slide, using Image Pro Plus software (Media Cybernetics, Bethesda, Maryland). For aortic sections, 4 different segments of each vessel were digitally photographed and analyzed in a blinded fashion at a magnification of 40 $\times$. The area and intensity of staining were quantified by image analysis using the Image Pro 4.01 software (Media Cybernetics, Silver Spring, Maryland) coupled to an Olympus BHS microscope/Spot RT digital camera (Olympus, Center Valley, Pennsylvania). After the images were calibrated for background lighting, integrated optical density (IOD) results were recorded as unweighted average optical densities per area, which was used to determine the concentration of antigen of interest. There were no differences between the results of statistical analysis using either IOD or area of

staining values. The results were expressed as percentage of IOD, which takes into account both the staining intensity and the area of staining.

Enzyme Immunoassay

Plasma corticosterone was determined by enzyme immunoassay using a commercial kit (Cayman Chemical, Ann Arbor, Michigan) with a detection limit of 30 pg/mL and a half maximal inhibitory concentration (IC_{50}) of 150 pg/mL in blood. Intra- and interassay coefficients of variation were 6.0% and 6.6%, respectively. Samples from 6 animals had to be pooled for P1 offspring in order to obtain enough plasma to run the assay. Pooling of samples was not necessary for the E20 dams.

Western Blot Analysis

Aortic tissues were sonicated in a lysis buffer and then analyzed for protein content using the bicinchoninic acid assay (Pierce, Rockford, Illinois). For each homogenate, 40 μ g of protein (pooled from 4 vessels from 4 animals from different litters) were separated on a 7.5% polyacrylamide gel, then transferred electrophoretically to Immobilon-P membranes (Millipore, Billerica, Massachusetts), blocked for 2 hours in 5% milk, and incubated overnight with antibodies against VEGF (sc-7269), VEGF-R1 (sc-316), MMP-2 (sc-10736), or MMP-9 (sc-10737; Santa Cruz Biotechnology) or antibodies against VEGF-R2 (9698S; Cell Signaling Technology, Beverly, Massachusetts). The VEGF antibody recognizes multiple isoforms of VEGF, although only the 21-kDa band, which corresponds to the predominant isoform of VEGF (VEGF₁₆₅), was analyzed in this study. The blots were quantified via enhanced chemiluminescence (Amersham, Van Nuys, California) using anti-mouse secondary antibodies conjugated to immunoglobulin G horseradish peroxidase and exposure to autoradiography film. Band densities were determined using scanning densitometry. Proteins were pooled from 4 P1 offspring derived from different litters to obtain a single protein sample, which was treated as $N = 1$. To ensure equal loading, protein blots were stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase or stained for total protein by Ponceau S dye.²⁶

Microvascular Endothelial Cell Culture

Microvascular endothelial cells were isolated as described previously.²⁷ Staining with von Willerbrand factor confirmed that endothelial cell purities were routinely >95%. Cells from passage 3 were used for branch counting. Culture wells were digitally photographed, divided into quadrants, and analyzed using "count object" feature of the Image Pro Plus software to determine branch number.

Statistics

Tests for normality were initially performed, and normally distributed data sets were analyzed by analysis of variance (Sigmastat, Systat Software, San Jose, California). Otherwise,

nonparametric tests (eg Kruskal-Wallis) were used. The Student-Newman-Keuls test was used for post hoc analysis. When male and female subsets did not differ significantly, the subsets were combined. Statistical significance implies $P < .05$ unless stated otherwise. Statistical power ≥ 0.8 was considered acceptable for all comparisons.

Results

Figure 1 summarizes the body weight results. In P1 pups, body weight was significantly less in MUN than in control offspring, confirming our prior studies.¹³ Offspring of MET-treated dams had body weights similar to those of control offspring. By 4 months of age, gender differences became significant. The MUN male offspring were heavier compared to males in the control and MET groups, whereas there were no differences in body weight between males in the MET and control groups. In contrast to males, no group differences in body weights were found among 4-month-old female offspring. Blood pressures determined by tail plethysmography in adult offspring also exhibited gender differences. In the MUN group, but not the MET group, adult male offspring had higher blood pressures than controls. No group differences in blood pressures were found among adult female offspring.

Figure 2 summarizes the corticosterone results. In MUN dams, but not in MET-treated dams, corticosterone levels were significantly greater than that in controls at E20. In the P1 offspring, corticosterone levels were significantly lower in both MUN and MET groups as compared to controls, and there were no gender differences. Corticosterone levels in MUN and MET offspring at P1 were not significantly different.

Figure 3 summarizes the effects of MUN and MET on the abundances of VEGF, VEGF-R1, and VEGF-R2 protein in P1 aortas. The expression of VEGF, VEGF-R1, and VEGF-R2 were lower in the MUN group as compared with controls. However, MET treatment only reversed the effect of MUN on VEGF and not the VEGF receptors. This suggests that MUN-induced increases in GC primarily affected VEGF expression rather than the receptors. Figure 4 summarizes the distribution of VEGF and VEGF-R2 in mesenteric arterioles as determined by immunohistochemistry; VEGF-R1 staining was too faint for an accurate analysis. Similar to aorta, MUN inhibited the expression of VEGF and VEGF-R2 and this inhibitory effect of MUN was blocked for both VEGF and VEGF-R2 by MET, thus suggesting that in mesenteric arterioles GC exerts effects on both VEGF and VEGF-R2.

To address the possibility that reduced VEGF and VEGF-R2 expression may cause reduced NO bioavailability,²⁸ we measured eNOS protein immunoreactivity in mesenteric arterioles. Similar to the expression profiles of VEGF and VEGF-R2, eNOS protein immunoreactivity was depressed relative to controls in MUN but not in MET animals (Figure 5).

The effects of MUN and MET on angiogenic potential are summarized in Figure 6. An index of angiogenesis is microvessel density in highly vascularized tissues such as skeletal muscle. To quantify angiogenic activity in skeletal muscle, we used

Figure 1. The effects of maternal undernutrition (MUN) on offspring body weight in 1-day-old neonates (P1) and adults. Corresponding adult systolic blood pressure values are shown in the bottom panel. For P1 males, control N = 24, MUN N = 22, and metyrapone (MET) N = 23. For P1 females, control N = 19, MUN N = 16, and MET N = 26. For adult males, control N = 11, MUN N = 11, and MET N = 26. For adult females, control N = 13, MUN N = 12, and MET N = 22. For systolic blood pressure, N = 6 in all the groups. Error bars indicate standard errors. Asterisks indicate $P < .05$.

Figure 2. Corticosterone levels in day 20 dams (N = 3 per group) were significantly elevated relative to control in the maternal undernutrition (MUN) group but not in the metyrapone (MET) group (left panel). In P1 offspring (control N = 7, MUN N = 5, and MET N = 7), corticosterone levels were depressed in both the MUN and the MET groups (right panel). Error bars indicate standard errors. Asterisks indicate $P < .05$.

factor VIII staining to identify microvascular endothelial cells. Representative sections demonstrating these microvessels are shown in Figure 6A. The results indicated that microvessel density in the anterior tibialis was significantly less than control in MUN but not in MET animals. To corroborate these findings, we used a second method to assess angiogenesis as indicated by the ability of endothelial cells to give rise to new vessels in vitro. Because the lung is a rich source of microvascular endothelial cells, we isolated these cells and determined their ability to generate new microvessel branches in vitro. The ability of lung microvascular endothelial cells to form new branches, in vitro, was significantly less than control in MUN but not in MET animals. These data support the interpretation that GC-induced inhibition of VEGF in endothelial cells of offspring results in reduced angiogenic potential and that this effect can be blocked by prevention of MUN-induced increases in GC in the MUN dams by MET.

Figure 3. Representative Western blot gel and summarized band densities demonstrating protein expression levels of VEGF, VEGF-R1, and VEGF-R2 in P1 aortas. N = 5 per group (each N is pooled protein from 4 different animals from different litters). Error bars indicate standard errors. Asterisks indicate $P < .05$. VEGF indicates vascular endothelial growth factor.

One consequence of reduced VEGF expression in offspring vessels could be decreased microvascular cell proliferation and apoptosis (rarefaction); therefore, mitotic activity was determined by PCNA staining, and apoptosis was assessed as the proportion of cells expressing active caspase 3. As indicated by immunostaining for PCNA in mesenteric arterioles (Figure 7, left panel), the percentage of cells undergoing mitosis was significantly less than control in MUN but not in MET animals. Conversely, the percentage of caspase 3-positive (apoptotic) cells was greater in MUN but not in MET animals, compared to the controls (Figure 7, right panel).

Owing to the obligatory involvement of extracellular matrix (ECM) remodeling in angiogenesis, we determined the expression of MMP-2 and MMP-9 proteins by Western blot in aortic specimens (Figure 8). Compared to controls, MUN reduced the expression of both MMP-2 and MMP-9; these effects were absent in MET animals.

Discussion

The results of this study demonstrate that MUN-induced inhibition of angiogenesis in offspring, as previously reported by us¹³ and others,²⁹ is due in part to exposure of fetal blood vessels to high maternal circulating GC levels. Evidence that GC levels were responsible for reduced angiogenesis in MUN dams was suggested by the ability of MET, an inhibitor of GC synthesis, to prevent the MUN phenotype. Our data also demonstrate that MUN inhibited the expression of VEGF and VEGF-R2 in both aorta and mesenteric arterioles, and this effect was associated with reduced angiogenic potential of the

endothelial cells in vitro and reduced microvessel density in the anterior tibialis of P1 offspring. These widespread inhibitory effects of MUN on angiogenesis were prevented by blockade of maternal GC synthesis by treatment with Met.

At birth, the inhibitory effects of undernutrition on offspring body weight were prevented by maternal MET treatment, suggesting that elevated GC of maternal origin has a role in the reduction of offspring bodyweight, confirming the findings of Smith and Waddell.²³ In adult offspring, increased weight in the MUN group exhibited gender differences, which were prevented by MET administered during pregnancy. The patterns of changes in adult offspring were similar for systemic blood pressure and body weight changes. Increased blood pressure in the adult male MUN offspring was prevented by MET, but in the female offspring there was only a decrease in body weight at birth, and by adulthood no differences in female body weight were detectable. Similarly, in the adult female offspring, there were no significant differences in blood pressure among groups. These gender differences in the metabolic and blood pressure characteristics of adult offspring in response to MUN are possibly secondary to a protective effects of estrogen later on in life in the female offspring and are in accordance with a number of other animal studies demonstrating a protective effect of female gender from an adverse uterine environment.³⁰ These previous studies have shown that moderate global dietary restriction during pregnancy can lead to increased blood pressure earlier and to a greater extent in male compared with female offspring.³¹ In a moderate protein restriction model of programming, male offspring showed a marked increase in blood pressure and decrease in the number of nephrons^{32,33};

Figure 4. Immunostaining results for metyrapone (VEGF; top row) and VEGF-R2 (middle row) in mesenteric arterioles from the 3 treatment groups. The bottom panel indicates summarized quantitative immunohistochemical data expressed as percentage of integrated optical density (IOD) for VEGF and VEGF-R2 (N = 8 per group). Error bars indicate standard errors. Asterisks indicate $P < .05$.

only severe protein restriction resulted in hypertension in both genders.³⁴

Glucocorticoids play a significant role in fetal programming of adult diseases.^{4,5} The GC administration during critical stages of gestation produces many of the same effects as undernutrition or protein restriction and results in adult offspring with the metabolic syndrome phenotype.⁴ Undernutrition during pregnancy stimulates increased synthesis of maternal GC⁸ as confirmed in this study by our data. Of note is our finding that corticosterone levels in P1 offspring were suppressed. The lower levels of corticosterone in these offspring are most likely secondary to global inhibition of adrenal steroidogenic enzymes in P1 offspring as previously reported by our group.³⁵ This global suppression of offspring steroidogenic enzymes in MUN offspring could be due to a negative feedback response to elevated maternal GC.³⁶ It is unclear as to why corticosterone levels were also lower in the MET offspring, but this suggests that maternal food restriction, independent of the influence of maternal corticosterone, has an inhibitory effect on fetal adrenal steroidogenesis. The placenta plays a vital role in

regulating the amount of GC that is delivered to the fetus by adjusting the expression of 11β -HSD-2. Either malnutrition or GC administration during pregnancy can inhibit the placental expression of 11β -HSD-2, thereby allowing increased transfer of active GC to the fetal compartment.^{37,38} Furthermore, placentas of 11β -HSD-2 null mice are smaller presumably due to reduced expression of VEGF in the placenta and the correspondingly reduced capillary surface area.³⁹

The GC receptors are expressed in all vessel types. In microvessels, both the GC and the mineralocorticoid receptors are expressed in all layers, and their abundances are greater in spontaneously hypertensive rats than in normotensive controls.⁴⁰ Undernutrition could reduce expression of placental 11β -HSD-2 and thereby increase transfer of active GC into the fetal compartment, which in turn could directly bind to vascular GC receptors and inhibit VEGF and VEGF receptor expression and ultimately angiogenesis. Correspondingly, numerous studies have demonstrated an inhibitory effect of GC on VEGF abundance and angiogenesis. Inhibitory effects of GC on VEGF expression have been demonstrated in renal carcinoma cells,⁴¹

Figure 5. Maternal undernutrition (MUN) depressed the abundance of endothelial nitric oxide synthase (eNOS) protein in mesenteric arterioles as determined by immunohistochemistry. This effect was prevented in the metyrapone (MET) group. Results are expressed as percentage of control integrated optical density (IOD; N = 8 per group). Error bars indicate standard errors. Asterisks indicate $P < .05$.

chondrocytes,^{42,43} retinal pigment epithelial cells,⁴⁴ glioma cells,⁴⁵ and neural microvessel morphogenesis *in vitro*.⁴⁶ In vascular smooth muscle cells, GC can inhibit platelet-derived growth factor (PDGF)-induced VEGF expression.⁴⁷ The inhibitory effects of GC on *VEGF* gene expression can also be reversed by specific GC receptor antagonists.^{45,48} However, the exact molecular mechanism by which GC inhibits *VEGF* expression is yet to be determined. Evidence for transcriptional regulation was provided by Finkenzeller et al.⁴⁹ These investigators showed dexamethasone-suppressed PDGF-induced induction of a stably transfected -2018 ± 50 -bp VEGF reporter gene construct in the mouse NIH-3T3 embryonic fibroblast cell line. However, this type of transcriptional regulation could not be demonstrated by Gille et al.⁵⁰ in cultured keratinocytes. Instead, the latter investigators showed that GC inhibited VEGF expression by increasing VEGF messenger RNA turnover. In human endothelial cells, GCs act directly to inhibit tube formation. These effects did not alter endothelial cell viability or migration but involved changes in cell morphology mediated in part by induction of thrombospondin 1.⁵¹

Glucocorticoid might also inhibit angiogenesis through epigenetic mechanisms. The GC can inhibit natural killer cell function by global inhibition of histone acetylation.⁵² Our own recent studies suggest that GC can inhibit angiogenesis indirectly through a micro RNA (miRNA)-mediated pathway whose targets are angiogenic factors.²³ In these studies, we identified a vascular miRNA (miR29c) whose expression was inhibited by *in utero* undernutrition. Equally important, one of the putative targets of this miRNA was VEGF. The MET treatment of dams blocked the inhibitory effect of maternal food restriction on offspring vascular miR29c expression (unpublished observation), suggesting an influence of maternal GCs on fetal vascular miRNA expression levels.

The ability of GC to activate apoptotic pathways is potentially another mechanism for inhibition of angiogenesis. We previously reported that blood vessels of undernourished offspring exhibit increased apoptosis in all layers of the arterial wall along with an inhibition of mitosis.¹³ In the present study, we confirmed our prior data and demonstrated that MET can block the effects of undernutrition on microvascular endothelial cell rates of mitosis and apoptosis. Several mechanisms could explain decreased endothelial cell turnover in food-restricted blood vessels. Elevated maternal GC could inhibit endothelial cell proliferation⁵³ or induce fetal endothelial cell apoptosis. This latter mechanism of GC-mediated endothelial cell rarefaction has been reported in a spontaneously hypertensive rat model and in rats administered exogenous GC.^{18,19} Alternatively, the increased rates of apoptosis and reduced proliferation of endothelial cell in food-restricted offspring blood vessels could be secondary to reduced expression of VEGF.⁵⁴ Microvessel rarefaction is one proposed mechanism by which inhibition of angiogenesis can lead to development of hypertension.¹⁵ Because VEGF is known to influence endothelial cell proliferation via the NOS pathway,^{55,56} another consequence of reduced expression of VEGF and its receptor in MUN offspring is reduced expression of eNOS protein (Figure 5) or eNOS activity leading to reduced NO bioavailability and ultimately hypertension.

Matrix metalloproteinases also play an important role in angiogenesis through degradation of the adventitial matrix, which allows endothelial cells to proliferate and expand into the interstitial matrix.^{57,58} These enzymes also serve to release growth factors such as VEGF that are typically bound to the ECM.⁵⁷ Thus, decreased expression of MMPs in the vessels of MUN offspring, as shown in this study (Figure 8), could contribute to the reduced expression of VEGF in these animals.¹³ The expression of MMPs is regulated by a variety of cytokines and growth factors such as VEGF,⁵⁹ and in reciprocal fashion, GC can inhibit the expression of MMPs in multiple cell types.⁶⁰⁻⁶³ Our data suggests that elevated maternal GC can inhibit the expression of fetal vascular MMPs and that this effect can be blocked by treatment of food-restricted dams with Met.

A main limitation of this study arises from uncertainty about the heterogeneity of MUN-induced effects in different vascular beds. For example, it is well known that endothelial cells from different vascular beds vary significantly in relation to their transcriptional profiles,⁶⁴ patterns of cell-to-cell adhesion, and responses to growth factors.⁶⁵ Another limitation is due to the possibility that MET may also inhibit aldosterone synthesis, which could potentially influence the levels of VEGF and angiogenesis. However, in contrast to GCs, most published reports indicate that aldosterone has a proangiogenic effect,⁶⁶⁻⁶⁸ thus supporting our hypothesis that the inhibitory effects of MUN on VEGF are primarily GC, rather than aldosterone, mediated.

In summary, maternal food restriction inhibits the expression of VEGF and both VEGF receptor subtypes in offspring P1 aortas and VEGF and VEGF-R2 receptor in mesenteric arterioles. Reduced VEGF and VEGF responsiveness in MUN offspring

Figure 6. Representative sections of microvessels stained for endothelial factor 8 (brown color). Sections from muscles harvested from the control (upper left), maternal undernutrition (MUN; upper right), and metyrapone-treated (MET; lower left) groups are included. Microvessel density values were determined using automated and standardized Image Pro software routines. The bar plots in the lower right quadrant indicate the results of the microvessel density measurements. Please note that MUN decreased microvessel density, and this effect was absent in the MET group. Sample sizes were N = 9 in the control and MET groups and N = 10 in the MUN group. The bar graphs also indicate the results of measurements of newly formed branch counts obtained from in vitro cultures of lung microvascular endothelial cells, included here for comparison (N = 3 culture wells with cells from 8 to 10 animals per culture well). MUN decreased branch counts, and this effect was absent in the MET group. Error bars indicate standard errors. Asterisks indicate $P < .05$.

Figure 7. The numbers of proliferating cell nuclear antigen (PCNA)-positive staining cells in mesenteric arterioles of the 3 treatment groups are shown in the left panel. Maternal undernutrition (MUN) decreased the numbers of PCNA-positive cells, and this effect was absent in the metyrapone (MET) group (N = 8 per group). Conversely, MUN increased the numbers of caspase-positive cells, and this effect was also absent in the MET group (N = 10 for control and MUN groups and N = 8 for the MET group). Error bars indicate standard errors. Asterisks indicate $P < .05$.

Figure 8. Representative Western blot and bar plot demonstrating the abundances of matrix metalloproteinase (MMP) 2 and MMP-9 in PI aortic lysates from the 3 study groups (N = 5 per group). PL; Ponceau S dye band density. Error bars indicate standard errors. Asterisks indicate $P < .05$.

could result in reduced angiogenesis, microvascular rarefaction, reduced capacity to synthesize NO, and, ultimately, adult-onset hypertension. Prevention of increased GC synthesis in MUN dams prevents the effects of undernutrition on VEGF and downstream consequences on endothelial cell proliferation, endothelial apoptosis, eNOS, and MMP expression. Future studies should address how these pathways interconnect and influence the relative contribution of each mechanism to inhibition of fetal angiogenesis in the food-restricted dam. These promising new directions should reveal new therapeutic opportunities to reverse the effects of maternal food restriction with VEGF administration.

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Declaration of Conflicting Interests

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