Folate Supplementation During Pregnancy Improves Offspring Cardiovascular Dysfunction Induced by Protein Restriction

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Abstract—Dietary protein restriction in the rat compromises the maternal cardiovascular adaptations to pregnancy and leads to raised blood pressure and endothelial dysfunction in the offspring. In this study we have hypothesized that dietary folate supplementation of the low-protein diet will improve maternal vascular function and also restore offspring cardiovascular function. Pregnant Wistar rats were fed either a control (18% casein) or protein-restricted (9% casein) diet ±5 mg/kg folate supplement. Function of isolated maternal uterine artery and small mesenteric arteries from adult male offspring was assessed, systolic blood pressure recorded, and offspring thoracic aorta levels of endothelial nitric oxide (NO) synthase mRNA measured. In the uterine artery of late pregnancy dams, vasodilatation to vascular endothelial growth factor was attenuated in the protein-restricted group but restored with folate supplementation, as was isoprenaline-induced vasodilatation (P<0.05). In male offspring, protein restriction during pregnancy led to raised systolic blood pressure (P<0.01), impaired acetylcholine-induced vasodilatation (P<0.01), and reduced levels of endothelial NO synthase mRNA (P<0.05). Maternal folate supplementation during pregnancy prevented this elevated systolic blood pressure associated with a protein restriction diet. With folate supplementation, endothelium-dependent vasodilatation and endothelial NO synthase mRNA levels were not significantly different from either the control or protein-restricted groups. Maternal folate supplementation of the control diet had no effect on blood pressure or vasodilatation. This study supports the hypothesis that folate status in pregnancy can influence fetal development and, thus, the risks of cardiovascular disease in the next generation. The concept of developmental origins of adult disease focuses predominately on fetal life but must also include a role for maternal cardiovascular function. (Hypertension. 2006;47:982-987.)

Key Words: diet ■ endothelium ■ hypertension, experimental ■ nitric oxide ■ pregnancy

Cardiovascular disease arises from complex interactions between genetic susceptibility and adverse environmental influences. Increasing evidence suggests that environmental, particularly nutritional, factors operate from the earliest stages of development. The concept of developmental origins of adult disease has been strengthened by observations in animals that an unbalanced diet in pregnancy leads to cardiovascular and metabolic dysfunction in the offspring. The offspring phenotype induced by such dietary manipulation has similarities with the human metabolic syndrome, thus, the risks of cardiovascular disease in the next generation. The concept of developmental origins of adult disease focuses predominately on fetal life but must also include a role for maternal cardiovascular function. (Hypertension. 2006;47:982-987.)

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pregnancy on cardiovascular function in both the mother and also in her offspring.

**Methods**

Animal procedures were in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986.

**Dietary Protocol**

Virgin female Wistar rats were mated and randomly assigned to 1 of 4 dietary groups when pregnant (P): control (P-C, 18% casein), protein restricted (P-PR, 9% casein), protein restricted + 5 mg/kg folate (P-PRF, 9% casein + 5 mg/kg folate), or control + 5 mg/kg folate (P-CF, 18% casein + 5 mg/kg folate). The control and protein-restricted diets, as described previously, and folate concentrations were based on guidelines for women of reproductive age and from previous studies. A subgroup (P-C, n=10; P-PR, n=10; P-CF, n=6; and P-PRF, n=8) for study in pregnancy was killed on day 18 or 19 of gestation (term, 21.5 days) by CO2 inhalation and cervical dislocation. The remainder of dams (C, n=7; PR, n=7; and CF, n=6) were delivered and fed standard chow postpartum. Litters were weaned, sexed, and culled to 8 at delivery. No more than 2 adult male offspring (O) from each litter, aged 136±4 days from the 4 groups (O-C, mothers fed 18% casein; O-PR, mothers fed 9% casein; O-PRF, mothers fed 9% casein +5 mg/kg folate; and O-CF, mothers fed 18% casein +5 mg/kg folate) were killed by CO2 inhalation and cervical dislocation. At all of the points, experimenters were blinded to the dietary groups.

**Blood Pressure Measurement**

Systolic blood pressure (SBP) was recorded in pregnant dams at day 16 of gestation and in offspring at 15 weeks postnatally by tail-cuff plethysmography (IITC blood pressure monitor, Linton Instruments) as described previously. To minimize any stressful response to this procedure, animals were handled by trained staff throughout their life and were made familiar with the recording equipment before measurements were made.

**Vascular Protocol: Maternal Uterine Arteries**

Uterine artery segments (UA, internal diameter, 488±12 μm) from pregnant dams were mounted on a wire myograph (J.P. Trading,) as described previously. Cumulative concentration response curves (CRCs) were measured for phenylephrine (PE, 1 nM to 100 μM/L). Then, after preconstriction with PE (EC80), cumulative CRCs to acetylcholine (ACH, 1 nM to 10 μM/L), vascular endothelial growth factor (VEGF; 10 pM to 3 nm), isoprenaline (ISO, 1 nM to 30 μM/L), calcitonin gene-related peptide (CGRP; 1 pm to 3 nM), and adrenomedullin (ADM, 1 pm to 30 μM/L) were produced concentration-dependent vasodilatation in the iso-PE-induced contraction. Cumulative CRCs to agonists were analyzed by fitting to a 4-parameter logistic equation using nonlinear regression to obtain the pEC50 and maximum response. Differences were assessed by 1-way ANOVA with Bonferroni post hoc correction (Prism 3.0, GraphPad Software Inc). When the curve produced by nonlinear regression was dissimilar to the unfitted data, curve-fitted data were not used. Where curves were not sigmoidal, the pEC50 was deemed inappropriate, and CRCs were compared using 2-way ANOVA (Prism 3.0, GraphPad Software Inc). Significance was accepted if P<0.05.

**Results**

**Maternal and Fetal Observations**

Maternal blood pressure at day 16 of gestation did not differ between the groups. At postmortem on days 18 to 19 of gestation litter size, fetal and placental weights were similar between the 4 groups (Table).

**Uterine Artery Reactivity**

In all of the arteries, the depolarizing KPSS wash produced a vasoconstriction that did not differ between the 4 groups. Similarly, the cyclic AMP-dependent vasodilator that was similar in all of the groups.

**Endothelial-Dependent Vasodilatation**

The endothelial-dependent vasodilators ACh and VEGF both produced concentration-dependent vasodilatation in the isolated uterine artery. Responses to ACh did not differ between the groups (pEC50: P-C, 7.8±0.08, n=9; P-PR, 7.77±0.18, n=10; P-PRF, 7.57±0.11, n=7; and P-CF, 7.50±0.02, n=6; P was not significant). In the P-PR group, maximal dilatation to VEGF was significantly attenuated compared with the control group. In the P-PRF group, responses to VEGF were similar to that of controls, as were the responses in the P-CF group (% maximal response; P-C, 66.3±5.3, n=8; P-PR,
In the presence of the NO synthase inhibitor L-NAME (100 μmol/L), VEGF-induced vasodilatation of the uterine artery was not altered compared with naïve preparations in any dietary group. Conversely, the presence of the cyclooxygenase inhibitor indomethacin (10 μmol/L) significantly attenuated the VEGF response in all of the groups except the P-PRF group. The presence of the 2 inhibitors in combination had little additional effect to indomethacin alone except in the P-PRF group (Figure 2).

ISO-, ADM-, and CGRP-Induced Vasodilatation

In all of the groups, the β-adrenoceptor agonist ISO produced a concentration-dependent vasodilatation. This was significantly shifted to the right in the P-PR compared with controls (pEC50: P-C, 7.89±0.04, n=7; and P-PR, 6.91±0.12, n=8; P<0.001). Supplementation of the maternal protein-restricted diet with folate restored the ISO response to be similar to controls, whereas the supplementation of folate to the control diet had no effect on the response (pEC50: P-C, 7.89±0.04, n=7; P-PRF, 7.75±0.03, n=6; and P-CF, 7.62±0.05, n=5; P was not significant; Figure 3a). In contrast, vasodilatation to ADM or CGRP was not significantly different in any group (Figure 3b and 3c).

Offspring

Birth weight was similar between the groups. SBP at 15 weeks was raised in O-PR rats compared with controls. The supplementation of maternal diet with folate did not alter blood pressure in the offspring of the O-CF group and restored blood pressure to controls.

**Figure 1.** Cumulative additions of the endothelial-dependent vasodilator VEGF to UA from near-term P-C (○, n=8), P-PR (●, n=10), P-PRF (▲, n=8), or P-CF (■, n=5) dams. Values are mean±SEM; *P<0.05% max response P-PR vs P-C, P-PRF, and P-CF.

**Figure 2.** Maximum response to the endothelial-dependent vasodilator VEGF (n=5 to 10) on UA of (a) P-C, (b) P-PR, (c) P-PRF, and (d) P-CF dams in the presence of L-NAME (100 μmol/L) alone (n=4 to 6), indomethacin (10 μmol/L) alone (n=4 to 5), and L-NAME and indomethacin together (n=4 to 5). Values are mean±SEM. *P<0.05 vs naive preparation.
pressure to control levels in the O-PRF group (SBP mm Hg: O-C, 108.0±2.0, n=11; O-PR, 124.0±3.0, n=9; O-PRF, 108.0±4.0, n=10; and O-CF, 113.0±3.0, n=9; P<0.01; Figure 4).

Mesenteric Artery Reactivity
Neither the vasoconstriction to KPSS nor to PE was different between the 4 groups.

Endothelial-Dependent Vasodilatation
In all of the groups, the endothelial-dependent vasodilator ACh produced a concentration-dependent vasodilatation. This was significantly shifted to the right in the O-PR compared with controls (pEC50: O-C, 7.80±0.11, n=11; and O-PR, 7.08±0.10, n=9; P<0.01). Supplementation of the maternal protein-restricted diet with folate produced an intermediate response that was not significantly different from either the control or the O-PR groups (pEC50: O-PRF, 7.31±0.16, n=9). Supplementation of the maternal control diet with folate tended to produce a rightward shift in the O-CF compared with controls, but this did not reach significance (pEC50: O-C, 7.80±0.11, n=11; and O-CF, 7.30±0.13, n=9; P>0.05; Figure 5a). In the presence of the NOS inhibitor L-NAME (100 μmol/L) and the cyclooxygenase inhibitor indomethacin (10 μmol/L), responses to ACh were significantly impaired except in the O-PR group where no effect of inhibition was noted (Figure 5b).

17 β-Estradiol–Induced Vasodilatation
The addition of the endothelial-independent vasodilator 17 β-estradiol produced a concentration-dependent vasodilatation in all 4 of the experimental groups. As with ACh, vasodilatation to 17 β-estradiol was significantly shifted to the right in the O-PR group compared with controls (pEC50: O-C, 6.23±0.14, n=6; and O-PR, 5.26±0.08, n=6; P<0.05). Supplementation of the maternal diet with folate did not alter the response to 17 β-estradiol in either the O-PRF or O-CF group compared with controls (pEC50: O-C, 6.23±0.14, n=6; O-PRF, 5.91±0.09, n=7; and O-CF, 6.00±0.21, n=6; P>0.05; Figure 5c).

eNOS mRNA
Thoracic aorta eNOS mRNA levels in O-PR rats were decreased compared with O-C but not compared with O-PRF (O-C, 1.20±0.16, n=8; O-PR, 0.75±0.04, n=7; and O-PRF, 0.86±0.10, n=10; P<0.05 O-C versus O-PR).

Discussion
In the present study, we report that supplementation of a low-protein diet with folate improves the vascular defects in pregnant dams and normalizes the blood pressure in her offspring, while having a modest effect on vascular function. These data provide a good example of how a micronutrient supplement can ameliorate the adverse effects of macronutrient imbalance in pregnancy.

We demonstrated previously that maternal protein restriction attenuates VEGF-induced vasodilatation of the uterine artery in near-term pregnant dams. In the present study, we have repeated that observation and demonstrated that supplementing the maternal diet with folate restores VEGF-induced dilatation of the uterine artery. This folate-induced restoration of maternal vasodilatation is most likely mediated through changes in the NO pathway. The restriction of dietary protein during pregnancy leads to a reduction in the NO release from the pregnant dams and normalizes the blood pressure in her offspring, while having a modest effect on vascular function. These data provide a good example of how a micronutrient supplement can ameliorate the adverse effects of macronutrient imbalance in pregnancy.

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Figure 3. (a) Cumulative additions of the β-adrenoceptor agonist isoprenaline to the uterine artery of P-C (●, n=7), P-PR (●, n=8), P-PRF (●, n=6), or P-CF (●, n=5) dams and maximal response to (b) ADM and (c) CGRP to UA from near-term P-C (●, n=7), P-PR (●, n=8), P-PRF (●, n=6), or P-CF (●, n=9) dams. Values are mean±SEM; **P<0.01 O-PRF vs O-C, P-PRF, and P-CF.

Figure 4. SBP in 15-week-old O-C (●, n=11), O-PR (●, n=9), O-PRF (●, n=10), or O-CF (●, n=9). Values are mean±SEM; **P<0.01 O-PR vs O-C, O-PRF, and O-CF.
NO release in the mesenteric arterial bed of such dams, effects on the Ach-mediated dilatation in the uterine artery have not been observed, either in the present study or our previous investigation. It remains possible, therefore, that alterations occur in different cell signaling pathways in different arterial beds, and perhaps, therefore, the defect lies not in NO production but rather the cAMP pathway. The main component of ISO-induced vasodilatation is the activation of adenylate cyclase producing a subsequent rise in cAMP. The present data confirm our previous findings\(^6\) that in the rat uterine artery the main component of VEGF-induced vasodilatation is PGI\(_2\) rather than NO and, as such, is mediated in the vascular smooth muscle by cAMP, not cGMP. Interestingly, the response to the structurally similar peptides ADM and CGRP, which act through both a cAMP-dependent\(^17\) and a cGMP/NO-dependent pathway,\(^18\) were not different between the groups. The similarity between the groups in response to these agonists may suggest that these dual pathways allow compensation for attenuations in one of the components.

The apparent importance of the PGI\(_2\)/cAMP pathway rather than the NO pathway in this model raises the possibility that the effect of folate in the present study is mediated independent of NO. One such possibility is folate acting to lower homocysteine (hcy) levels,\(^19\) which are known to be elevated in this model.\(^7\)\(^,\)\(^20\) Any such lowering of hcy would undoubtedly reduce hcy-mediated vascular damage by oxidative stress.\(^21\) Alternatively, folate may be protective, because it can also act as an antioxidant.\(^11\) However, this argument assumes that the protein-restricted dams rapidly become folate deficient, and we do not know this.

Restriction in dietary protein during pregnancy in the rat has been shown to lead to raised blood pressure and endothelial dysfunction in the offspring.\(^2\) The present study demonstrates that maternal supplementation of the protein-restricted diet with folate prevents the onset of hypertension and goes some way to restoring endothelial function in the offspring. How folate might influence the development of the fetus and ultimately prevent raised blood pressure and endothelial dysfunction in adult life is important to understanding the mechanisms that underpin the developmental origins hypothesis. The influence of folate on fetal adaptive responses and on long-term cardiovascular health in the offspring may be mediated via several mechanisms. The ability of folate to improve maternal vascular function, as discussed above, may prove to be one important mechanism, improving uterine blood flow and allowing an adequate nutrient supply to the developing fetus. This idea, however, has not been tested directly. Previous studies show impaired uteroplacental perfusion and attenuated dilatation of the uterine artery in dietary-restricted pregnant rats\(^6\)\(^,\)\(^22\) and the experimental reduction of such perfusion leads to cardiovascular dysfunction in later life.\(^23\) Furthermore, in our model, we do not observe any intrauterine growth restriction of the fetus whether or not folate status is altered, suggesting that protein provision to the fetus is adequate to sustain normal growth.

However, an additional mechanism is possible. The availability of folate will affect fetal deoxynucleotide triphosphate pools and DNA synthesis rates in the fetus,\(^24\)\(^,\)\(^25\) whereas disturbed fetal S-amino acid metabolism may affect DNA methylation patterns at critical periods in development, producing epigenetic effects on gene expression and contributing to the functional defects observed.\(^26\)\(^–\)\(^28\) Others have reported abnormalities of global DNA methylation in offspring of dams exposed to low protein in pregnancy or after uteroplacental insufficiency.\(^29\)\(^,\)\(^30\) Maternal dietary folate supplementation was shown to prevent obesity, insulin resistance, and cancer in the offspring of the Agouti mouse.\(^28\)

The importance of the model would be enhanced if differential effects on the methylation of specific genes, as opposed to changes in global DNA methylation, could be shown. We showed recently that changes in the peroxisome proliferator-activated receptor \(\alpha\) and the estrogen receptor \(\alpha\) gene expression in the liver of offspring of the low-protein fed rat were accompanied by reciprocal changes in DNA methylation of the promoter region of these genes. Moreover, the effects of this protein restriction were prevented by maternal dietary folate supplementation.\(^15\)

In the vasculature, 1 potential candidate gene for altered methylation because of maternal protein restriction is the estrogen receptor \(\alpha\), which is known to be differentially methylated and is intrinsically linked to the expression of eNOS and the availability of NO.\(^31\) The present study demonstrates attenuated responses to estrogen in the O-PR but not in O-PRF offspring. Similarly, eNOS mRNA levels were decreased in the O-PR but...
not O-PRF offspring. Such alterations in eNOS expression have been linked to both raised blood pressure and endothelial dysfunction, in both eNOS knockout mice\textsuperscript{32,33} and in the offspring of global nutritionally restricted dams\textsuperscript{34}. The absence of any direct measurement of eNOS mRNA expression from the mesenteric arteries in the present study prevents a direct link to the endothelial dysfunction reported. However, indirect evidence suggests a role for altered eNOS in the finding that the ACh response in O-PR appears resistant to eNOS and cyclooxygenase blockade. Such a response is suggestive of a downregulation of the NO pathway and perhaps a corresponding compensatory upregulation of the EDHF pathway. However, that maternal folate supplementation did not return both the ACh or eNOS mRNA expression to control levels perhaps suggests that such changes in vascular reactivity may be the result of, rather than the cause of, persistently elevated blood pressure.

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**References**


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