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 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. In the rat, we have demonstrated previously that maternal protein restriction during pregnancy leads to impaired endothelial function in the uterine and mesenteric arteries of the dam in late pregnancy and that supplementation of the maternal diet with glycine reverses this effect. An adequate supply of dietary folate in early pregnancy has long been recognized to be necessary for normal embryo development, and supplementation lowers the incidence of congenital defects. However, folate is known to have direct beneficial effects on the cardiovascular system and, in particular, the NO pathway. Folate has been specifically shown to enhance NO production through mechanisms possibly involving the enhanced regeneration of the eNOS cofactor tetrahydrobiopterin (BH4) or through its antioxidant properties. This could be of particular importance, because we have demonstrated previously a decreased release of NO in protein-restricted pregnant dams. We, therefore, hypothesized that folate supplementation would prevent the adverse effects of dietary imbalance during pregnancy.

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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,12 and folate concentrations were based on guidelines for women of reproductive age and from previous studies.13 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; PRF, n=7; and CF, n=6) were allowed to deliver and fed standard chow postpartum. Litters were weighed, 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 (ITTC blood pressure monitor, Linton Instruments) as described previously.12 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 being 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.6 Cumulative concentration response curves (CRCs) were measured for phenylephrine (PE, 1 nM to 100 μM/L). Then, after preconstriction with PE (EC50), cumulative CRCs to acetylcholine (ACH, 1 nM to 10 μM/L), vascular endothelial growth factor (VEGF; 10 pM to 3 nM), calcitonin gene-related peptide (CGRP; 1 pm to 3 nM), and adrenomedullin (ADM, 1 pM to 30 nM/L), calcium gene-related peptide (CGRP; 1 pm to 3 nM), and adrenomedullin (ADM, 1 pM to 30 nM/L) were conducted. To investigate which factors were involved in the VEGF-induced vasodilatation, VEGF responses were repeated in the presence of the NO synthase inhibitor L-N-nitro-l-arginine methyl ester (l-NAM; 100 μM/L), the cyclooxygenase inhibitor indomethacin (10 μM/L), and the combination of both l-NAM (100 μM/L) and indomethacin (10 μM/L).

Vascular Protocol: Adult Offspring
Mesenteric Arteries
Mesenteric artery segments (internal diameter, 307.4±4.4 μm) from adult male offspring were mounted on the wire myograph as described previously.2 As above, CRCs were measured for PE (1 nM to 100 μM/L). After preconstriction with PE (EC50), cumulative CRCs to ACh (1 nM to 10 μM/L) and 17 β-estradiol (1 nM to 10 μM/L) were measured. To further investigate the factors involved in ACh-induced vasodilatation, responses to ACh were repeated in the presence of l-NAM (100 μM/L) and indomethacin (10 μM/L). In contrast to the uterine artery studies on the pregnant dams, these inhibitors were given only in combination and not independently; this was based on our previous observations that the prostacyclin (PGI2) and, therefore, cyclooxygenase-sensitive component of the ACh response is negligible in this particular vascular bed. All of the drugs and chemicals were obtained from Sigma (Poole), except human recombinant VEGF (Genentech Inc).

Analysis of Endothelial NO Synthase mRNA Levels
Endothelial NO synthase (eNOS) mRNA levels in the thoracic aorta were determined relative to 18s ribosomal RNA using real-time PCR and the following primers and probes: forward primer 5’-CCATT-TACTGCAAAGGCTGACT-3’, reverse primer 5’-GCTGATTTCGCTGCTGT-3’, and probe 5’-FAM-TCTCCACAGAAAGAATTTG-TAGCCTGGAACATCT-3’-TAMRA (Applied Biosystems).

Calculations and Statistical Analysis
Data are expressed as mean±SEM. Constrictor responses were calculated as percentage of maximum induction contraction by 125 mmol/L KPSS and relaxant responses as percentage inhibition of 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, calculation of 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 α1-adrenoceptor agonist PE produced a concentration-dependent vasoconstriction 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, 8.02±0.08; 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 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 produced 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 uterine artery in near-term pregnant dams. In the present study, we report that supplementation of a protein-restricted 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).

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=5) dams. Values are mean±SEM; *P<0.01 P-PR 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,7 effects on the Ach-mediated dilatation in the uterine artery have not been observed, either in the present study or our previous investigation.8 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.16 The present data confirm our previous findings6 that in the rat uterine artery the main component of VEGF-induced vasodilatation is PGI2 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-dependent17 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 PGI1/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 above in the absence (♂, n=9 to 11) and presence of l-NAME (100 μmol/L) and indomethacin (10 μmol/L; ♂, n=8 to 11). Values are mean±SEM; **P<0.05 vs naive preparation; ***P<0.001 vs naive preparation, different letters indicate significance (P<0.05) between the groups. (c) Cumulative additions of the endothelial-dependent vasodilator ACh in MA from 19-week-old O-C (♂, n=6), O-PR (♂, n=6), O-PRF (♂, n=7), and O-CF (♀, n=6) offspring. Values are mean±SEM; *P<0.05 pEC50 O-PR vs O-C, O-PRF, and O-CF.

The importance of the model would be enhanced if differential effects on the methylation of specific genes, as oppose to changes in global DNA methylation, could be shown. We showed recently that changes in the peroxisome proliferator-activated receptor α and the estrogen receptor α gene expression in the liver of offspring of 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 α, 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.

Perspectives
To date, the consequences of folate supplementation in human pregnancy have been considered in terms of protecting against major developmental abnormalities, such as neural tube defects, and in the treatment of anemia. Our findings suggest that maternal folate status has wider implications for cardiovascular function in the mother during pregnancy and for the developmental origins of health and disease in her offspring in the long term.

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