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

Christopher Torrens, Lee Brawley, Frederick W. Anthony, Caroline S. Dance, Rebecca Dunn, Alan A. Jackson, Lucilla Poston, Mark A. Hanson

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.1,2 The concept of developmental origins of adult disease3 has been strengthened by observations in animals that an unbalanced diet in pregnancy leads to cardiovascular and metabolic dysfunction in the offspring.4 The offspring phenotype induced by such dietary manipulation has similarities with the human metabolic syndrome, providing further evidence for a developmental component to the etiology of this syndrome and giving models for investigating the underlying mechanisms.5

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 pregnancy6 and that supplementation of the maternal diet with glycine reverses this effect.7 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.8 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 production9,10 through mechanisms possibly involving the enhanced regeneration of the eNOS cofactor tetrahydrobiopterin (BH4) or through its antioxidant properties.10,11 This could be of particular importance, because we have demonstrated previously a decreased release of NO in protein-restricted pregnant dams.7

We, therefore, hypothesized that folate supplementation would prevent the adverse effects of dietary imbalance during

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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, were based on guidelines for women of reproductive age and from previous studies. As above, CRCs were measured for PE (1 nM to 100 mol/L), acetylcholine (ACh, 1 nM to 10 mol/L), and the combination of both L-NAME (100 mol/L) and indomethacin (10 mol/L). The remainder of dams (C, n = 10; P-PR, n = 10; P-CF, n = 10; and P-PRF, n = 9) were 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 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 (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 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. Cumulative concentration response curves (CRCs) were measured for phenylephrine (PE, 1 nM to 100 μmol/L). Then, after preconstriction with PE (EC₅₀), cumulative CRCs to acetylcholine (ACh, 1 nM to 10 μmol/L), vascular endothelial growth factor (VEGF; 10 pM to 3 μmol/L), isoprenaline (ISO; 1 nM to 30 μmol/L), calcium gene-related peptide (CGRP; 1 pm to 3 nM), and adrenomedullin (ADM, 1 pm to 30 pmol/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 N²-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L), the cyclooxygenase inhibitor indomethacin (10 μmol/L), and the combination of both L-NAME (100 μmol/L) and indomethacin (10 μmol/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. As above, CRCs were measured for PE (1 nM to 100 μmol/L). After preconstriction with PE (EC₅₀), cumulative CRCs to ACh (1 nM to 10 μmol/L) and 17 β-estradiol (1 nM to 10 μmol/L) were measured. To further investigate the factors involved in ACh-induced vasodilatation, responses to ACh were repeated in the presence of L-NAME (100 μmol/L) and indomethacin (10 μmol/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-TACTGCAAAGCAGACT-3', reverse primer 5'-GGTGGATTT-GCTGCTCTGT-3', and probe 5'-FAM-TCTCCACAGAAAGAATTGTAGCCTGGAAACATCT-3'-TMRA (Applied Biosystems).

Calculations and Statistical Analysis
Data are expressed as mean ± SEM. Constrictor responses were calculated as percentage of maximum contraction induced by 125 μmol/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, 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, Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Litter Size</th>
<th>Fetal Weight</th>
<th>Placental Weight</th>
<th>Fetal:Placental Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-C</td>
<td>11.2 ± 0.9</td>
<td>2.00 ± 0.16</td>
<td>0.46 ± 0.05</td>
<td>4.74 ± 0.49</td>
</tr>
<tr>
<td>P-PR</td>
<td>11.4 ± 0.6</td>
<td>1.99 ± 0.16</td>
<td>0.38 ± 0.02</td>
<td>5.20 ± 0.29</td>
</tr>
<tr>
<td>P-PRF</td>
<td>11.3 ± 0.7</td>
<td>1.91 ± 0.15</td>
<td>0.38 ± 0.01</td>
<td>5.07 ± 0.34</td>
</tr>
<tr>
<td>P-CF</td>
<td>11.4 ± 0.6</td>
<td>1.76 ± 0.15</td>
<td>0.41 ± 0.02</td>
<td>4.34 ± 0.29</td>
</tr>
</tbody>
</table>
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 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 mesenteric arteries, and folate is capable of upregulating eNOS levels, as well as reducing the activity of the endogenous eNOS inhibitor asymmetrical dimethylarginine. Alterations in the NO pathway may also impact on the other vasodilators for which changes were noted in the present study. ISO-induced vasodilatation, for example, has been shown to involve an endothelial NO pathway.

However, whereas perturbations in the NO pathway may be a promising avenue for investigation, it is worth noting that any deficiency in uterine artery NO production would be expected to be reflected in an impaired ACh response. Whereas we have previously reported impaired ACh vasodilatation and reduced

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 O-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 P-PR vs P-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 that in the rat uterine artery the main component of VEGF-induced vasodilatation is PGI2 rather than NO. One such possibility is folate acting to lower homocysteine (hcy) levels, which are known to be elevated than the NO pathway in this model raises the possibility that the effect of folate in the present study is mediated independently of NO. Three such effects are folate acting to lower homocysteine (hcy) levels, which are known to be elevated in this model. Any such lowering of hcy would undoubtedly reduce hcy-mediated vascular damage by oxidative stress. Alternatively, folate may be protective, because it can also act as an antioxidant. 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. 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 and the experimental reduction of such perfusion leads to cardiovascular dysfunction in later life. 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, 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. Others have reported abnormalities of global DNA methylation in offspring of dams exposed to low protein in pregnancy or after uteroplacental insufficiency. Maternal dietary folate supplementation was shown to prevent obesity, insulin resistance, and cancer in the offspring of the Agouti mouse.

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 α and the estrogen receptor α 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.

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. 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...
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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 and in the offspring of global nutritionally restricted dams. 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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References

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