Altered Vascular Function in Fetal Programming of Hypertension

Delphine Lamireau, MD; Anne Monique Nuyt, MD; Xin Hou, MD, PhD; Sylvie Bernier, PhD; Martin Beauchamp, MSc; Fernand Gobeil, Jr, PhD; Isabelle Lahaie, MSc; Daya R. Varma, MD, PhD; Sylvain Chemtob, MD, PhD

Background and Purpose—Reduced endothelium-dependent vasorelaxation partly due to loss of nitric oxide (NO) bioavailability occurs in most cases of chronic hypertension. Intrauterine nutritional deprivation has been associated with increased risk for hypertension and stroke, associated with relaxant dysfunction and decreased vascular compliance, but the underlying mechanisms are not known. The present studies were undertaken to investigate whether endothelial dysfunction associated with altered NO-dependent vasodilatation pathways is also observed in a model of in utero programming of hypertension.

Methods—Pregnant Wistar rats were fed a normal (18%), low (9%), or very low (6%) protein isocaloric diet during gestation. Vasomotor response of resistance cerebral microvessels (<50 μm) was studied in adult offspring of dams fed the 18% and 9% protein diets by a video imaging technique. Endothelial NOS (eNOS), soluble guanylate cyclase (sGC), and KCa channel expression were measured by Western blot. NO synthase (NOS) activity was measured enzymatically as well as in situ by NADPH diaphorase staining.

Results—Litter size and survival to adulthood were not affected by the diets. Birth weights of offspring of dams fed the 6% diet were markedly lower than those of dams fed the 9% diet, which were marginally lower than those of controls. Systolic blood pressures of adult offspring of mothers in the 6% and 9% groups were comparably greater (156±2 and 155±1 mm Hg, respectively) than that of control offspring (137±1 mm Hg); we therefore focused on the 9% and 18% groups. Cerebral microvessel constriction to thromboxane A2 mimetic and dilation to carba–prostaglandin I2 did not differ between diet groups. In contrast, vasorelaxation to the NO-dependent agents substance P and acetylcholine was diminished by 50% in low protein–exposed offspring, but eNOS expression and activity were similar between the 2 diet groups. Vasorelaxant response to the NO donor sodium nitroprusside was also decreased and was associated with reduced (by 50% to 65%) cGMP levels and sGC expression. cGMP analogues caused comparable vasorelaxation in the 2 groups. Expression of KCa (another important mediator of NO action) and relaxation to the KCa opener NS1619 were unchanged by antenatal diet.

Conclusions—Maternal protein deprivation, which leads to hypertension in the offspring, is associated with diminished NO-dependent relaxation of major organ (cerebral) microvasculature, which seems to be largely attributed to decreased sGC expression and cGMP levels. The study provides an additional explanation for abnormal vasorelaxation in nutrient-deprived subjects in utero. (Stroke. 2002;33:2992-2998.)

Key Words: blood circulation ■ guanylate cyclase ■ hypertension ■ infant, low birth weight ■ nitric oxide ■ rats

Epidemiological studies reveal that the risks of cardiovascular diseases such as hypertension, stroke, and coronary heart disease are inversely related to birth weight and independent of genetic factors and lifestyle; the mechanisms underlying this programming of diseases are currently incompletely understood. It has been suggested that a poor nutrient supply at a critical period of early development leads to permanent alteration of the developing vascular structures or functions. This concept has been supported by animal studies demonstrating an association between nutritional deficit during intrauterine life and increased blood pressure in adulthood.

Studies in humans have reported endothelial dysfunction, decreased vascular compliance, and atherosclerosis in children and adults with low birth weight. Similarly, chronic hypertension is associated with reduced endothelium-dependent vascular relaxation due to part in loss of nitric oxide (NO) generation before the onset of vascular dysfunc-
tion.\textsuperscript{7} NO is produced by endothelial nitric oxide synthase (eNOS) and plays a major role in the regulation of blood pressure and vascular tone.\textsuperscript{8} NOS inhibitors increase blood pressure in animals and humans,\textsuperscript{9} and mice with a disrupted eNOS gene are hypertensive.\textsuperscript{10}

The present studies were undertaken to test the hypothesis that hypertension and vascular dysfunction associated with in utero nutrient deprivation are secondary to “programmed” alterations in NO-dependent vasorelaxant pathways in microvasculature of a major organ, namely, brain. For this purpose, we used an animal model of fetal programming of hypertension in which mild restriction of protein intake of the pregnant rat leads to offspring with high blood pressure.\textsuperscript{3} In these hypertensive rats, we studied the NO-dependent vasodilatation pathway of cerebral resistance microvessels because of their role as major regulators of cerebral circulation.\textsuperscript{11} Our findings confirm impaired NO-mediated vasodilatation in hypertensive rats exposed to a low protein diet and reveal that this involves reduced soluble guanylate cyclase (sGC) expression and cGMP levels, whereas NO synthase (NOS) expression and function are normal. These findings provide an uncommon mechanism of microvascular dysfunction associated with chronic hypertension (secondary to in utero protein deprivation).

Materials and Methods

Animals

Animals were used according to a protocol of the Animal Care Committee of the Hôpital Sainte-Justine in accordance with the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Virgin Wistar rats (weight, 225 to 250 g) were mated overnight and on the day of conception were allocated to be fed with isocaloric diets containing either 18% (control), 9% (low), or 6% (very low) protein (casein).\textsuperscript{12} Within 12 hours of delivery, dams were returned to regular rat chow, and 4 to 5 pups per litter were weighed. Pups were weaned at 4 weeks of age to regular chow.

Systolic blood pressure of the offspring was determined from 7 to 12 weeks of age by the tail-cuff method (Harvard) by the same person. In a separate group of 10- to 12-week-old animals in the 9% group, the angiotensin-converting enzyme inhibitor enalaprilat (0.9 mg/kg per day)\textsuperscript{13} (n=3) or saline (n=2) was infused (Alzet osmotic pump, Durect Corporation). Blood pressure was determined daily. After 1 week of treatment, brain tissues were collected to study vasomotor response.

Isolation of Brain Tissues

Rats (9 to 12 weeks old) were decapitated, and brains were rapidly removed and placed in cold Krebs’ buffer to which 1.5 U/mL heparin was added. For biochemical measurements, tissues were frozen in liquid N\textsubscript{2} and stored at \textdegree-80°C.

Slices of brain (1 mm thick) exposing the pial microvessels were prepared as previously described\textsuperscript{14} to study the relatively undisturbed cerebral vasculature (40 to 60 \textmu m).

Vasomotor Response

Brain microvessels were visualized and recorded with a video camera (model CCD72, MTI) mounted on a dissecting microscope (model M-400, Nikon), as previously reported.\textsuperscript{14} Vascular diameter was measured with a digital image analyzer (Sigma Scan software, Jandel Scientific) and repeated 3 times with a variability of <1%.

Responses to vasorelaxant agents were determined on tissues precontracted with the thromboxane A\textsubscript{2} (TXA\textsubscript{2}) mimetic U46619 (0.3 \mu mol/L) to 50% of maximum contraction; the contraction to U46619 was not modified by the maternal diets. Vascular diameter was recorded before and after topical application of the following agents: acetylcholine, substance P, and prostacyclin (prostaglandin I\textsubscript{2}, [PGI\textsubscript{2}]); the NO donor sodium nitroprusside (SNP); a stable cGMP analogue 8-bromo cGMP\textsuperscript{15}; a selective protein kinase G stimulant, \beta-phenyl-L-N\textsuperscript{2}-etheno-8-bromoguanosine-3',5'-monophosphate (8-bromo PET cGMP)\textsuperscript{16}; and the K\textsubscript{Ca} channel agonist NS1619 in the absence or the presence (30 minutes of pretreatment) of GC inhibitor 1H-(1,2,4) oxizadolo (4,3-a) quinoxaline-1-one (ODQ) (0.1 mmol/L) and the K\textsubscript{Ca} channel blocker ibetironit (100 mmol/L).

Preparation of Microvessels

Cortical microvessels were isolated as previously reported.\textsuperscript{17} Briefly, cortical tissue was collected in ice-cold PBS (pH 7.4), cut into small pieces, and centrifuged at 1000 rpm for 10 minutes at 4\textdegree C. Pellets were suspended in PBS, mixed with Ficol 400 (20%) at a 1:1 (vol/vol) ratio, and centrifuged at 13 000 rpm for 20 minutes at 4\textdegree C, then washed 3 times in PBS.

Determination of NOS Activity

NOS activity of isolated cortical microvessels was measured by quantifying the conversion of L-arginine to L-citrulline with the use of a commercially available kit (Bioxynex NOS Assay Kit, Oxis International). Briefly, protein extracted from microvessels was incubated with radiolabeled L-arginine in the presence or in the absence of 1 mmol/L NOS inhibitor L-N-nitro-arginine methyl ester. The reaction was terminated by the addition of 50 mmol/L HEPES buffer containing 5 mmol/L EDTA. Radiolabeled L-citrulline was counted after removal of excess L-arginine with an equilibrated resin and centrifugation.

NADPH-Diaphorase Histochemistry

NADPH-diaphorase reactivity was performed as previously described.\textsuperscript{18} Briefly, tissue from cortex was fixed by immersion in 4% buffered paraformaldehyde containing 0.1% \beta-NADPH, 0.3% Triton X-100, 0.5 mmol/L MgCl\textsubscript{2}, 0.01 mol/L sodium azide, and 0.1% nitroblue tetrazolium. After they were processed, the slides were counterstained with neutral red. The intensity of staining in blood vessels was analyzed digitally with the software ImagePro+4.1 (Media Cybernetics) and Photoshop 5 (Adobe).

Western Blotting

Western blotting analysis of microvessels lysates was performed as described.\textsuperscript{19} Twenty-five micrograms of solubilized protein was probed with the following antibodies: anti-eNOS monoclonal antibody (1:1000); anti-sGC polyclonal antibody (1:1000); and anti-BK\textsubscript{Ca} channel polyclonal antibody (1:1000).

cGMP Radioimmunoassay

cGMP was measured by radioimmunoassay on protein extracted from homogenates of cortical tissue with the use of a commercially available kit, as previously reported.\textsuperscript{19}

Chemicals

The following agents were purchased: 8-bromo PET cGMP and 8-bromo cGMP (Biolog); SNP, acetylcholine, substance P, ibuprofen, EDTA, HEPES, \beta-NADPH, Triton X-100, nitroblue tetrazolium, ODQ, and U46619 (Sigma Chemical); enalaprilat (Vasotec, Merck Frosst); NS1619 (Research Biochemicals International); Ficol and cGMP assay kits (Amersham); anti-eNOS monoclonal antibody (Transduction Laboratories); anti-sGC polyclonal antibody (Cayman Chemicals); anti-BK\textsubscript{Ca} channel polyclonal antibody (Alomone Labs); and horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse IgG and Super Signal Western blot analysis system (Pierce). Other high-purity chemicals were purchased from Fischer Scientific.

Statistical Analysis

All results are expressed as mean \pm SEM. Results were analyzed with the Student t test or 2-way ANOVA, factoring for concentrations and
Results

Effects of Low Protein Diet on Animal Weight and Litter Size

Net weight gain during pregnancy of dams in the 9% (144±7 g; n=18) and control (18%) (142±5 g; n=19) groups was similar but was reduced for dams in the 6% group (125±11 g; n=5). Dietary protein affected neither the litter size (18%: 14.8±0.7; n=8; 9%: 14.9±0.9; n=7; 6%: 14.3±0.8; n=7) nor the survival rate of offspring during the 14 weeks of the study period. Birth weight of the 9% group (5.3±0.1 g; n=14) tended to be slightly (but not significantly) less than that of the control (18%) group (5.6±0.2 g; n=14); pups born to dams in the 6% group were significantly lighter (4.5±0.1 g; n=14) than those in the other groups.

Effects of Antenatal Diet on Blood Pressure

The systolic blood pressure of offspring of mothers fed a low protein diet was higher than that of the control (18%) throughout the study period (Figure 1). We focused on offspring of the 9% and 18% groups because the very low protein diet did not cause further increases in blood pressure but was detrimental to fetal and maternal weight gain.

Effects of TXA2 Mimetic, PGI2, and NO-Releasing Agents on Brain Microvessel Vasomotor Response

Constriction to the TXA2 mimetic U46619 and dilation to carba-PGI2 did not differ between diet groups (Figure 2). In contrast, maximum relaxation evoked by NO-releasing agents (Figure 3) was markedly decreased by approximately 50% in the 9% group; EC50 values were increased for acetylcholine but only marginally so for substance P in rats of the 9% group (Table).

eNOS in Brain Microvessels

eNOS protein expression, NOS activity, and NADPH diaphorase reactivity did not differ between the diet groups (Figure 3). There is convincing epidemiological evidence that maternal protein deficiency is associated with vascular dysfunction and

Relative Contribution of GC and KCa Channels in NO-Induced Relaxation

Because vasorelaxant effects of NO can be mediated to a significant extent by KCa channels in addition to cGMP, we investigated the relative contribution of these factors. The role of KCa may also be of interest because its activity may be augmented in a compensatory manner in hypertension. Inhibition of both GC (with ODQ) and KCa channel (withiberiotoxin) abolished SNP-evoked vasorelaxation regardless of diet (Figure 5A). Blockade of KCa or of GC inhibited to a comparable extent (25% to 30%) the SNP-induced response in both groups. Correspondingly, there was no difference in immunoreactive KCa protein expression (Figure 5B) or in the vasodilatation evoked by the KCa opener NS1619 between the groups (Figure 5C and Table).

Discussion

There is convincing epidemiological evidence that maternal protein deficiency is associated with vascular dysfunction and
hypertension in adult life.\textsuperscript{23} Since endothelial dysfunction with impaired NO production is an important pathophysiological element underlying hypertension and atherosclerosis, we tested the hypothesis that NO-dependent vasodilatation is altered in hypertension programmed during fetal life. Our results reveal that hypertension in adult rats induced by exposure to a low protein diet during intrauterine life is indeed associated with an altered NO-dependent vasorelaxation, which cannot be attributed to decreased NO production but rather to changes in the cGMP pathway.

We have reproduced a rat model in which pregnant dams are fed a diet containing 9% protein.\textsuperscript{12} Although relative to

<table>
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<tr>
<th>Agents</th>
<th>EC(_{50}) (nmol/L) 18% Protein</th>
<th>EC(_{50}) (nmol/L) 9% Protein</th>
<th>Maximum Relaxation (%) 18% Protein</th>
<th>Maximum Relaxation (%) 9% Protein</th>
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<tr>
<td>Substance P</td>
<td>0.99±0.20</td>
<td>2.46±0.37*</td>
<td>45.3±3.2</td>
<td>21.4±2.2*</td>
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<tr>
<td>Acetylcholine</td>
<td>0.51±0.20</td>
<td>10.42±2.93*</td>
<td>92.2±4.9</td>
<td>28.5±3.1*</td>
</tr>
<tr>
<td>SNP</td>
<td>0.65±0.10</td>
<td>0.33±0.20</td>
<td>177.5±16.0</td>
<td>96.0±4.6*</td>
</tr>
<tr>
<td>8-bromo cGMP</td>
<td>2.11±0.21</td>
<td>6.51±0.38</td>
<td>117.7±6.9</td>
<td>105.4±10.0</td>
</tr>
<tr>
<td>8-bromo PET cGMP</td>
<td>0.43±0.20</td>
<td>1.26±0.26</td>
<td>122.20±12.4</td>
<td>110.8±10.8</td>
</tr>
<tr>
<td>NS1619</td>
<td>461.2±13.3</td>
<td>540.3±11.5</td>
<td>61.4±4.1</td>
<td>55.5±3.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM of 3 to 4 experiments. EC\(_{50}\) values were calculated from dose-response curves of concentrations of agents ranging from 10\(^{-12}\) to 10\(^{-5}\) mol/L. Maximum relaxation is expressed as percent reversal of U46619 (0.3 µmol/L)-induced constriction.

\(^*p<0.05\) compared with corresponding value in 18% protein group.

**Figure 3.** Representative immunoblot and relative densitometry of eNOS protein in isolated cerebral microvessels (A), NOS activity (B), and NADPH-diaphorase staining of cortical brain slices (C) in 9- to 12-week-old rats in the low protein (9%) and control (18%) groups. A, Representative immunoblot of Western analysis of eNOS (25 µg of protein was loaded); arrow points to eNOS 140-kDa protein. Right panel represents compiled immunoreactive densitometry relative to that of the 18% protein diet set at 100%. B, NOS activity in isolated brain microvessels, measured as Ca\(^{2+}\)-dependent Nω-nitro-L-arginine–sensitive production of \([^{3}H]\)L-citrulline from \([^{3}H]\)L-arginine. Values are mean±SEM of 3 experiments for each group. C, Brain slices were fixed for NADPH-diaphorase staining of blood cortical vessels; arrows point to the NADPH-diaphorase–reactive vessels. Tonality densitometry was analyzed as described in Materials and Methods. Lighter arbitrary tonality units correspond to reduced densitometry (histogram).
the control diet this is a 50% reduction in protein content, the 9% diet provided 75% of the basic protein requirements of a pregnant rat.25 Birth weights of pups of dams fed a diet containing 9% protein are slightly but not significantly reduced, as reported by others.26,27 The hypertension observed in animals exposed to a low protein diet therefore implies fetal programming, which is defined as a permanent or long-term change in the physiology and morphology of organs in response to a specific stimulus at a critical period in development.28 Along these lines, although offspring of dams fed a low protein diet exhibited only a modest reduction in mean outer vessel diameter (42.5 versus 44.1 μm in controls), eutrophic remodeling of vessel structure associated with increased media-to-lumen ratio may contribute to these observations, as reported in other hypertensive conditions.29 NO is a potent endogenous vasodilator. Endothelial dysfunction with decreased synthesis of endothelium-derived NO is present in hypertensive patients7 and may initiate or contribute to diseases such as atherosclerosis, stroke, and hypertension.8 In humans with lower birth weight, vascular dysfunction has been suggested by altered response to acetylcholine,30 decreased arterial distensibility,5 and increased risk of atherosclerosis.6 Our findings reveal a decreased vasorelaxation to NO-dependent mechanisms in offspring of dams fed low protein diets due to reduced activity of the GC pathway. This impaired vasorelaxation to NO is not secondary to the hypertension per se since it persisted in rats in the 9% group with normalized blood pressure. eNOS expression and activity were unaltered by diets, whereas expression and activity of sGC, the main target of NO, were reduced in the

Figure 4. A, Vasorelaxant response to SNP on brain pial microvessels of low protein (9%) and control (18%) groups. Experimental preparations are similar to those of Figure 2. Data are mean±SEM of 4 experiments. *P<0.05 compared with control (ANOVA). B, Vasorelaxant response to SNP on brain pial microvessels of animals in the low protein (9%) group treated for 7 days with the angiotensin-converting enzyme inhibitor enalaprilat or with saline. Data are mean±SEM of 3 experiments. C, cGMP levels in isolated cerebral microvessels. Data are mean±SEM of 3 to 4 experiments. *P<0.01 compared with control. D, Representative immunoblot with relative densitometry of α-sGC in isolated cortical brain microvessels. Arrow points to α-sGC 97.3-kDa protein. Right panel represents compiled densitometry of the immunoblot relative to that of the 18% protein diet set at 100%. Values are mean±SEM of 3 experiments for each group. *P<0.05 compared with control. E, Vasorelaxant response to 8-bromo cGMP and 8-bromo PET cGMP on brain pial microvessels of 9- to 12-week-old rats in low protein and control groups. Experimental preparations are described in Figure 2. Data are mean±SEM of 5 to 6 experiments.
low protein diet group. Since sGC is primarily located in the smooth muscle of vasculature, our observations disclose an uncommon feature in the pathogenesis of hypertension, in this case associated with a low protein diet during fetal development involving primarily a smooth muscle rather than endothelial functional defect. Of interest, decreased sGC expression and cGMP production as a mechanism for impaired vasodilation have been described in the black population\(^{31}\) (susceptible to hypertension), after subarachnoid hemorrhage,\(^{32}\) and in pulmonary hypertension.\(^{33}\) Similarly, decreased vasodilator potency of SNP on sGC activity\(^{34}\) and downregulation of components of the sGC-dependent pathway\(^{35}\) have been demonstrated in aged and spontaneously hypertensive rats. The underlying mechanisms and regulatory processes that govern sGC levels and function are not well known but may involve cAMP, phosphodiesterases,\(^{32}\) nerve growth factor, and cytokines,\(^{36}\) all of which may participate in atherosclerosis and vascular dysfunction.

Although NO-evoked relaxation of pial arteries has been documented to be independent of \(K_{Ca}\) in newborn pig,\(^{37}\) others have reported \(K_{Ca}\) to mediate a significant portion of NO-evoked relaxation,\(^{20,21}\) including NO-induced vasodilation of rat cerebral arteries independent of cGMP.\(^{38}\) Our findings support the latter inference, such that abolition of NO-elicited relaxation required inhibition of both \(K_{Ca}\) and GC. On the other hand, one would have anticipated a relatively increased contribution of \(K_{Ca}\) in the low protein group,\(^{22}\) but this was not the case. Indeed, acute inhibition of either GC or \(K_{Ca}\) pathways yielded only a small diminution of NO-evoked relaxation, suggesting compensation by the alternate pathway. However, since \(K_{Ca}\) expression and vasorelaxant activity were unaltered by diet, it may be possible that its endogenous activity is diminished or that the activity of other related \(K_{Ca}\) channels is unaltered or augmented, as reported in certain forms of hypertension.\(^{39}\)

In conclusion, our findings demonstrate impaired NO-mediated vasodilation of major resistance cerebral microvessels in a model of fetal programming of hypertension by protein deprivation and reveal that this involves an uncommonly described mechanism, specifically reduced sGC.
expression and cGMP levels with normal NOS expression and function. This impaired NO-dependent vasodilatation, which is unrelated to NO production and which seems to involve smooth muscle cell dysfunction, is an uncommonly described mechanism associated with chronic hypertension and may provide a pathophysiological explanation for relaxation dysfunction in subjects previously exposed in utero to nutrient deficiency.

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