Microvascular rarefaction and decreased angiogenesis in rats with fetal programming of hypertension associated with exposure to a low protein diet in utero

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Running title: Microvascular rarefaction in programmed hypertension

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Acknowledgments and Funding

This work was supported by grants from the Canadian Institutes of Health Research (AMN, MB), the Hospital for Sick Children Foundation (PH and AMN) and the Heart and Stroke Foundation of Canada (AMN). Patrick Pladys was supported by an Institut National de Santé et Recherche Médicale (France) and the Canadian Institutes of Health Research (INSERM-CIHR) research fellowship and by the Conseil Régional de Bretagne. Anne Monique Nuyt and Pierre Hardy are recipients of fellowship from the Fonds de la Recherche en Santé du Québec. Sonia Brault is recipient of a training award from the CIHR, Daniella Checchin from the Foundation Fighting for Blindness and Karine Bibeau from the Natural...
Sciences and Engineering Research Council of Canada. Sylvain Chemtob is a recipient of a Canadian Institutes of Health Research Scientist award and a Canada Research Chair (perinatology).
Microvascular rarefaction and decreased angiogenesis in rats with fetal programming of hypertension associated with exposure to a low protein diet during gestation
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ABSTRACT

In hypertension, increased peripheral vascular resistance results from vascular dysfunction with or without structural changes (vessel wall remodeling and/or microvascular rarefaction). Humans with lower birth weight exhibit evidences of vascular dysfunction. The current studies were undertaken to investigate whether in utero programming of hypertension is associated with in vivo altered response to vasoactive agents and/or abnormal vascular structure.

Offspring of Wistar dams fed a normal (CTRL) or low (LP) protein isocaloric diet during gestation were studied. Mean arterial blood pressure (MABP) response to AngII was significantly increased and depressor response to sodium nitroprusside (SNP) infusions significantly decreased in male LP adult offspring relative to CTRL; response to phenylephrine infusion was not different between groups. No arterial remodeling was observed in male LP compared to CTRL offspring. Capillary and arteriolar density was significantly decreased in striated muscles from LP offspring at 7 and 28 days of life, but was not different in late fetal life (day 21 of gestation (E21)). Angiogenic potential of aortic rings from LP newborn (day of birth, P0) was significantly decreased. Striated muscle expressions (Western blots) of AngII AT1 receptor subtype, endothelial nitric oxide synthase, angiopoietin 1 and 2, Tie 2 receptor, vascular endothelial growth factor (VEGF) and receptor (VEGFR-2), and platelet derived growth factor C at E21 and P7 were unaltered by antenatal diet exposure.

In conclusion, blood pressure responses to AngII and to nitric oxide donor SNP are altered and microvascular structural changes prevail in this model of fetal programming of hypertension. The capillary rarefaction is absent in the fetus and appears in the neonatal period, in association with decreased angiogenic potential. The study suggests that intrauterine protein restriction increases susceptibility to postnatal factors resulting in microvascular rarefaction which could represent a primary event in the genesis of hypertension.
KEY WORDS:
Fetal programming, hypertension, microvascular rarefaction, angiotensin, nitric oxide
INTRODUCTION

Substantial evidence from epidemiological studies indicates that the risks of hypertension, stroke and coronary heart disease in later life may arise from early events occurring in the pre/perinatal period (5; 43). These observations have raised the hypothesis that the physiological or metabolic adaptations of the fetus to a period of deprivation result in permanent alteration in the programming of the developing cardiovascular structures or functions, inducing responses that produce dysfunction in later life. The concept of fetal programming of disease is supported by animal models, which have shown that the restriction of dietary protein or a global restriction in nutrients during pregnancy gives rise to offspring with elevated blood pressure (36; 52) and vascular dysfunction (11; 29; 35).

Studies of the offspring of dams fed a low protein (LP) diet during gestation suggest that the renin angiotensin system plays a key role in the development and the maintenance of the elevated blood pressure (37; 55). Blockade of angiotensin II (AngII) formation or of AngII AT1 receptor subtype during the first weeks of life prevents later elevation of blood pressure. This permanent effect is not observed when adult animals are treated (37; 59; 60). The activity of angiotensin converting enzyme, which generates AngII from its precursor angiotensin I, is elevated in LP offspring (although AngII levels are not consistently found increased) (37). Taken together, these findings could suggest increased sensitivity of LP offspring to circulating AngII.

In clinical and experimental hypertension, increased peripheral vascular resistance results from vascular dysfunction with or without structural changes (19; 34). Changes in vascular structure can comprise arterial remodeling and reduced density of arterioles and capillaries (termed “rarefaction”) (reviewed by (9)). Humans with lower birth weight exhibit vascular dysfunction as suggested by altered response to acetylcholine (51), decreased arterial distensibility (13; 44) and increased risk of atherosclerosis (44). Impaired vasorelaxant response to nitric oxide (NO) donor, acetylcholine and bradykinin has been found ex vivo in rats with hypertension associated with in utero exposure to protein restriction (11; 35) or to 50% global undernutrition (21). Programmed hypertension has also been associated with increased pressor response to AngII in anesthetized female LP offspring (46). Whether in utero programming of hypertension is associated with in vivo altered response to vasoactive agents in non anesthetized LP rat offspring and with abnormal vascular structure is unknown.

To address this question blood pressure response to NO donor, phenylephrine and AngII were studied in adult male rats with elevated blood pressure associated with exposure to a low protein diet during intrauterine life. Carotid and mesenteric arteries and renal
arterioles from adult offspring were examined for vessel wall remodeling. Microvascular density was studied in peripheral muscle, kidney and brain during development. As our results reveal microvascular rarefaction in LP exposed offspring, we also examined whether angiogenic potential was altered in this model of fetal programming of hypertension.
METHODS

Animals

Animals were used according to a protocol approved by the Animal Care Committee of Sainte-Justine Hospital in accordance with the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. Virgin Wistar rats (initial weight 225-250g) were mated overnight and on the day of conception (determined by the presence of a vaginal plug), were allocated to feed ad libidum on a diet containing either 18% (control group: CTRL, n = 13) or 9% (low protein group: LP, n = 15) casein diet (37). All diets contained 5g/kg methionine to avoid sulphur deficiency and were made isocaloric with starch and sucrose supplement. The dams were weighed on the first day of gestation and weekly thereafter. Three dams in each diet group were anesthetized with intraperitoneal ketamine (65 mg/kg) and xylazine (7 mg/kg) at 21 days of gestations (E21, term is 22 days) and the fetal tissues harvested (brain and tibialis anterior muscles for microvessel density studies, see below). Within 12 h of delivery, the other dams were returned to regular rat chow. On the first day of life (P0), 6 pups per diet group (one pup per litter) were sacrificed and the aortas harvested (for angiogenesis studies). At P7, 10 pups per diet group were sacrificed and tibialis anterior muscles harvested (one pup per litter, n = 6 were used for microvessel density and n = 4 for Western blots, see below). At P28, 6 pups per diet group (one pup per litter) were sacrificed and tibialis anterior muscles harvested (for microvessel density). At 9-12 weeks of age, in vivo blood pressure studies were performed in 8 LP and 6 CTRL offspring (one per litter per diet group); a second group of adult offspring (12 LP and 10 CTRL, one per litter per diet group) were sacrificed for tissue sampling (kidneys in 12 LP and 10 CTRL; carotid and mesenteric arteries, brain and tibialis anterior muscles in n = 6 each group). A third group of adult offspring (n = 13 each group, at least one per litter) were sacrificed by decapitation and the first drops of blood obtained for plasma renin activity measurements. At P28 and 9-12 weeks of age, only male offspring were studied; sex could not be determined on examination at earlier study time points.

Net weight gain during pregnancy, litter size, birth weight and survival rate of the offspring during the 12 weeks of the study period did not differ between groups, similarly to results previously reported (35). Pups were weaned at 4 weeks of age to regular rat chow.

Experimental procedures

In vivo blood pressure studies

Surgical Preparation: Male offspring (9-12 weeks old) were anesthetized as above. Under sterile conditions, polyethylene catheters (PE50, Plastics Ones Inc, VA) were inserted
into a femoral artery and vein, tunneled subcutaneously to the back of the neck, threaded through a flexible metal spring and connected to a dual-channel swivel mounted directly above the cage (Lomir Biochemical Inc, QC). The spring was anchored onto a fabric jacket that was adjusted to the rat front legs and thorax. This set up allowed the rat freedom of movement within the cage. Each rat was given a dose of intravenous Cefazolin (25 mg/kg) and allowed to recover for 24 h before experiments were performed. We have previously verified that the blood pressures obtained are not different whether animals are studied after a 24 h vs. four days recovery period (55).

On the day of the experiment, the rats were brought in their cage to the laboratory and allowed to adapt for one hour. During each experiment, mean arterial blood pressure (MABP) was monitored continuously using a pressure transducer (The Perceptor, Namic, Glens Falls, NY) aligned to the level of the heart and a Grass recorder (Astro-Med Inc, West Warwick, RI). The signal was displayed and simultaneously recorded on a computer via a Grass PVA-1A 8-channel analog-to-digital conversion board using the software Polyview (version 2.3, Astro-Med).

In LP and CTRL offspring, resting MABP was recorded for a period of 15 min before, and 20 min after blockade of endogenous AngII formation by the angiotensin converting enzyme inhibitor enalaprilat (150 µg/kg iv)(61). MABP responses to continuous intravenous infusions of AngII (10, 20 and 40 µg/kg/min), phenylephrine (20 and 40 µg/kg/min) or NO donor sodium nitroprusside (SNP; 10, 20 and 40 µg/kg/min) were then determined. Each infusion rate was maintained until MABP was stabilized for at least 1min. A 30-min recovery period was allowed for MABP to return to resting values before the alternative drug was administered.

Studies of vascular structure

Tissue collection

Arterial remodeling was studied in carotid and mesenteric arteries from LP and CTRL adult offspring (9-12 week old). Under general anesthesia, the mesenteric vascular bed was infused at a constant pressure of 100 mmHg with saline and frozen in situ with powdered dry ice. Branch II of the mesenteric arteries were then removed and stored at −80°C. Carotid arteries were also collected, snap frozen in liquid nitrogen and stored at −80°C.

Morphological measurements of microvessel density were performed in E21, P7, P28 and adult (9-12 week old) CTRL and LP rat offspring. The tissues studied included the tibialis anterior muscle (E21, P7 and P28) (method adapted from (15)), kidney (adult) (method
adapted from (27)) and brain (E21 and adult) (method adapted from (7)). Animals were sacrificed by decapitation; the tissues were quickly removed, frozen in powdered dry ice and stored at –80°C.

Tissue sections (8 µm carotid and mesenteric arteries, transversal 12 µm for muscles, coronal 20 µm for brain, 8 µm transhilus longitudinal for kidneys) were cut on a cryostat (Microm Cryostat, Germany) at –25°C, thaw-mounted on microscope slides (Superfrost, VWR Scientific, PA) and fixed in ½ 70% ethanol-½ acetone for 10 min. Vessel sections were stained with hematoxyline eosin. Muscle, brain and kidney sections were pre-incubated for 20 min at room temperature with PBS Triton1%, then overnight in the same buffer containing 1/100 BS-1 lectin (Griffonia Simplicifolia lectin; Sigma Chemicals) or anti-rat CD31 (Serotec) both for endothelial staining, and 1/100 anti-smooth muscle actin monoclonal (Sigma); the antibodies were TRITC or FITC labeled for immunofluorescence. The tissue sections were subsequently transferred through three 10-min washes in 4°C PBS then mounted on microscope slides with aqueous mounting gel (Biomeda, Foster city, CA).

**Analysis of vascular structure**

**Mesenteric and carotid arteries**: The internal diameter, wall thickness and media to lumen ratio were calculated using a standardized method (49).

**Microvasculature**: The images of the microvasculature of tissue sections obtained from an epifluorescence microscope (Nikon E800) were analyzed using a computerized image-analysis system (Image-Pro plus, Media Cybernetics). **Muscle**: The density of capillaries and small arterioles were studied on sections obtained from the mid belly of each tibialis anterior muscle. A square counting frame (area 0.3 mm²) was placed over the image (total magnification x 100). Within each muscle section, analyses were performed on four specific areas (two in the cortex and two in the core region of the muscle). The mean fiber cross sectional area was calculated for each area studied and the capillary density expressed in number of capillaries/mm² and in number of capillaries/muscle fiber. **Kidney**: The number and the size of glomerular capillaries (stained by anti-CD31) were measured on at least six glomeruli per animal studied. In order to avoid analysis of tangential section of the glomeruli, only glomeruli with sizes within one standard deviation of the average glomeruli area located underneath the cortex cortices (calculated from 20 glomeruli area measurements per kidney section) and with a clearly identifiable afferent arteriole (stained by anti-smooth muscle actin) were included in the analysis. The internal diameter, media thickness and media to lumen ratio were measured on six afferent arterioles per animal studied when these arterioles were clearly
associated with a glomerulus and having patent lumen (28). The number of glomeruli expressing AngII AT_1 receptor subtype per kidney trans-hilus longitudinal section were also examined using quantitative in vitro angiotensin receptor autoradiography with 125I-[Sar1, Ile8]AngII receptor binding as described (66). Brain: In newborn rats, the length and the density of the cerebral blood vessels were measured in each animal in four areas (0.3 mm^2 each) located in the forebrain (cortex and caudate putamen) and in the midbrain (periaqueductal grey matter and hypothalamus). In adult animals measurements were performed in five areas of 0.5 mm^2: two in the cortex (close to the plane of symmetry and lateral), two in the corresponding subcortical areas and one in the caudate putamen. The subcortical area was not studied in newborn because it is not sufficiently well delimited at that age.

**Evaluation of nephron number**

Method adapted from (40). Kidneys (adults, n = 5 per group, all different litters) were fixed in formalin, embedded in paraffin wax and cut transversally (coronal) in 5 pieces. From each piece, 6 to 9 sections (4 μm) were consecutively collected and stained with hematoxylin-phloxin-saffron. For each section, total surface area and total number of nephrons (identified by their glomeruli) were counted. Values from all sections were averaged for each kidney piece; nephron count and surface area varied less than 5% from one section to another within each piece. Results from the 5 kidney pieces were added to obtain a total surface area and a total number of nephron counted per kidney studied.

**Angiogenesis**

To examine whether angiogenic potential was altered in LP offspring, the microvascular network arising from aortic rings cultured in three-dimensional matrix gel was studied (8). CTRL and LP groups were simultaneously studied under the same experimental conditions. Aortas were removed from P0 offspring (average of 18 hours of life) and immediately transferred to a culture dish containing ice-cold serum-free EGM-EBM2 media for endothelial culture (Clonetics). The peri-aortic fibroadipose tissue was carefully removed without damaging the aortic wall. One millimeter long aortic segments (approximately 6 per aorta) were cut and rinsed in 3 consecutive washes of EGM-EBM2. The aortic rings were then individually embedded in 0.35 ml of basement membrane matrix gel containing extracellular matrix proteins and growth factors (Matrigel™, BD Biosciences) in a 24-well tissue culture plate (Becton Dickinson), and each well was covered with 0.5 ml of EGM-EBM2 medium. The plates were kept at 37°C in a humidified environment and examined on day 2 and 4 with an inverted microscope (Nikon); all images were digitized under the same
conditions (light, contrast, magnification). The quantification of angiogenesis was performed using image-analysis software (Image-Pro plus, Media Cybernetics). On each image, an orthogonal 75 µm grid was applied. The aortic ring and neovessel formation areas were then determined by tracing polygonal figures. The external limit of the neovessel formation area was determined by linking the outmost vessels, with a limit of one neovessel (or point) per 75x75 µm square. The total area covered by the neovessel formation (minus the area of the aortic ring per se), its average width and the number of microvessels observed at the external limit of the neovessel formation area were calculated.

**Western blotting**

Western blot analysis of the expression of factors that can modulate angiogenesis was performed on muscles of E21 (LP: n = 4 from 3 litters and CTRL: n = 5 animals from 3 litters, tibialis anterior muscles from both limbs were combined for each animal) and P7 (n = 4 animals each group) offspring from both diet groups. Protein extraction of cells was performed as previously described (58). For each experiment, tissues were pooled and blots were performed on equal amounts of total crude extract of protein. The antibodies used on the muscle were the polyclonal anti-vascular endothelial growth factor (VEGF) (Chemicon International), polyclonal anti-VEGF receptor (VEGF-R2) (Santa Cruz Biotechnology), polyclonal anti-angiopoietin 1 (id), polyclonal anti-angiopoietin 2 (id), polyclonal anti-platelet derived growth factor C (PDGF-C) (id), polyclonal anti-tie2 angiopoietin receptor (id), polyclonal anti-AngII AT1 receptor subtype (id) and monoclonal anti-endothelial NO synthase (BD Biosciences Pharmingen). Polyclonal anti-GAPDH (Santa Cruz Biotechnology) was used as internal control.

**Plasma renin activity**

Plasma renin activity was measured indirectly by radio immuno assay of the angiotensin I generated during a 2-h incubation period. The antibody used was purchased from Peninsula Laboratories (Belmont, CA) (25).

**Drugs and chemicals**

The following agents were purchased: sodium nitroprusside, phosphate buffer, EDTA, AngII, bacitracine and bovine serum albumin (Sigma Chemical, MO); 3-[125I]iodotyrosyl4 Sar1 Ile8 Angiotensin II (125I-[Sar1, Ile8]AngII, Amersham Biosciences); enalaprilat (Vasotec, Merck Frosst); Ketamine (Ayerst); Xylazine (Bayer); Cefazolin (Novopharm).

**Statistical analysis**

Values are expressed as mean ± SEM and compared with Student’s t-test or Mann-Whitney test as appropriate. Concentration response curves were compared by two way
repeated measures ANOVA and by Student’s t-test or Mann-Whitney test as appropriate. Significance was set at $p < 0.05$. 
RESULTS

Effect of antenatal diet exposure on in vivo vasomotor responses of adult male offspring

MABP of LP exposed animals was increased (125 ± 3 mmHg in LP vs. 109 ± 3 mmHg in CTRL, p < 0.05). Plasma renin activity was significantly increased in the LP group (0.61 ± 0.06 pmol angiotensin I conversion/ml/h in LP vs. 0.41 ± 0.06 in CTRL, n=13 each group). Systemic administration of enalaprilat significantly reduced MABP of LP without altering CTRL; there was no statistically significant difference in MABP between groups after enalaprilat (Figure 1). After blockade of endogenous AngII formation, the MABP pressor response to AngII was significantly increased and depressor response to SNP significantly decreased in LP rats relative to CTRL (Figure 1). In contrast, vasopressor response to phenylephrine infusion did not differ between diet groups (LP (n = 8) vs. CTRL (n = 6) at 20 µg/kg/min: 158 ± 9 vs. 159 ± 8 mmHg and at 40 µg/kg/min: 168 ± 7 vs. 171 ± 8 mmHg).

Effects of antenatal diet exposure on vascular structure

Arterial remodeling

Lumen diameter, media cross sectional area, media thickness and media to lumen ratio of the carotid and mesenteric arteries and of the renal afferent arterioles from adult offspring did not differ between groups (Table 1).

Microvasculature

Muscle: Within the tibialis anterior muscle, capillary density was significantly decreased in LP offspring at P7 and P28 but not at E21 (Figure 2 A, B and C). This postnatal vascular rarefaction remained significant when capillary density was expressed in number of capillaries per muscle fiber (E21: 0.15 ± 0.01 LP vs. 0.13 ± 0.003 CTRL; P28: 1.20 ± 0.06 LP vs. 1.39 ± 0.04 CTRL, p<0.05 at P28) or in capillary length density (E21: 8.8 ± 0.3 LP vs. 8.7 ± 0.1 mm/mm² CTRL; P7: 10.9 ± 0.7 LP vs. 13.8 ± 0.6 mm/mm² CTRL, p<0.05 at P7). At P28, density of the arterioles (12-50 µm) was also significantly decreased in LP offspring (7.9 ± 0.3 vs. 9.4 ± 0.9 arterioles/mm² in CTRL, p < 0.05).

Kidney: The adult offspring capillary density per glomeruli was unaltered by antenatal diet exposure (Table 2 and Figure 3). Renal weight (LP: 2.1 ± 0.3 g, n = 12 vs. CTRL 2.1 ± 0.2 g, n = 10), number and density of glomeruli expressing AT1 receptor, and the overall AT1 receptor binding density (per kidney section) also did not differ between diet groups. In 5 adults per diet group, evaluation of nephron number was also performed: the kidney to body weight ratio (LP: 0.34 ± 0.01 vs. CTRL: 0.34 ± 0.01 %), the total number of nephron counted per kidney (LP: 785 ± 24 vs. CTRL: 690 ± 50) and the total transversal surface area studied per kidney (LP: 343 ± 11 vs. CTRL: 326 ± 13 mm²) did not differ between groups. There was
no correlation between the glomerular area and the glomerular capillary density within each group.

**Brain:** The number of capillaries per mm$^2$ in the cortex was not different between groups at E21 and in the adults; however, the capillary density (in mm/mm$^2$) and accordingly the mean capillary length were lower in the E21 LP offspring ($4.0 \pm 0.7$ mm/mm$^2$ in LP vs. $5.5 \pm 0.4$ mm/mm$^2$ in CTRL, and $36 \pm 9$ μm in LP vs. $56 \pm 10$ μm in CTRL, both $p < 0.05$, $n = 6$ each group), but were similar in the adult offspring ($n = 6$ each group). The capillary density (mm/mm$^2$, n/mm$^2$) and length in the other brain areas studied (caudate putamen, hypothalamus and periaqueductal grey matter at E21; forebrain sub cortical area and caudate putamen in the adult) were unaffected by antenatal diet exposure.

**Angiogenesis**

Neonatal aortic rings embedded in matrigel gave rise to microvessels in both groups. The first microvascular sprouts were visible within 48 h of culture. On day 4, the neovessel formation area, the average microvessel length and the number of microvessels at the external limit of the neovessel formation area were all significantly lower in the LP group (Figure 4).

**Western blotting (tibialis anterior muscle) at E21 and P7**

Protein expression of AngII AT$_1$ receptor subtype, endothelial NO synthase, angiopoietin 1 and 2, Tie 2 receptor, VEGF, VEGFR-2 and PDGF-C at E21 ($n = 5$ and 4 for LP and CTRL respectively) and at P7 ($n = 4$ each group) were found unaltered by antenatal diet exposure (data not shown).
DISCUSSION

Our results demonstrate that the elevated blood pressure of adult rat after intrauterine protein restriction is associated with altered blood pressure response to AngII and NO donor and with microvascular rarefaction. The current studies also reveal that microvascular rarefaction is not present during fetal life and appears early during the neonatal period, in association with decreased angiogenesis.

Hypertension in offspring of LP fed dams is seen equally in male and female (39; 64). However the mechanisms underlying the increased blood pressure are probably sex-specific (47). McMullen et al. have shown that LP male but not female hypertension at 4 weeks of age is glucocorticoid mediated. Renal expression of AngII receptors in LP offspring also differ according to gender (see below and (47)). We have restricted these studies to male offspring at P28 and in the adults; therefore the results of those age groups cannot be extended to female offspring. However, sex could not be determined at E21 and P7 and one can therefore suppose that the microvascular rarefaction observed applies to both gender.

In vivo response to vasoactive agents

The findings of elevated plasma renin activity and the significant lowering effect of angiotensin converting enzyme inhibitor on resting blood pressure in LP offspring are in agreement with previously published reports (37; 60). In addition, we demonstrate that increased circulating renin is not the sole element involved in the role of RAS in maintaining programmed hypertension. After blockade of endogenous AngII formation, we found that pressor responses to AngII infusion are enhanced in male LP offspring, as previously shown in anesthetized female LP offspring (22; 46). In the current studies, this response seems specific to AngII as blood pressures achieved with phenylephrine infusion were unaffected by antenatal diet exposure.

Circulating AngII can increase blood pressure through central and peripheral (vascular) effects. We have previously shown that male LP offspring have increased expression of AT1 receptors in brain cardiovascular regulating areas (55); these receptors can increase efferent sympathetic activity and arterial blood pressure when activated. Peripherally, preliminary studies have shown that vascular reactivity to AngII was increased in adult LP offspring (54). Vascular response to AngII can be enhanced by an increase in the expression of AngII AT1 receptor subtype, through a decrease in the expression of AngII AT2 receptor subtype which favors vasodilatation or through changes in AngII-mediated signal transduction at or beyond the level of the cell membrane receptor as it has been described in other forms of chronic hypertension (62).
The exact role of RAS in the programming of hypertension is still debated and can be considered as a causal factor (37; 60) or as an early associated event (16; 17). Blockade of AngII formation or losartan administration (AT₁ receptor antagonist) during the first weeks of life in LP offspring prevents later elevation of blood pressure (60). Studies in fetal sheep indicate that the role of the renin angiotensin system in maintaining blood pressure and in the pressor response to AngII infusion is greater in growth restricted than in control animals (16; 17). However, Woods et al showed reduced renin and AngII levels in the kidneys of P0 LP offspring (65).

The impaired hypotensive response to NO-donor SNP in rats with in utero programmed hypertension is in agreement with previous reports by us and others showing ex vivo decreased vasorelaxation to NO-dependent mechanisms in LP offspring (11; 35) due to reduced activity of the guanylate cyclase pathway (35).

**Vascular structural changes**

Analysis of the current data reveal that while blood pressure responses to AngII and SNP infusion were significantly different between study groups, the difference remained relatively constant and was not attenuated even at high doses. These observations suggest that in addition to altered vasoreactivity, vascular structural changes prevail. We did not observe significant remodeling in carotids, renal afferent arterioles and mesenteric arteries which are among the vessels where remodeling associated with chronic hypertension is mostly seen (49). These results are supported by a report of unchanged internal diameter of mesenteric arteries in a similar model of programmed hypertension (11). Nonetheless, absence of arterial remodeling is unusual in hypertension (31; 49). The difference in MABP we and others observe between the LP offspring and CTRL animals (37)–which is not as major what is encountered in spontaneously hypertensive rats for example- could explain this finding since comparison between different models of experimental hypertension show a positive correlation between media to lumen ratio and blood pressure values (49). One can also hypothesize that arterial remodeling is not yet apparent at 9-12 weeks of age.

The current studies demonstrate microvascular rarefaction in a major site of peripheral resistance (i.e. the striated muscle) in 7 and 28 day old, but not in late fetal rats with programmed hypertension, suggesting an early postnatal disruption in normal microvessel development. Rarefaction is an important common characteristic of various microvascular beds in hypertension in both human and animal models and is generally considered a consequence rather than a cause of the increased blood pressure (42). However it can be postulated from our data that microvascular rarefaction is a primary event in the development
of programmed hypertension (42). Supporting this are reports of microvessel rarefaction at early stages or prior to the development of hypertension in high risk individuals (2; 3; 50). Whether blood pressure is elevated in newborn LP offspring is unknown. Evaluation of blood pressure and vasoreactivity in newborn rat has not been reported but studies of newborn of larger animals and humans demonstrate that blood pressure at birth correlates mainly to actual birth weight, independently of adverse intra uterine environment conditions (1). Therefore the increase in arterial blood pressure (resulting in major part from activation of RAS) could be an adaptation mechanism allowing sufficient capillary recruitment to maintain adequate oxygen delivery to the peripheral tissues (24).

Microvascular rarefaction can result from reduced formation (impaired angiogenesis) or active disappearance. Response to angiogenic stimuli was altered in newborn LP aortic rings whereas expression of key factors modulating angiogenesis was not different between diet groups at E21 and P7. This suggests that antenatal diet exposure modifies factors other than those examined or increases susceptibility to postnatal elements leading to impaired growth or vessel degeneration (12). Disappearance of microvessels can result from vasoobliteration secondary to endothelial cell death. Reduced NO bioactivity and increased renin angiotensin system activity associated with this model as well as an increased oxidative stress (20; 21) in early life could be postulated to impact on microvessels formation and survival (6; 18; 32). Vascular endothelial cells are particularly susceptible to free radical-induced peroxidation injury and death (10; 33). In addition, peroxidation product isoprostane causes greater microvessel vasoconstriction in young than in adult animals (30). Vasoconstriction and endothelial cell damage lead to degenerative processes in terminal arterioles and capillaries inducing anatomical rarefaction (26; 63). Reactive oxygen species could also contribute to rarefaction through impaired angiogenesis. Indeed, VEGF and angiopoietin-1 require NO to mediate their effect (4). Reduced NO bioavailability by reactive oxygen species scavenging could reduce microvascular development.

The role of AngII in primary microvascular rarefaction is controversial. AngII can have proangiogenic and mitotic effect in adult through AT1 receptors (18). On the other hand, AT2 receptors -which inhibit angiogenesis (6)- markedly predominate in peripheral tissues at birth (23). Excess reactive oxygen species production (including by AngII) could also counterbalance the proangiogenic effect of AngII-AT1. Therefore one can speculate that tissue specific excess AngII in early development contributes to local microvascular rarefaction.

In contrast with the postnatal microvessel rarefaction found in skeletal muscle, a decreased density in cortical brain microvasculature was observed only in late fetal LP
offspring. Decreased cortical capillary density has been described in 3 day old LP offspring (7) and in 30 day old pups underfed after birth (53). Our results confirm the negative impact of antenatal maternal protein restriction on brain angiogenesis and are the first to report that this rarefaction excludes midbrain microvasculature. The fact that microvascular density normalizes by adulthood is in agreement with data reported by Conradi et al. (14), but not Bennis-Taleb (7); the latter report differed in that female offspring were studied, using a photographic emulsion perfusion method to identify the capillaries.

Glomeruli arteriolar structure and capillary density were not significantly modified by antenatal protein restriction. We also did not find a difference in kidney weight, kidney to body weight ratio, nephron density and in the number of glomeruli expressing AT₁ receptor. Although the method used does not evaluate the total nephron count, it was reported to reliably reflect it (40). Decrease in total glomeruli number and glomeruli hypertrophy has been reported in association with “programmed” hypertension by most (45; 48; 56; 65) but not by all studies (38). Indeed, feeding a LP diet in the first week only of gestation resulted in elevated blood pressure in the offspring even though nephron count was unchanged compared to control (40; 41). The current findings support the hypothesis that alteration in renal development might not play the sole etiologic role in programming hypertension. AngII receptor expression has been studied in the kidneys at 4 weeks: renal AT₂ mRNA expression is decreased of female but not male LP offspring whereas AT₁ mRNA expression was found unchanged (46). However, renal AT₁ protein expression studied by Western and binding was found increased in 4 week old male LP offspring (56; 57). Therefore from the current results one could postulate that the total number of glomeruli is indeed decreased (and not evidenced by our counting method) but that the proportion expressing AT₁ is increased in LP offspring. Alternatively, as it has been suggested that glomeruli could be relatively immature in LP kidneys, differing levels of maturity could account for altered proportion of glomeruli expressing AT₁ (57); however as renal renin angiotensin system has been reported to be no longer suppressed by 4 weeks of age (57) and as our studies were performed in 9-12 weeks LP offspring, we believe this is improbable.

In conclusion, our findings demonstrate alteration in blood pressure response to AngII and to NO donor, and microvascular structural changes at a major site of peripheral resistance in a model of fetal programming of hypertension associated with antenatal exposure to a low protein diet. More importantly, our results reveal that striated muscle capillary rarefaction is absent in the fetus and appears in the neonatal period. As we demonstrate that angiogenesis is decreased in LP exposed newborn rats, we postulate that intra uterine protein restriction
increases susceptibility to postnatal elements resulting in decreased angiogenesis and that this microvascular rarefaction represents a primary event in the genesis of hypertension.
Table 1. Effect of antenatal diet exposure on adult arterial structure (n = number of animals studied per group).

<table>
<thead>
<tr>
<th>Artery</th>
<th>CTRL (n = 6)</th>
<th>LP (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carotid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen diameter (µm)</td>
<td>885 ± 11</td>
<td>815 ± 34</td>
</tr>
<tr>
<td>Media thickness (µm)</td>
<td>55.2 ± 3.5</td>
<td>53.9 ± 2.8</td>
</tr>
<tr>
<td>Media/lumen ratio (%)</td>
<td>6.1 ± 0.3</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Cross-sectional area (X 1000 µm²)</td>
<td>79.7 ± 6.2</td>
<td>72 ± 6.6</td>
</tr>
<tr>
<td><strong>Mesenteric artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen diameter (µm)</td>
<td>242 ± 9</td>
<td>288 ± 9</td>
</tr>
<tr>
<td>Media thickness (µm)</td>
<td>24.9 ± 1</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>Media/lumen ratio (%)</td>
<td>10.1 ± 0.6</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>Cross-sectional area (X 1000 µm²)</td>
<td>10.1 ± 0.4</td>
<td>12.4 ± 0.8</td>
</tr>
<tr>
<td><strong>Renal afferent arteriole</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen diameter (µm)</td>
<td>10.9 ± 0.79</td>
<td>10.3 ± 0.71</td>
</tr>
<tr>
<td>Media thickness (µm)</td>
<td>1.3 ± 0.1</td>
<td>1.24 ± 0.1</td>
</tr>
<tr>
<td>Media/lumen ratio (%)</td>
<td>12.4 ± 0.7</td>
<td>12.4 ± 0.6</td>
</tr>
</tbody>
</table>
Table 2. Effect of antenatal diet exposure on adult renal glomeruli and capillary structure (n = number of animals studied per group)

<table>
<thead>
<tr>
<th></th>
<th>CTRL (n = 10)</th>
<th>LP (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of capillaries per glomeruli section</td>
<td>89 ± 6</td>
<td>86 ± 8</td>
</tr>
<tr>
<td>Capillary diameter (µm)</td>
<td>4.2 ± 0.1</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Capillary density (/mm² of glomeruli area)</td>
<td>6436 ± 361</td>
<td>5627 ± 654</td>
</tr>
<tr>
<td>Mean glomeruli area (µm²)</td>
<td>14155 ± 1032</td>
<td>15900 ± 1167</td>
</tr>
<tr>
<td>Number of glomeruli expressing AT₁ / mm² of cortical area</td>
<td>5.31 ± 0.21</td>
<td>5.26 ± 0.43</td>
</tr>
<tr>
<td>Number of glomeruli expressing AT₁ / kidney section</td>
<td>297 ± 9</td>
<td>276 ± 7</td>
</tr>
</tbody>
</table>


Figure legends

Figure 1. MABP response to blockade of endogenous angiotensin II (AngII) formation with angiotensin converting enzyme inhibitor enalaprilat (150 µg/kg iv), followed by continuous intravenous infusions of AngII (10, 20 and 40 µg/kg/min) or of NO donor sodium nitroprusside (SNP; 10, 20 and 40 µg/kg/min). * = p < 0.05 vs. CTRL at the same dose.

Figure 2. A and B. Representative illustration of the capillary supply in the tibialis anterior muscle of 28 day old (P28) offspring of dams fed a control (CTRL, A) vs. low protein (LP, B) diet during gestation. Capillary endothelial cells were stained with a fluorescent anti rat CD31 antibody (see Methods section). Bar scale = 100 µm. C. Compiled capillary density (/mm²) in tibialis anterior muscle of 21 day fetuses (E21, term is 22 days), 7 day old newborn (P7) and P28 LP and CTRL offspring. * = p < 0.05 vs. CTRL.

Figure 3. A. Representative autoradiographic distribution of 125I-[Sar¹, Ile⁸]AngII binding sites (glomeruli, arrows) in a kidney trans-hilus longitudinal section. B. Representative illustration of capillary (arrows) density in glomeruli. Capillary endothelial cells and afferent arteriole (arrow head) were stained with fluorescent anti rat CD31 and anti rat smooth muscle actin antibodies respectively (see Methods section). Bar scale = 25 µm.

Figure 4. A and B. Representative illustrations of neovessels arising from aortic rings after 4 days of culture in three-dimensional matrix gel. Rings were obtained from newborn (day of birth) offspring of dams fed a control (CTRL, A) or low protein (LP, B) diet. Bar scale = 200 µm. C. Compiled analysis of the angiogenic potential of aortic rings from CTRL and LP newborns (n = 6 each group), cultured simultaneously under the same experimental conditions. Four rings per animal were studied. The total area covered by the neovessel formation (minus the area of the aortic ring per se and expressed relatively to the perimeter of the aortic ring), its average width and the number of microvessels observed at the external limit of the neovessel formation area (expressed relatively to the perimeter of the aortic ring) were calculated. * = p < 0.05 vs. CTRL.
Figure 1

LP: low protein diet exposed (9% casein), n = 8
CTRL: control (18% casein), n = 6
*: p< 0.05 vs ctrl in the same condition
Figure 2

A

B

C

Capillary density (cell/mm²)

E21  P7  P28

Control  Low protein

*
Figure 4

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>area of neovessel formation (mm$^2$/mm)</td>
<td>0.45±0.03</td>
<td>0.35±0.02 *</td>
</tr>
<tr>
<td>average width (µm)</td>
<td>300±13</td>
<td>246±9 *</td>
</tr>
<tr>
<td>number of microvessels at the external limit</td>
<td>62±2</td>
<td>56±2 *</td>
</tr>
</tbody>
</table>