Exaggerated vasomotor response to angiotensin II in rats with fetal programming of hypertension associated with exposure to a low protein diet during gestation

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Running title: Vasomotor response to AngII in programmed hypertension

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ABSTRACT:

The renin angiotensin system plays a key role in the initiation and maintenance of elevated blood pressure associated with altered intra uterine milieu. The current studies were undertaken to verify whether vascular response to AngII is increased in adult offspring of low protein fed dams (LP) compared to control (CTRL) and if so, to examine underlying mechanism(s).

AngII-induced contraction of carotid rings was increased in LP (Emax relative to maximal response to KCl 80 mM: 230 ± 3% LP vs. 201 ± 2% CTRL, p<0.05). In both groups, contraction to AngII was mediated solely by AT1R. Responses to thromboxane A2 analogue U46619 and to KCl 80 mM under step increases in tension were similar between groups. Endothelium depletion enhanced contraction to AngII in both groups, more so in LP. Blockade of endothelin formation had no effect on response to AngII and angiotensin 1-7 did not elicit vasomotor response in either group. Superoxide dismutase (SOD) analogue Tempol normalized LP without modifying CTRL response to AngII.

Basal levels of superoxide (aortic segments, lucigenin-enhanced chemiluminescence and fluorescent dye hydroethidine) were higher in LP. AngII further increased superoxide production in LP only and this was inhibited by co-incubation with diphenylene iodonium or apocynin (inhibitor of NADPH oxidase complex). AT1R expression in carotid arteries was increased in LP whereas SOD expression was unchanged.

In conclusion, vasoconstriction to AngII is exaggerated in this model of developmental programming of hypertension, secondary to enhanced vascular production of superoxide anion by NADPH oxidase with concomitant increase of AT1R expression.

KEY WORDS:
Fetal programming, hypertension, oxidative stress, angiotensin, isolated vessels
INTRODUCTION:

Epidemiological studies have revealed that the risk of cardiovascular diseases such as hypertension, stroke and coronary heart disease in later life seem inversely related to birth weight (23) and that this relation is independent of genetic factors and lifestyle (1; 3). These observations led to the postulate that perturbations (of nutrition for example) at a critical period of early development could lead to permanent alterations in the programming of the developing cardiovascular structures or functions (2). Animal studies support the concept of developmental programming of hypertension (17; 19; 25; 46).

The renin-angiotensin system (RAS) has been shown to play a key role in the initiation and maintenance of elevated blood pressure associated with altered intra uterine milieu. 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 (41; 42). We and others have demonstrated that in adult offspring of dams fed a low protein (LP) diet during gestation, plasma renin activity is increased, elevated blood pressure is normalized by angiotensin converting enzyme inhibitor (ACEi) and pressor response to infusion of AngII is increased (20; 26; 33; 34). Circulating AngII increases blood pressure through peripheral and central mechanisms. We have previously demonstrated increased expression of AngII AT1R in brain cardiovascular regulating areas of adult male LP offspring and normalization of their blood pressure with intracerebroventricular injection of the ACEi enalaprilat or of the AT1R antagonist Losartan (33).

The current studies were undertaken to verify whether peripheral vascular response to AngII is also increased in adult male LP offspring compared to control (CTRL) and to explore potential mechanisms underlying the exaggerated vasoconstriction to AngII. For this purpose, vasomotor responses of carotid arteries rings (organ chambers) were studied. Increased vasomotor response to AngII could be observed in the presence of vascular remodeling and/or increase in the expression of AngII AT1R subtype, decrease in the expression of AngII AT2R subtype which favors vasodilatation, or 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 (45). Also, increased vasoconstriction can be encountered in the presence of defective vasodilatation, such as decreased endothelium (nitric oxide)-mediated vasodilatation classically reported in many human cases or animal models of chronic hypertension. To explore
these potential mechanisms, the following studies were undertaken: As we and others reported unchanged histological measurements of arteries (media, lumen) from LP offspring (6; 34), we verified whether tensional force capacity was also unaffected by antenatal diet exposure. To sort the role of AT\(_1\)R vs. AT\(_2\)R in vasomotor response to AngII, specific antagonists were used; however, expression of AT\(_2\)R has not been reported in non lesioned carotid arteries. Activation of AT\(_1\)R can lead to enhanced formation of endothelin as well as superoxide, mostly through activation of NADPH oxidase, both of which can enhance vasoconstriction (40; 44). Alternatively, AngII can lead to vasodilatation through metabolite Ang1-7 and through stimulation of NO production (5; 9). The latter potential mechanisms were studied using specific blockers. Defective endothelium-mediated vasodilatation was studied in vessel rings denuded of their endothelium. As our results support our primary hypothesis and reveal that the exaggerated constriction to AngII is normalized by superoxide dismutase (SOD) analogue Tempol, the vascular production of superoxide anion was also evaluated.
MATERIALS AND 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 = 12) or 9% (low protein group: LP, n = 11) casein (20). All diets contained 5g/kg methionine to avoid sulphur deficiency and were made isocaloric with starch and sucrose supplement. All dams were weighed daily and had free access to food and water. Within 12 hours of delivery, dams were returned to regular chow. Pups were weaned at 4 weeks of age to regular chow and male were studied at 9-12 weeks of age. Unless specified otherwise, one animal per litter was used for the different studies.

Experimental procedures

Ex vivo vascular reactivity studies

Freshly excised carotid arteries from anesthetized (intraperitoneal ketamine (65 mg/kg) and xylazine (7 mg/kg)) offspring were placed in ice cold modified Krebs bicarbonate solution (KBS) of the following composition (in mM): NaCl 118, KCl 4.7, NaHCO₃ 25, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, and dextrose 11. They were cleaned of adherent connective tissue and precisely cut into rings of same length (4 mm). Four to eight rings from one rat were used for one experiment. The n presented with each Figure represent the number of animals studied. In a separate series of experiments, endothelium was removed in half the rings by gently rubbing the internal lumen with a blunt needle (20 G, Becla Dickinson). Rings were suspended horizontally between two stainless steel wires in organ chambers that contained 20 ml of KBS maintained at 37°C and aerated continuously with 95% oxygen and 5% carbon dioxide. The tension of the preparations was recorded with a linear force transducer on a computerized data acquisition system (Kent Scientific, Litchfield, CT). The rings were progressively stretched to a preload tension of 19.0 mN and allowed to equilibrate for 30 min with frequent washing and tension adjustments. After stabilization, rings were repeatedly exposed to KCl (80 mM) to test their viability and to determine a standard contractile response for each of them. When response to KCl was stable, endothelial integrity was then assessed in all experiments by a characteristic
relaxation response to Carbachol (100 µM) after contraction evoked by phenylephrine (1 µM). Rings were then allowed to recover for 60 min after which cumulative concentration-response curves were generated with AngII (1 pM to 1 µM) in presence and absence (half the rings tested each condition for each experiment) of PD 123319 (0.1 µM; AngII AT2R antagonist) or Losartan (1 µM; AngII AT1R antagonist), phosphoramidon (1 µM, neutral endopeptidase (NEP) 24.11 and endothelin converting enzyme inhibitor), Tempol (1 mM, SOD analogue). All drugs were added to the bath 30 minutes before cumulative concentration-response curves. Cumulative concentration-response curves were also generated with the thromboxane A2 mimetic U46619 (1 pM to 1 µM) and with angiotensin (1-7) (Ang(1-7), 1 pM to 1 µM). For the determination of Ang(1-7) vasorelaxant responses, rings were precontracted with U46619 (0.3 µM) added to the organ chamber 15 minutes before. For the experiments in which the endothelium had been mechanically removed, absence of vasorelaxation to Carbachol after normal vasoconstriction to KCl and phenylephrine was verified prior to the generation of the concentration-response curve to AngII. To verify whether antenatal diet exposure was associated with changes in tensional force capacity, we also studied vasoconstriction to KCl (80 mM) under different tension applied to the carotid arteries rings (48).

**Evaluation of vascular production of superoxide anion by chemiluminescence**

Vascular superoxide production was estimated using lucigenin-enhanced chemiluminescence, as described (4; 24). Briefly, in an additional set of experiments, aortas were removed from anesthetized rats and cut into segments. Aortic rings (5 mm) were placed in polypropylene tubes each containing 0.5 ml of Krebs and lucigenin (5 µM). Chemiluminescence counts (expressed in relative light units) were measured (LS 6500 Multi-purpose Scintillation Counter, Beckman Coulter) during 15 min (one reading/min) after preincubation for 30min with Krebs solution, Krebs solution + AngII (1 µM), Krebs solution + AngII (1 µM) + diphenylene iodonium (DPI) (100 µM, inhibitor of flavin containing enzyme) or Krebs solution + AngII (1 µM) + apocynin (1 mM, inhibitor of the assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex). Background counts (Krebs solution alone) were subtracted from the counts obtained with vascular rings. Dry weight was obtained for each ring for normalization of activity.

**Evaluation of vascular production of superoxide anion by hydroethidine**
Superoxide levels were also measured by the oxidative fluorescent dye hydroethidine, as described previously (27). Cells are permeable to hydroethidine and, in the presence of superoxide anion, hydroethidine is oxidized to fluorescent ethidium bromide, in which form it is trapped by intercalation with DNA. This method provides detection of superoxide anion levels intra and extra cellular. Briefly, unfixed frozen aorta segments were cut into 12 µm thick sections with a cryostat (Microm Cryostat, Germany) at −20 °C and thaw-mounted on microscope slides (Superfrost, VWR Scientific, PA). Hydroethidine (2 µM) was applied to each tissue section and coverslipped. Then, slides were incubated in a light-protected humidified chamber at 37 °C for 30 min. Images were obtained with a laser scanning confocal microscope (LSM 510 laser scanning microscope, Zeiss) equipped with a argon laser. Fluorescence was detected with a 514-nm long-pass filter. The LP and CTRL offspring were processed and imaged in parallel.

**Western blotting**

Western blot analysis of AngII AT1 receptor subtype and of SOD enzyme was performed on carotid arteries of 9-12 weeks old male (LP: n = 4 from 3 litters and CTRL: n = 5 animals from 3 litters). Frozen arterial tissues were disrupted using mortar and pestle in liquid nitrogen. Proteins were extracted with RIPA buffer supplemented with Nonidet P40 (1%), Na3VO4 (1 mM), NaF (1 mM), EDTA (1 mM) and 1x solution of cocktail protease inhibitors (Roche). Samples were sonicated, centrifuged and supernatant recovered for determination of protein content by Bradford assay using BSA as standard. For each experiment, tissues were pooled and blots were performed on equal amounts (30 µg) of total crude extract of protein. Polyclonal anti-actine (Santa Cruz Biotechnology) was used as internal control. Equal loading of proteins for SDS-PAGE was also verified by Ponceau staining. The antibodies used were the polyclonal anti-AngII AT1 receptor subtype (Santa Cruz Biotechnology) and polyclonal anti-Cu/Zn SOD (Calbiochem).

**Chemicals**

The following agents were purchased: ketamine (Ayerst, Montreal, QC); xylazine (Bayer); AngII, Ang(1-7), apocynin, phosphoramidon, Carbachol, phosphate buffer, EDTA, DPI, bovine serum albumin, PD123319 and U46619 (Sigma Chemical, St Louis, MO); Tempol (Fluka Chemika), Losartan was a gift of Merck Frosst Canada and Du Pont, Kirkland, QC.

**Statistical analysis**
Cumulative concentration-response curves were analyzed by computer fitting to a four-parameter sigmoid curve using the Prism 3 program (GraphPad, San Diego, CA) to evaluate the EC$_{50}$ and Emax, the maximum asymptote of the curve. For the comparison of superoxide levels detected by chemiluminescence under control conditions versus in the presence of AngII +/- DPI or apocynin, analysis was performed using ANOVA. Comparison of superoxide levels between groups was performed using unpaired Student’s t test. All values are expressed as means +/- SE. A p value < 0.05 was considered significant.
RESULTS

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

The maximal constriction generated by AngII (Emax) was significantly increased in LP compared to CTRL offspring; EC₅₀ value was not different between groups (Figure 1A). Administration of Losartan nearly totally inhibited the vasoconstriction to AngII, whereas administration of PD123319 was without effect in both groups (Figure 1B and 1C).

The vasoconstriction in response to KCl at different tension was not different between groups, indicating that the tensional force was not different between the groups (Figure 2A). Vasoconstriction in response to U46619 (Figure 2B) was not altered by the different antenatal diet exposure.

Vasomotor response to AngII was significantly enhanced after removal of the endothelium in both groups. Percent increase in Emax for LP offspring rings in absence versus in the presence of endothelium was significantly more than for CTRL offspring rings (Figure 3A and B).

Phosphoramidon which blocks both endothelin converting enzyme and neutral endopeptidase 24.11 (which converts angiotensin I to Ang(1-7)) did not modify cumulative response curves to AngII in either group (Figure 4A and B).

As AngII can be metabolized by angiotensin converting enzyme 2 to vasodilator Ang(1-7), its potential role in modulating vasoconstriction to AngII was examined. Cumulative doses of Ang(1-7) elicited no vasomotor effect in either group. Integrity of the vasorelaxant properties of the rings was verified at the end of each experiment with Carbachol (100 µM) (Figure 4C and D).

Effect of Tempol on ex vivo vasomotor response to AngII

In the presence of Tempol, maximal vasoconstriction to AngII of LP offspring ring was significantly decreased to values similar to CTRL. Tempol did not modify vasomotor response to AngII of CTRL offspring (Figure 5A and B).

Effect of antenatal diet exposure on vascular production of reactive oxygen species

Superoxide production in the aortic artery was measured using lucigenin-enhanced chemiluminescence (Figure 6A). LP offspring basal levels of superoxide were significantly higher than CTRL. AngII further increased significantly superoxide generation in LP offspring only; co-incubation of the aortic segments with DPI or apocynin prevented the increased in superoxide generation observed in the presence of AngII. In the CTRL group, we did not observe
differences in the levels of superoxide between baseline conditions and in the presence of AngII +/− DPI or apocynin.

Superoxide production evaluated by the oxidative fluorescent dye hydroethidine was also markedly increased in LP aorta and localized in vascular smooth muscle cells (Figure 6 B and C).

**Western blotting**

AT_1R expression in carotid arteries was significantly increased in LP whereas SOD expression was not different between groups (Figure 7).
**DISCUSSION**

In the current studies we demonstrate exaggerated vascular (carotid arteries) response to AngII in adult male offspring of dams fed a low protein diet during gestation. This enhanced vascular response to AngII seems specific as response to another vasoconstrictor U44619, analogue of thromboxane A2, was found unaltered in LP compared CTRL offspring. This vasomotor response is mediated by AngII AT1R subtype, which expression is increased in LP offspring carotid arteries, and by the production of superoxide anions. We found no difference between groups in the carotid arteries expression of SOD. We show that production of superoxide anions is significantly increased in baseline conditions and in response to AngII in LP offspring aortic wall, and that this increased production of superoxide anion is mediated by NADPH oxidase. Our results also show that the exaggerated carotid arteries vasoconstriction to AngII is not caused by increase in tensional force capacity of the vessel, by a defect in endothelium released relaxing factor or by secondary release of endothelin. Finally, the current results show that in both diet groups vasomotor response to AngII is not mediated by either AT2R or the Ang(1-7) Mas receptor.

Many studies have demonstrated the key role of the RAS in the initiation and maintenance of hypertension associated with intra-uterine protein restriction. In adult LP offspring, plasma renin activity is increased and blood pressure is normalized by ACEi (20; 33; 34). The activity of ACE is elevated in LP offspring (although AngII levels are not consistently found increased) (20; 21), which could suggest increased sensitivity of LP offspring to circulating AngII. Supporting this are reports of increased blood pressure response to intravenously infused AngII in LP adult offspring (26; 34), and the current data examining carotid arteries confirm this is the case.

Vascular hyper responsiveness to AngII, more markedly so than to other vasoconstrictor, has been reported in human and experimental animal models of hypertension (45). In developmentally programmed hypertension, other studies have also found no modification in Emax response to U46619, as well as to phenylephrine in pial microvessels and mesenteric arteries (6; 16).

Increased vasomotor response to AngII can be observed in the presence of vascular remodeling and/or increase in the expression of AngII AT1R subtype, decrease in the expression of AngII AT2R subtype which favors vasodilatation, or changes in AngII-mediated signal
transduction (45). Alternatively, increased vasoconstriction can be encountered in the presence of defective vasodilatation.

Increased tensional force capacity seem absent in adult LP offspring. Vasomotor response to another potent constrictor U46619 is not different between LP and CTRL offspring. Constriction response curves of both groups to KCl under increasing tensions applied to the rings were nearly identical. These functional data are supported by previous reports by us and others showing no difference between groups in lumen diameter, media cross sectional area, media thickness and media to lumen ratio of the carotid and mesenteric arteries {((6; 34).

We identified the AT1R as the sole receptor implicated in the carotid artery vasoconstriction response to AngII for the two groups. Losartan nearly completely inhibited the vasoconstriction to AngII whereas PD123319 did not modify the response to AngII, suggesting that AT2R is not or functionally weakly expressed in rat carotid arteries (30; 32). However we cannot exclude a role for AT2R in the resulting hypertension or in the AngII in vivo pressor response in this model of programmed hypertension. Indeed, although not in vessels, renal AT2R mRNA expression is decreased in 4 week old female (but not male) LP offspring (26).

Carotid expression of AT1R is increased in LP offspring. Based on the Western blot analysis performed on whole vessels and the vasomotor response elicited by AngII in the absence of endothelium, we conclude that AT1R is present on the vascular smooth muscle cells. AT1R had previously been studied in 4 week old male LP kidneys where protein expression (Western and binding analyses) was found increased (37)(Vehaskari 2004) but mRNA expression unchanged (26). In another model of fetal programming of hypertension associated with a 50% global nutrient restriction of the dam during gestation, AT1R mRNA expression is also reported unchanged in mesenteric arteries of adult offspring (10). The underlying factors leading to enhanced AT1R vascular expression in LP offspring are unknown. One of the most potent element affecting AngII receptor expression if AngII itself. In vitro and in vivo, AngII decreases vascular smooth muscle cell AT1R expression (13; 22). Even though ACE and plasma renin activities are increased in adult LP offspring, circulating AngII has been reported as unchanged or inconsistently increased in LP adult offspring (18). However renal tissue AngII is decreased in newborn (47); it could therefore be postulated that this decrease in neonatal AngII, if it applies also to circulating AngII, could permanently program vascular AT1R expression. The fact that others have reported unchanged mRNA allows one to consider posttranscriptional and/or
posttranslational changes in the regulation of AT1R expression in LP offspring. Factors leading to enhanced expression of AT1R could also comprise corticosteroids. Exposure of the fetus to elevated levels of corticosteroids has been shown to play a role in programming of hypertension (25). Blood pressure responsiveness to AngII but not to noradrenaline is enhanced in fetal sheep after cortisol infusion (43). In adult rats, glucocorticoids can increase pressor response to AngII and AT1R expression on vascular smooth muscle cells (39). Whether vascular AT1R expression is increased from early in development is unknown.

Increased response to AngII in the absence of endothelium raises the possibility that AngII stimulates liberation of endothelial relaxing factor(s). Activation of AT1R located on endothelial cells can modulate the release of other vasoactive molecules such as nitric oxide (5), prostacyclin (11) and vasoconstrictor endothelin. We and others reported decreased vasorelaxation to nitric oxide-dependent mechanisms in programmed hypertension in vivo (34) and ex vivo (6; 16). Vasomotor response to prostacycline was studied in adult LP and CTRL offspring pial microvessels and was found unaltered (16). In the current studies, vasoconstriction in response to AngII was increased for the two diet groups in the endothelium-depleted compared to intact carotids. This suggests either that AngII stimulates the release of vasorelaxant factors and/or that there is constitutive release of endothelial derived relaxing factors. If increased carotid vasomotor response to AngII had been secondary to defective endothelium derived relaxing factors, the difference between groups in their vasoconstriction to AngII would have been less in the absence of endothelium. On the contrary, the increase in the vasoconstriction response was significantly more in LP offspring suggesting that if some defective endothelium mediated relaxation prevails, exaggerated response of vascular smooth muscle cells to AngII predominates in LP carotid arteries rings. Carotid arteries are conductance and not resistance vessels, and properties of the different vascular beds or vessel type differ (29). Therefore the observation that defective endothelium mediated vasorelaxation does not play a (major) role in the carotid responses to AngII cannot be considered as contradictory with previously published studies (6; 16; 34).

AngII can lead to the synthesis of endothelin (36). Studies realized in the presence of phosphoramidonsuggest that endothelin does not play a role in AngII-mediated vasoconstriction of the carotid arteries. These data are also in agreement with the observation that vasoconstriction was enhanced in the absence of endothelium: if endothelin had mediated part of the response
observed to AngII, vasoconstriction to AngII would have decreased in the absence of endothelium.

Phosphoramidon also blocks NEP 24.11 which metabolizes angiotensin I to form Ang(1-7). Ang(1-7) can also be formed from AngII under the action of ACE2. Ang(1-7) through activation of the G protein-coupled Mas receptor can counteract the effects of AT1R and result in vasodilatation (9; 38). We observed no vasomotor effect of Ang(1-7) in both groups, indicating that vasoconstriction in response to AngII is not modulated by Ang(1-7) and that Mas receptor seem absent from carotid arteries (38). We are not aware of studies demonstrating the presence (or absence) of Mas receptor in rat carotid arteries.

Animals and humans studies have shown that increased vascular reactive oxygen species, especially superoxide anion, contributes significantly to the vascular dysfunction present in hypertension (28). SOD mimetic can normalize blood pressure and regional blood flow in AngII-infused rats (31). Normalization by SOD mimetic Tempol of the enhanced vasoconstriction to AngII in LP offspring suggests indeed an increased vascular production of superoxide anion. This is supported by the lucigenin-enhanced chemiluminescence revealing a marked increase in the aortic production of superoxide anion in the presence of AngII in the LP group only, which is inhibited by the addition of DPI. The latter element suggests that superoxide anion is generated through a flavin containing enzyme. Among all the potential flavin-containing enzymatic sources of reactive oxygen species, a functional role in adult hypertension has been recognized for NADH/NADPH oxidases, uncoupled nitric oxide synthase and xanthine oxidase as well as for the mitochondrial electron transport chain (7; 49). The nearly complete inhibition of superoxide production in the presence of apocynin indicates that NADPH oxidase is the main source of superoxide in LP offspring aorta, both in baseline conditions and after stimulation by AngII. These results are in agreement with many reports showing that, in adults with chronic hypertension, membrane-bound NADH and NADPH oxidases are the most significant ROS source in the vascular wall (14; 49) and that AngII enhances the vascular production of ROS (mostly superoxide) essentially through the activation of NADH and NADPH oxidases {Thannickal, 2000 1061 /id; Touyz, 2004 990 /id; Touyz, 2004 1077 /id; }. In another model of fetal programming of hypertension, AngII was shown to increase superoxide production through NAPDH oxidase in mesenteric arteries; the latter studies however did not examine the vasomotor
response to AngII (10) but did show that apocynin normalized defective vasodilatation to bradykinin and acetylcholine.

These results provide a basic mechanism by which increased vascular reactivity to AngII prevails in adult LP offspring. However, it is unknown from our and previously published data whether oxidative stress was present early in life and could have initiated vascular dysfunction and hypertension. This can be hypothesized considering the key role of AngII in the development of “programmed” hypertension (see introduction). AngII can increase NADPH oxidase activity and expression of its components (8; 12; 15; 28; 35). In turn, NADH and NADPH oxidase enhance the production of ROS through activation of xanthine oxidase, the auto-oxidation of NADH, and the inactivation of SOD (8; 12; 15; 28; 35). Hence increased RAS activity contributes significantly to augmented ROS and could lead to vascular dysfunction and vascular structural changes such as microvascular rarefaction (34).
Figure Legends:

**Figure 1**: Vasoconstrictor response to angiotensin II (AngII) of carotid artery rings from 9-12 week old male rats exposed during gestation to a low protein (LP) or control (CTRL) diet (panel A) * p<0.05 compared with CTRL. Role of AngII receptor subtypes in mediating vasoconstriction was studied in the presence of Losartan (1 µM) and PD123319 (0.1 µM), respectively AT1 and AT2R antagonists (LP in panel B and CTRL in panel C). Data are mean ± SEM of n = 6 rats per group.

**Figure 2**: Vasoconstrictor response of carotid artery rings from 9-12 week old male rats exposed during gestation to a low protein (LP) or control (CTRL) diet to KCl (80 mM) under increasing tension (panel A, n = 5 rats per group) and to thromboxane A2 analogue U46619 (panel B, n = 6 rats per group). Data are mean ± SEM.

**Figure 3**: Vasoconstrictor response to angiotensin II (AngII) of carotid artery rings with (+) or without (-) endothelium from 9-12 week old male rats exposed during gestation to a low protein (LP, panel A) or control (CTRL, panel B) diet. Constriction is expressed relative (percent) to the response elicited by KCl (80 mM). * p<0.05 compared with the response with intact endothelium in the same diet group. Data are mean ± SEM of n = 6 rats per group.

**Figure 4**: Vasoconstrictor response to angiotensin II (AngII) of carotid artery rings from 9-12 week old male rats exposed during gestation to a low protein (LP, panel A,) or control (CTRL, panel B) diet in the presence or not of phosphoramidon (1 µM) (LP: n = 6 rats with and n = 7 without phosphoramidon; CTRL: n = 7 rats with and n = 9 without phosphoramidon). Constriction is expressed relative (percent) to the response elicited by KCl (80 mM). C and D: Cumulative dose response curve to angiotensin 1-7 (Ang(1-7)) of carotid artery rings from 9-12 week old LP (panel C) or CTRL (panel D) male rats (n = 5 rats per group). At the end of each experiment, integrity of the rings was tested by the addition to Carbachol (100 µM) to the organ chambers. Relaxation is expressed as percent reversal of U46619 (0.3 µM)-induced vasoconstriction. Data are mean ± SEM.
Figure 5: Vasoconstrictor response to angiotensin II (AngII) of carotid artery rings from 9-12 week old male rats exposed during gestation to a low protein (LP, panel A) or control (CTRL, panel B) diet in the presence (+) or not (-) of superoxide dismutase mimetic Tempol (1 mM). Constriction is expressed relative (percent) to the response elicited by KCl (80 mM). * p<0.05 compared with the response without Tempol in the same diet group. Data are mean ± SEM of n = 6 rats per group.

Figure 6: A: Superoxide levels measured by lucigenin-enhanced chemiluminescence in aortas from 9-12 week old male rats exposed during gestation to a low protein (LP, black columns, n = 6) or control (CTRL, white columns, n = 4) diet in baseline conditions, after a 5 minutes preincubation with angiotensin II (AngII, 1 µM) or with AngII plus diphenylene iodonium (DPI, 100 µM) or apocynin (1 mM). RLU: relative light units. Data are mean ± SEM. * p<0.05 compared with baseline. ** p<0.05 compared with CTRL in same conditions. & p<0.05 compared with response to AngII alone for the same group. B and C: Representative sections of aorta from 9-12 week old male LP (panel B) or CTRL (panel C), after treatment with hydroethidine (2 µM) (see Methods). Images were obtained with a laser scanning confocal microscope (LSM 510 laser scanning microscope, Zeiss) equipped with an argon laser. Fluorescence was detected with a 514-nm long-pass filter. Bar scale = 10 µm.

Figure 7: A: Representative immunoblots of Western analysis of angiotensin II AT1 receptor subtype and of superoxide dismutase (SOD) (30 µg of protein was loaded) in whole carotid arteries (n = 4 LP and n = 5 CTRL); the arrows point to the AT1 40-kDa protein, the SOD 16-kDa protein and the beta actin 40-kDa protein. The panel B represents compiled immunoreactive densitometry relative to that of the CTRL protein diet set at 100%.
Figure 1
Figure 2

A

Vasoconstriction (mN) vs. tension (mN)

CTRL
LP

B

Vasoconstriction (mN) vs. U46619 [log mol/L]

CTRL
LP
Figure 3

A

B
Figure 4

A

B

C

D

[Graphs showing vasoconstriction and vasodilation responses to AngII and Ang(1-7) in LP and CTRL conditions with and without phosphoramidon.]
Figure 5

A

B

**AngII [log mol/L]**

**Vasoconstriction (%KCl 80mM)**
FIGURE 6

A

![Bar graph showing lucigenin signal (RLU/mg dry weight) for baseline, AngII, AngII + DPI, and AngII + apocynin treatments.](image)

- **CTRL**
- **LP**

B

![Image B](image)

C

![Image C](image)
Figure 7

A

\[ \text{CTRL} \quad \text{LP} \]

\[ \text{AT1} \]

\[ \text{SOD} \]

\[ \text{beta-actin} \]

B

<table>
<thead>
<tr>
<th>Densitometry (% of CTRL)</th>
<th>CTRL</th>
<th>LP</th>
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<tbody>
<tr>
<td>AT(_1)</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>SOD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates significant difference.
Reference List


