

Uteroplacental insufficiency programmes vascular dysfunction in non-pregnant rats: compensatory adaptations in pregnancy

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Key points

- Uteroplacental insufficiency programmes uterine vascular dysfunction in female offspring born growth restricted. The vascular adaptations in these female offspring when they in turn become pregnant are poorly understood.
- Females born small and later become pregnant have compensatory vascular adaptations, such that the increased uterine and renal arterial stiffness observed in the non-pregnant state was resolved in late pregnancy.
- Vascular smooth muscle and endothelial function was normal in pregnant growth restricted female offspring. There was a reduced sensitivity to angiotensin II, but an increased sensitivity to phenylephrine in uterine arteries during pregnancy, and enhanced endothelium-mediated relaxation in uterine and mesenteric arteries. Importantly, arteries of growth restricted females adapted to these changes.
- Pregnancy was associated with increased outside and internal diameters in uterine and mesenteric arteries, but not renal and femoral arteries, and being born growth restricted did not alter this process.
- These findings may assist our understanding of the maternal vascular adaptations to pregnancy in growth restricted female offspring.

Abstract Intrauterine growth restriction is a risk factor for cardiovascular disease in adulthood. We have previously shown that intrauterine growth restriction caused by uteroplacental insufficiency programmes uterine vascular dysfunction and increased arterial stiffness in adult female rat offspring. The aim of this study was to investigate vascular adaptations in growth restricted female offspring when they in turn become pregnant. Uteroplacental insufficiency was induced in WKY rats by bilateral uterine vessel ligation (Restricted) or sham surgery (Control) on day 18 of pregnancy. F0 pregnant females delivered naturally at term. F1 Control and Restricted offspring were mated at 4 months of age and studied on day 20 of pregnancy. Age-matched non-pregnant F1 Control and Restricted females were also studied. Wire and pressure myography were used to test endothelial and smooth muscle function, and passive mechanical wall properties, respectively, in uterine, mesenteric, renal and femoral arteries of all four groups. Collagen and elastin fibres were quantified using polarized light microscopy and qRT-PCR. F1 Restricted females were born 10–15% lighter than Controls ($P < 0.05$). Non-pregnant Restricted females had increased uterine and renal artery stiffness compared with Controls ($P < 0.05$), but

this difference was abolished at day 20 of pregnancy. Vascular smooth muscle and endothelial function were preserved in all arteries of non-pregnant and pregnant Restricted rats. Collagen and elastin content were unaltered in uterine arteries of Restricted females. Growth restricted females develop compensatory vascular changes during late pregnancy, such that region-specific vascular deficits observed in the non-pregnant state did not persist in late pregnancy.

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Abbreviations Ang II, angiotensin II; EDHF, endothelium-derived hyperpolarising factor; eNOS, endothelial nitric oxide synthase; E_{\max} , maximal response; Indo, indomethacin; ID, internal diameter; LEV, levromakalim; NO, nitric oxide; pD_2 , $-\log EC_{50}$; PE, phenylephrine; PSS, physiological saline solution; PGI₂, prostacyclin; $K_{Ca}2.3$, small-conductance calcium-activated potassium channel; $K_{Ca}3.1$, intermediate-conductance calcium-activated potassium channel; SNP, sodium nitroprusside; WT, wall thickness.

Introduction

Intrauterine growth restriction occurs in about 7–10% of pregnancies and is a major cause of perinatal morbidity and mortality. Uteroplacental insufficiency is the leading cause of intrauterine growth restriction in the Western world and is characterised by compromised uteroplacental blood flow and reduced oxygen and nutrient delivery to the developing fetus. Epidemiological and experimental studies have shown a strong association between low birth weight, an indicator of intrauterine growth restriction, and risk of higher blood pressure and cardiovascular disease in adulthood (Barker *et al.* 2006). Uteroplacental insufficiency causes fetal growth restriction in both male and female offspring. However, there is a sexually dimorphic adult cardiovascular phenotype, with males but not females developing hypertension and glomerular hypertrophy (Grigore *et al.* 2008; Moritz *et al.* 2010).

During pregnancy, the maternal cardiovascular system undergoes remarkable adaptive changes. At a systemic level, increased blood flow to the uteroplacental circulation is achieved by elevating maternal blood volume and increasing cardiac output (Poston *et al.* 1995; Thornburg *et al.* 2000). To accommodate the increased blood supply, vascular tone is shifted in favour of vasodilatation. Accordingly, vascular responsiveness to vasopressors is attenuated in some vascular beds and vasodilator responses are enhanced *via* endothelium-dependent and -independent mechanisms (Magness *et al.* 2001; Gillham *et al.* 2003). The increase in endothelium-dependent vasodilatation is mediated by nitric oxide (NO), prostacyclin (PGI₂) and endothelium-derived hyperpolarising factor (EDHF). In addition, one of the most dramatic changes that occurs during pregnancy is remodelling of the uterine vasculature to ensure adequate uteroplacental perfusion to the developing fetus (Osol & Mandala, 2009).

During normal pregnancy, the uterine artery vascular wall undergoes hypertrophic and hyperplastic changes. Accordingly, the main uterine artery doubles in size (outside and internal diameters) in pregnant humans and increases 2- to 3-fold in rodents. Importantly, inappropriate adaptation of the uterine vasculature in pregnancy is associated with compromised uteroplacental blood flow, intrauterine growth restriction and pregnancy complications, including pre-eclampsia (Reslan & Khalil, 2010).

Our laboratory uses a rat model of uteroplacental insufficiency induced by bilateral uterine vessel ligation in late gestation, which results in offspring that are born 10–15% smaller (Wlodek *et al.* 2005, 2007, 2008). We have previously shown that 18-month-old virgin growth restricted female offspring have impaired uterine endothelial function manifested by reduced EDHF-mediated relaxation (Mazzuca *et al.* 2010). These rats have reduced uterine artery diameter and increased wall stiffness, and this is associated with increased proportion of thick, less compliant collagen and elastin fibre content (Mazzuca *et al.* 2010).

There is little information regarding the vascular adaptations to pregnancy in females who themselves had been born small. Thus, we have extended our studies to investigate whether or not adult females born growth restricted have impaired vascular adaptations in their pregnancy in the absence of any further uteroplacental insufficiency or other challenge. We predicted that the physiological challenge of pregnancy would exacerbate existing vascular phenotypes in growth restricted females. Given the regional heterogeneity in vascular function, this study aimed to provide a more comprehensive analysis of the vascular adaptations to pregnancy following growth restriction in female offspring by studying uterine, mesenteric, renal and femoral arteries.

Methods

Ethics

All procedures were approved by The University of Melbourne Pharmacology, Physiology, Biochemistry & Molecular Biology and Bio21 Institute Animal Ethics Committee. All of the authors have read the article 'Reporting ethical matters in *The Journal of Physiology*: standards and advice' (Drummond, 2009), and our experiments comply with the policies and regulations.

Animals

Wistar-Kyoto rats were housed in an environmentally controlled room with a 12 h light–dark cycle and access to food and tap water *ad libitum*. Virgin female rats aged 12–19 weeks were mated with normal males (Wlodek *et al.* 2007; O'Dowd *et al.* 2008). On day 18 of gestation, F0 pregnant rats were randomly allocated to a sham (offspring termed Control) or uteroplacental insufficiency (offspring termed Restricted) group ($n = 10/\text{group}$). The Restricted group underwent bilateral uterine vessel (artery and vein) ligation surgery (Wlodek *et al.* 2005, 2007; O'Dowd *et al.* 2008; Moritz *et al.* 2009; Mazzuca *et al.* 2010). The F0 pregnant females delivered naturally at term on day 22 of gestation. We have previously shown that uteroplacental insufficiency reduces total (male and female) litter size (Gallo *et al.* 2012). Body weights from Control and Restricted female offspring (F1) were recorded at post-natal days 1, 6, 14 and 35. At 12 weeks of age, F1 Control and Restricted female offspring were allocated to either non-pregnant or pregnant groups (1/litter/group; $n = 10/\text{group}$). Those allocated to the pregnant group were weighed and mated (12–19 weeks) with different normal males. Mating a Restricted female with a normal male resulted in a ~100% pregnancy success rate on the first attempt at mating. It is important to note that the pregnant offspring did not undergo uteroplacental insufficiency or sham surgery or any other experimental manipulation. At day 20 of gestation, pregnant female rats and age matched non-pregnant rats were weighed and anaesthetized with intraperitoneal injection of ketamine (100 mg kg⁻¹ body weight) and Ilium Xylazil-20 (30 mg (kg body weight)⁻¹). The main uterine and small mesenteric, renal and femoral arteries were isolated and used for vascular function studies (Mazzuca *et al.* 2010). Remaining uterine and renal artery were immediately snap frozen and stored at -80°C for later molecular analysis. The remainder of the uterine artery tissue was immersion fixed with 10% neutral buffered formalin for histological analysis (Mazzuca *et al.* 2010).

In vitro vascular reactivity

Rings of uterine (outside diameter (OD) ~350 μm), mesenteric (OD ~300 μm), renal (OD ~400 μm),

femoral (OD ~400 μm) arteries ~1–2 mm in length were mounted on a four channel wire myograph (Model 610 M, Danish Myo Technology, Aarhus, Denmark) for measurement of isometric tension (Mazzuca *et al.* 2010). Vascular segments from all rats came from the same location along the vascular tree. We used the main uterine artery, 2nd order mesenteric arteries, renal interlobar arteries and the saphenous branch of the femoral artery. Functional reactivity experiments were performed on the same vascular segment.

Briefly, arteries were bathed in physiological saline solution (PSS; mM: 120 NaCl, 5 KCl, 2.5 CaCl₂, 25 NaHCO₃, 11 glucose, 1 KH₂PO₄, 1.2 MgSO₄) bubbled with 95% O₂ and 5% CO₂ at ~36°C. Prior to the commencement of each experiment the viability of the smooth muscle in each segment was tested using a brief application of high K⁺ PSS (isotonic replacement of Na⁺ with 100 mM K⁺). Endothelial function was tested using 10⁻⁵ M acetylcholine (ACh) applied for 2 min in arteries precontracted with phenylephrine (PE).

To test smooth muscle contraction, arteries were exposed to cumulative concentrations of PE (10⁻⁹–10⁻⁴ M) and angiotensin II (Ang II, 10⁻¹⁰–10⁻⁷ M). Contractions were either expressed as a percentage of the contraction evoked by high K⁺ PSS or by the maximum contraction evoked by that constrictor for that individual artery. Endothelial function was studied in arteries submaximally precontracted (~70% of maximal) with PE (3 × 10⁻⁷ – 3 × 10⁻⁶ M). The level of precontraction was not different in arteries from non-pregnant or pregnant Control and Restricted groups. The endothelium was stimulated with increasing concentrations of ACh (10⁻⁹–10⁻⁵ M). To elucidate the role of EDHF, ACh curves were repeated in the presence of N^ω-nitro-L-arginine methyl ester (L-NAME, 2 × 10⁻⁴ M) and indomethacin (Indo, 10⁻⁶ M) to block nitric oxide synthase (NOS) and cyclooxygenase (COX), respectively. Smooth muscle relaxation was tested using the NO donor sodium nitroprusside (SNP, 10⁻⁹–10⁻⁵ M) and ATP-sensitive potassium (K_{ATP}) channel opener levcromakalim (LEV, 10⁻⁹–10⁻⁶ M).

Passive mechanical wall properties

In separate segments of arteries from these rats, uterine, mesenteric, renal and femoral arteries (3–4 mm long) were assessed for their passive mechanical wall properties using a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA), as previously described (Mazzuca *et al.* 2010). Briefly, arteries were continuously superfused at ~15 ml min⁻¹ with 0 mM Ca²⁺-free, 1 mM EGTA PSS, gassed with 95% O₂, 5% CO₂ at ~36°C. Each artery was pressurised from 0 to 200 mmHg, with 10 mmHg increases in intraluminal pressure. Arterial dimensions (length, OD

and wall thickness (WT)) were measured at each 10 mmHg increment. Wall stress and strain were derived as follows: wall stress (kPa) = (intraluminal pressure \times internal diameter)/(2 \times wall thickness); wall strain = (internal diameter – internal diameter extrapolated to 10 mmHg pressure)/internal diameter extrapolated to 10 mmHg pressure (Wigg *et al.* 2001). The media-to-lumen ratio was determined by calculating wall thickness (WT)/internal diameter (ID).

Histological examination of collagen and elastin in uterine arteries

Transverse fixed segments of uterine artery were cut at 5 μ m thickness from two locations along the artery from both non-pregnant and pregnant groups (Mazzuca *et al.* 2010). Collagen and elastin fibres were stained with picosirius red or Gomori fuschin aldehyde, respectively. Artery sections were examined under a polarised microscope (Olympus10 BX51 Pol) using conventional bright-field illumination and circular crossed-polarised light setup. Total collagen and elastin content were quantified using bright-field (Mazzuca *et al.* 2010). The proportion of thick (visualised as red, orange and yellow fibres under circular crossed-polarised light) and thin (green fibres) collagen fibres were quantified with circular crossed-polarised light (Mazzuca *et al.* 2010).

Gene expression analysis in uterine and renal arteries

RNA from frozen uterine and renal arteries was extracted and converted to cDNA and gene expression quantified using fluorescence-based qRT-PCR primers and TaqMan probes on the Rotor Gene 3000 system (Corbett Research, Mortlake, VIC, Australia) as previously described (Moritz *et al.* 2009; Mazzuca *et al.* 2010). Only the uterine and renal arteries were selected for molecular analysis as the largest vascular effects between non-pregnant and pregnant groups were detected in these arteries. The primer-probe design strategy was to situate primers and probes within the protein-coding region, exon spanning where possible to avoid genomic DNA contamination. The genes of interest included collagen I, III, IV (α 1), elastin, fibronectin, endothelial NOS, small-conductance calcium-activated potassium channel ($K_{Ca2.3}$) and intermediate-conductance calcium-activated potassium channel ($K_{Ca3.1}$). Optimal concentrations for all primers and probes were 300 and 100 nM, respectively. Relative quantification of gene expression was performed by the comparative CT ($\Delta\Delta C_T$) method with ribosomal 18S as the endogenous control. 18S expression in the uterine and renal artery was not different between Control and Restricted from either the pregnant or non-pregnant groups as verified by the cycle threshold values (C_T)

(Control vs. Restricted: Uterine, 18S, $P = 0.60$; Renal, $P = 0.40$; by two-way ANOVA). Although 18S was used as our housekeeper, we also verified the validity of our results with other common housekeepers such as cyclophilin A and β -actin. The C_T value of these housekeepers also did not change between Control and Restricted groups. Primer and probe sequences are given in the online Supplemental Material (Table S1).

Drugs and chemicals

The following agents were used: angiotensin II (Auspep, Melbourne, VIC, Australia); acetylcholine, indomethacin, levcromakalim, N^{ω} -nitro-L-arginine methyl ester, phenylephrine (Sigma-Aldrich, Castle Hill, NSW, Australia); ilium xylazil-20 (Troy Laboratories, Pty Ltd, Smithfield, NSW, Australia); ketamine (Parnell Laboratories, Pty Ltd, Alexandria, NSW, Australia); primers and probes (Biosearch Technologies, Novato, CA, USA); sodium nitroprusside (Ajax Chemicals, Auburn, SA, Australia); Sirius red F3B and DPX (BDH Laboratory Supplies, Poole, UK); Superscript III RT (Invitrogen, VIC, Australia); Tri Reagent (Ambion, Inc., Austin, TX, USA).

Statistical analysis

Values are expressed as means \pm SEM with n representing the number of offspring from separate mothers per group. Concentration–response curves were constructed using Prism (v.5.01; GraphPad Software, San Diego, CA, USA) and sigmoidal curves fitted to the data using the least squares method. Contractions to Ang II and PE were expressed as a percentage of contraction to high- K^+ PSS or as a percentage of their own maximum contraction evoked by that constrictor. Relaxations evoked by ACh, SNP and LEV were expressed as a percentage of pre-contraction evoked by PE. Where appropriate, data were analysed using a two-way ANOVA to determine the main effects of uteroplacental insufficiency (Control and Restricted) and pregnancy (non-pregnant and pregnant), or Student's unpaired t test, using SPSS (v18, SPSS Inc., Chicago, IL, USA). Two-way ANOVA with repeated measures was performed for vascular dose–response curves. If significant interactions were observed, then individual group means were compared, with the level of significance adjusted by Bonferroni's method to account for multiple comparisons, or Student's unpaired t test were performed for *post hoc* comparisons. For dose–response curves, if a main effect or significant interaction was observed, the EC_{50} was determined and the pD_2 ($-\log EC_{50}$) and maximum response (E_{max}) were then compared between groups. In renal arteries, higher concentrations of ACh tended to cause contraction, and the EC_{50} and E_{max}

Table 1. F1 female body weights

	Group allocation			
	Non-pregnant		Pregnant	
	Control	Restricted	Control	Restricted
F1 body weight (g)				
Postnatal day 1	4.03 ± 0.07	3.55 ± 0.09*	4.00 ± 0.08	3.42 ± 0.07*
Postnatal day 6	8.53 ± 0.27	7.28 ± 0.49*	7.67 ± 0.42	6.15 ± 0.35*#
Postnatal day 14	20.57 ± 0.50	19.57 ± 1.40	20.20 ± 1.03	17.82 ± 1.07
Postnatal day 35	73.67 ± 1.45	67.33 ± 3.15	66.80 ± 2.20	63.48 ± 1.87
Mating	—	—	204 ± 4	203 ± 7
Postmortem	212 ± 5	214 ± 4	288 ± 5	278 ± 9#
Pregnancy weight gain	—	—	84 ± 3	75 ± 3

Values are expressed as means ± SEM; $n = 9\text{--}11/\text{group}$. * $P < 0.05$ vs. Control (main effect); # $P < 0.05$ vs. non-pregnant (main effect). — indicates no measure.

values for ACh-induced relaxation were derived from sigmoidal curves fitted to the relaxation component of the dose–response curve (Mazzuca *et al.* 2010). Stress–strain relationships, OD and ID, WT, media-to-lumen ratio, histological examination of collagen and elastin were analysed using a two-way ANOVA or Student's unpaired t test, where appropriate. Results were considered significant when $P < 0.05$.

Results

Body weights

Uteroplacental insufficiency in F0 females reduced F1 female body weight at postnatal day 1 (by ~15%, $P < 0.05$, Table 1) compared with sham surgery Controls. Restricted female offspring remained lighter for the first 6 days of postnatal life (by 12–17%, $P < 0.05$, Table 1) but there was no difference in body weight by day 14 when compared with Controls (Table 1). Body weight was not different between Control and Restricted at the time of mating (4 mo) or at postmortem (Table 1). As expected, body weight was greater in pregnant compared to age-matched non-pregnant rats (+29%, $P < 0.05$) with no differences between Control and Restricted groups (Table 1).

Smooth muscle contraction

Sensitivity or maximal contraction evoked by Ang II or PE in uterine, mesenteric, renal and femoral arteries was not different between Control and Restricted females from either non-pregnant or pregnant groups (Fig. 1, Tables 2 and 3). Sensitivity to Ang II was reduced in uterine arteries of the pregnant groups compared with non-pregnant groups ($P < 0.05$; Fig. 1A, Table 3). Ang II did not evoke significant contraction in mesenteric, renal or femoral arteries (<0.2% of K^+ -PSS contraction) from either non-pregnant or pregnant animals (Tables 2

and 3). In late pregnancy there was an increase in sensitivity to PE (by ~6-fold) in uterine arteries compared with non-pregnant groups ($P < 0.05$; Fig. 1B, Tables 2 and 3), with no changes in sensitivity to PE in mesenteric, renal and femoral arteries (Fig. 1C–E, Tables 2 and 3).

Maximal absolute contraction evoked by K^+ -PSS, PE or Ang II (mN mm^{-1}) was not different in arteries from Control and Restricted groups (Table 2). Pregnancy was associated with a ~50% increase in maximal absolute Ang II-mediated contraction in the uterine artery compared with non-pregnant groups ($P < 0.05$; Table 2) with no differences in mesenteric, renal and femoral arteries (Table 2). Absolute contractions to K^+ -PSS or PE were smaller in uterine and femoral arteries of the pregnant groups when compared with those from non-pregnant ($P < 0.05$, Table 2), but were unchanged for the mesenteric and renal arteries (Table 2).

Endothelium-dependent and –independent relaxation

In the absence of blockers, sensitivity and maximal total endothelium-dependent relaxation evoked by ACh was not different in uterine, mesenteric, renal or femoral arteries between Control and Restricted groups (Fig. 2A–D, Tables 2 and 3). However, in the renal artery, higher concentrations of ACh (10^{-6} – 10^{-5} M) evoked contraction that was greater in Restricted compared with Controls for both non-pregnant and pregnant groups (10^{-5} M, $P < 0.05$, Fig. 2C). There was a pregnancy-induced increase in sensitivity to ACh in the uterine (by ~2.5-fold, $P < 0.0001$; Fig. 2A, Table 3) and mesenteric arteries (by ~1.3-fold, $P < 0.05$; Fig. 2B, Table 3) but not in renal or femoral arteries compared with arteries from non-pregnant rats (Fig. 2C and D, Tables 2 and 3).

The relaxation attributed to EDHF was revealed following the blockade of NOS and COX with L-NAME and Indo, respectively. In the presence of L-NAME and

Indo, concentration–relaxation curves to ACh were shifted to the right in the uterine, mesenteric and renal arteries of all four groups (Fig. 2E–G). In femoral arteries, ACh failed to evoke relaxation in the presence of L-NAME and Indo, reflecting the absence of EDHF-mediated relaxation (Fig. 2H; Tables 2 and 3) (Wigg *et al.* 2001; Mazzuca *et al.* 2010). In late pregnant rats, sensitivity to ACh and maximal EDHF-mediated relaxation in the uterine arteries was greater (pD_2 , ~4-fold; E_{max} , 41%) compared with arteries from the non-pregnant groups ($P < 0.05$; Fig. 2E, Tables 2 and 3). Furthermore, the constriction evoked by higher concentrations of ACh (10^{-6} – 10^{-5} M) in uterine arteries from non-pregnant animals was absent in arteries from the pregnant groups (Fig. 2E). In mesenteric arteries, there was a modest leftward shift (by ~1.5-fold) for the EDHF-mediated relaxation in pregnant compared with non-pregnant rats ($P < 0.05$; Fig. 2F, Table 3) with no differences in maximal relaxation (Table 2). Pregnancy was without effect on the EDHF-mediated relaxation in renal arteries of either group (Fig. 2C, Tables 2 and 3).

Sensitivities and/or maximal relaxations evoked by SNP or K_{ATP} channel opener LEV were not different in any of the arteries of all experimental groups (Tables 2 and 3).

Passive mechanical wall properties

Stress–strain relationships for uterine and renal arteries of the non-pregnant Restricted group were shifted to the left compared with those for non-pregnant Controls ($P < 0.05$; Fig. 3A and C), indicating increased arterial wall stiffness. There were no differences in the stress–strain relationships for the mesenteric and femoral arteries in non-pregnant Restricted compared with non-pregnant Controls (Fig. 3B and D). Both pregnant Control and Restricted females had similar stress–strain relationships for all arteries (Fig. 3A–D). In late pregnant rats, the stress–strain relationships for all arteries were shifted to the left compared with those from non-pregnant ($P < 0.05$; Fig. 3A–D) indicating increased arterial stiffness.

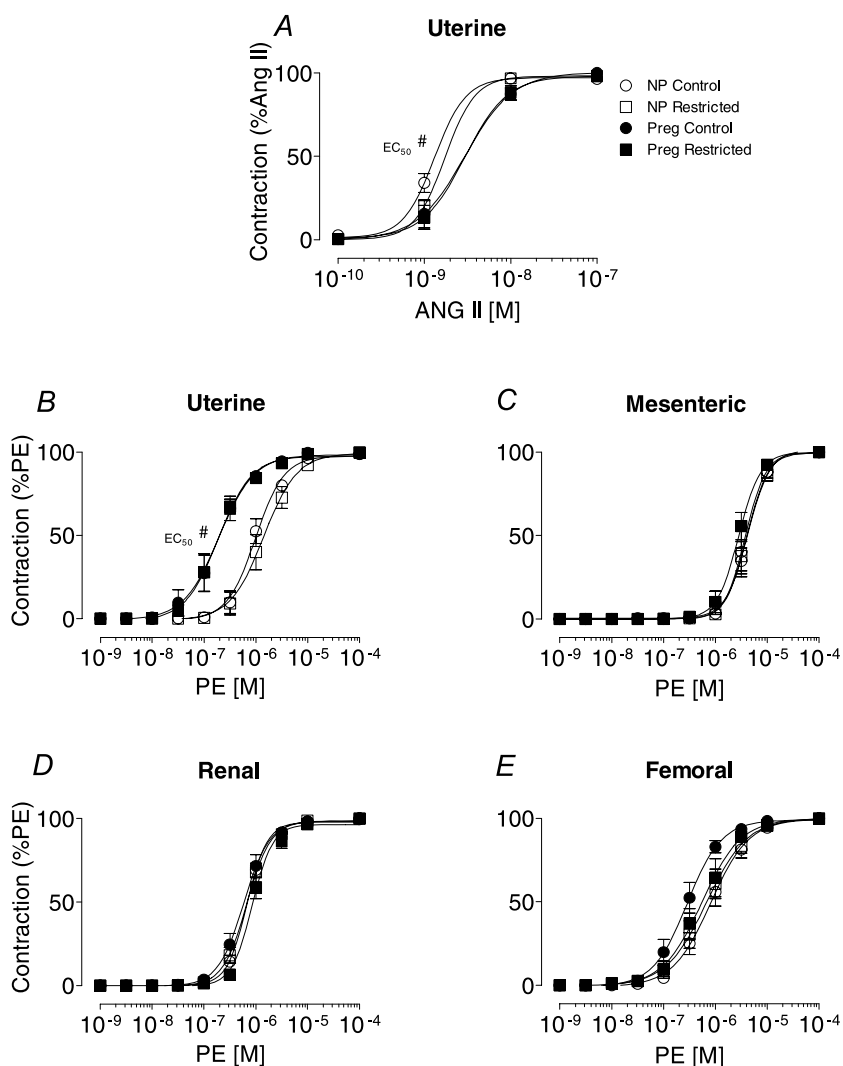


Figure 1. Smooth muscle contraction in arteries

A, concentration–contraction curves to Ang II in uterine arteries (A) from non-pregnant and pregnant (E20) rats. B–E, concentration–contraction curves to PE in uterine (B), mesenteric (C), renal (D) and femoral (E) arteries from non-pregnant and pregnant (E20) animals. Values are expressed as means \pm SEM; uterine ($n = 8$ – 10 /group); mesenteric ($n = 6$ – 10 /group); renal ($n = 8$ – 10 /group); femoral ($n = 6$ – 10 /group). # $P < 0.05$ vs. non-pregnant (main effect).

Table 2. Smooth muscle and endothelial function in arteries

Arteries	Non-pregnant Control				Non-pregnant Restricted			
	Uterine	Mesenteric	Renal	Femoral	Uterine	Mesenteric	Renal	Femoral
Smooth muscle function								
100 mmol l ⁻¹ K ⁺ (mN mm ⁻¹)	10.5 ± 0.2	7.0 ± 0.4	11.2 ± 0.6	13.4 ± 0.7	11.3 ± 0.3	6.5 ± 0.3	10.6 ± 0.3	14.1 ± 0.6
Maximum PE (mN mm ⁻¹)	11.3 ± 0.2	6.5 ± 0.3	11.5 ± 0.6	13.4 ± 0.5	11.8 ± 0.4	6.2 ± 0.3	10.9 ± 0.3	14.1 ± 0.8
Maximum Ang II (mN mm ⁻¹)	5.5 ± 0.6	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	5.5 ± 0.4	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.03
Ang II, E _{max} (% K ⁺)	55 ± 6	—	—	—	49 ± 4	—	—	—
PE, E _{max} (% K ⁺)	108 ± 2	93 ± 3	103 ± 1	101 ± 2	105 ± 2	95 ± 2	103 ± 2	99 ± 2
SNP, E _{max} (%)	96 ± 1	97 ± 1	88 ± 2	86 ± 2	93 ± 1	95 ± 1	89 ± 2	81 ± 4
LEV, E _{max} (%)	95 ± 1	91 ± 1	80 ± 4	85 ± 7	94 ± 1	97 ± 1	85 ± 3	89 ± 3
Endothelium-dependent relaxation								
ACh, E _{max} (%)	96 ± 1	99 ± 1	96 ± 1	90 ± 2	95 ± 1	98 ± 1	94 ± 1	90 ± 2
ACh+ L-NAME+Indo, E _{max} (%)	34 ± 9	95 ± 1	90 ± 1	—	53 ± 10	94 ± 2	87 ± 2	—
Arteries	Pregnant Control				Pregnant Restricted			
	Uterine	Mesenteric	Renal	Femoral	Uterine	Mesenteric	Renal	Femoral
Smooth muscle function								
100 mmol l ⁻¹ K ⁺ (mN mm ⁻¹)	8.5 ± 0.5#	6.7 ± 0.5	9.3 ± 0.6	11.0 ± 0.9#	8.9 ± 0.5#	6.0 ± 0.5	10.9 ± 0.8	13.1 ± 0.9#
Maximum PE (mN mm ⁻¹)	10.3 ± 0.6#	6.4 ± 0.5	9.5 ± 0.6	11.1 ± 1.2#	10.2 ± 0.5#	6.4 ± 0.5	10.3 ± 0.7	12.5 ± 0.8#
Maximum Ang II (mN mm ⁻¹)	8.2 ± 1.0#	0.06 ± 0.05	0.1 ± 0.04	0.04 ± 0.04	8.2 ± 0.9#	0.09 ± 0.06	0.1 ± 0.05	0.09 ± 0.08
Ang II, E _{max} (% K ⁺)	88 ± 4#	—	—	—	83 ± 3#	—	—	—
PE, E _{max} (% K ⁺)	122 ± 2#	94 ± 3	104 ± 4	100 ± 2	114 ± 1#	105 ± 3	95 ± 2	94 ± 4
SNP, E _{max} (%)	99 ± 1	97 ± 2	92 ± 2	81 ± 8	95 ± 1	98 ± 2	86 ± 2	79 ± 5
LEV, E _{max} (%)	91 ± 1	97 ± 1	84 ± 4	93 ± 1	87 ± 2	94 ± 1	75 ± 5	77 ± 7
Endothelium-dependent relaxation								
ACh, E _{max} (%)	99 ± 0.4	100 ± 0.2	96 ± 1	94 ± 3	98 ± 1	100 ± 0.4	93 ± 1	94 ± 5
ACh+ L-NAME+Indo, E _{max} (%)	86 ± 2#	98 ± 1	89 ± 2	—	83 ± 2#	92 ± 2	86 ± 2	—

Responses in arteries from non-pregnant and pregnant (E20) animals. Values are expressed as mean ± SEM; uterine ($n = 8-10/\text{group}$); mesenteric ($n = 6-10/\text{group}$); renal ($n = 8-10/\text{group}$); femoral ($n = 6-10/\text{group}$). E_{max}, maximal response. # $P < 0.05$ vs. non-pregnant (main effect). — indicates no response.

In late pregnancy, absolute OD and ID of the uterine arteries were significantly larger (by 42% and 44%, respectively) compared with those from non-pregnant rats ($P < 0.05$; Fig. 4A, Table 4). For the mesenteric arteries, OD and IDs were modestly larger in pregnant rats compared with non-pregnant rats (by 12%, $P < 0.05$; Fig. 4B, Table 4). Absolute WT was reduced in the uterine artery of non-pregnant Restricted females ($P < 0.05$; Table 4). During pregnancy, uterine artery WT in Restricted females increased to become similar to that in their Control pregnant counterparts. The media-to-lumen ratio (at 100 mmHg) was smaller in uterine arteries of non-pregnant Restricted compared with

non-pregnant Controls ($P < 0.05$; Table 4). In pregnant Controls, there was a uterine artery specific decrease in the media-to-lumen ratio compared with non-pregnant Controls ($P < 0.05$; Table 4).

Quantitative histological analysis of collagen and elastin in uterine arteries

There were no differences in total collagen and elastin content or in the proportion of thick and thin birefringent collagen fibres in the media or adventitia in uterine arteries of Restricted compared with Controls (Fig. 5A–D). Interestingly, pregnancy caused a reduction in total collagen

Table 3. Sensitivities (pD₂) to vasoconstrictors and vasodilators in arteries

Arteries	Non-pregnant Control				Non-pregnant Restricted			
	Uterine	Mesenteric	Renal	Femoral	Uterine	Mesenteric	Renal	Femoral
pD ₂								
AngII (% K ⁺)	8.92 ± 0.23	—	—	—	8.66 ± 0.14	—	—	—
PE (% K ⁺)	6.01 ± 0.04	5.38 ± 0.03	6.17 ± 0.01	6.08 ± 0.05	5.87 ± 0.07	5.39 ± 0.03	6.17 ± 0.03	6.16 ± 0.07
Ang II (% max Ang II)	8.83 ± 0.15	—	—	—	8.77 ± 0.09	—	—	—
PE (% max PE)	6.01 ± 0.04	5.38 ± 0.04	6.17 ± 0.01	6.09 ± 0.05	5.85 ± 0.06	5.39 ± 0.03	6.17 ± 0.03	6.17 ± 0.07
SNP	7.77 ± 0.04	7.45 ± 0.07	7.90 ± 0.06	7.51 ± 0.11	7.68 ± 0.04	7.30 ± 0.07	7.82 ± 0.06	7.33 ± 0.10
ACh	7.71 ± 0.04	8.13 ± 0.02	8.04 ± 0.03	7.53 ± 0.05	7.71 ± 0.03	8.08 ± 0.04	7.88 ± 0.03	7.61 ± 0.05
ACh+ L-AME+Indo	6.99 ± 0.05	7.28 ± 0.02	7.42 ± 0.03	—	6.92 ± 0.07	7.18 ± 0.03	7.40 ± 0.03	—
Arteries	Pregnant Control				Pregnant Restricted			
	Uterine	Mesenteric	Renal	Femoral	Uterine	Mesenteric	Renal	Femoral
pD ₂								
AngII (% K ⁺)	8.55 ± 0.12	—	—	—	8.54 ± 0.11	—	—	—
PE (% K ⁺)	6.71 ± 0.06#	5.42 ± 0.05	6.24 ± 0.01	6.53 ± 0.05	6.68 ± 0.05#	5.55 ± 0.03	6.09 ± 0.02	6.26 ± 0.08
Ang II (% max Ang II)	8.53 ± 0.09#	—	—	—	8.53 ± 0.10#	—	—	—
PE (% max PE)	6.71 ± 0.06#	5.42 ± 0.05	6.23 ± 0.03	6.54 ± 0.05	6.72 ± 0.05#	5.55 ± 0.03	6.07 ± 0.02	6.27 ± 0.07
SNP	7.71 ± 0.04	7.57 ± 0.09	7.99 ± 0.06	7.60 ± 0.20	7.88 ± 0.07	7.54 ± 0.15	7.95 ± 0.08	7.67 ± 0.19
ACh	8.12 ± 0.02#	8.23 ± 0.03#	8.05 ± 0.03	7.36 ± 0.08	8.07 ± 0.05#	8.22 ± 0.02#	7.94 ± 0.04	7.43 ± 0.10
ACh+ L-NAME+Indo	7.58 ± 0.02#	7.36 ± 0.03#	7.55 ± 0.04	—	7.55 ± 0.06#	7.48 ± 0.04#	7.45 ± 0.03	—

Responses in arteries from non-pregnant and pregnant (E20) animals. Values are expressed as means ± SEM; uterine ($n = 8-10$ /group); mesenteric ($n = 6-10$ /group); renal ($n = 8-10$ /group); femoral ($n = 6-10$ /group). Max, maximal contraction. # $P < 0.05$ vs. non-pregnant (main effect). — indicates no response.

(by 5%) and elastin content (by 8%) in the adventitia of uterine arteries, compared with non-pregnant groups ($P < 0.05$; Fig. 5A and B). Pregnant rats had fewer thick collagen fibres (by 11%), and more thin collagen fibres (by 10%) in the adventitia compared with their non-pregnant counterparts ($P < 0.05$; Fig. 5D). There were no changes in the proportions of thick and thin collagen fibres in the media between non-pregnant and pregnant groups (Fig. 5C).

Uterine and renal artery gene expression

There were no differences in the relative mRNA expression of collagens type I ($\alpha 1$), III ($\alpha 1$), IV ($\alpha 1$), elastin, fibronectin, eNOS, K_{Ca}2.3 and K_{Ca}3.1 in uterine or renal arteries between Control and Restricted offspring (data not shown).

Discussion

In this study, uteroplacental insufficiency programmed region-specific vascular dysfunction in non-pregnant growth restricted females. Arterial wall stiffness was increased in the uterine and renal arteries, but was unaffected in mesenteric and femoral arteries of non-pregnant Restricted females. In late pregnancy, growth restricted females had compensatory vascular adaptations, such that uterine and renal arteries were no longer stiffer than Controls. Furthermore, vascular

smooth muscle and endothelial function was essentially preserved in all arteries of non-pregnant and late pregnant Restricted females. Overall, this study highlights that females born growth restricted experience normal vascular adaptations to pregnancy such that the differences in arterial wall stiffness and wall thickness in the non-pregnant group did not persist in late pregnancy.

Passive mechanical wall properties

Uterine and renal arteries of Restricted virgin females have increased passive wall stiffness. One of the most striking findings of this study was that differences in arterial wall stiffness between Control and Restricted groups disappeared with pregnancy. In fact, the Restricted pregnant females exhibited normal vascular adaptations such that the stress–strain and pressure–diameter relationships were indistinguishable compared with late pregnant Controls. The fact that Restricted females do not have increased arterial stiffness in the major resistance beds including the mesenteric arteries may in part explain why these females do not develop high blood pressure during pregnancy (Gallo *et al.* 2012).

Another important point is that pregnancy caused a leftward shift in the stress–strain relationships for uterine, mesenteric, renal and femoral arteries when compared with non-pregnant, suggesting an increase in arterial stiffness. Increased stiffness of uterine and renal arteries in

pregnancy is consistent with previous studies in pregnant rats and sheep (Griendling *et al.* 1985; Gandley *et al.* 1997; St Louis *et al.* 1997; Gandley *et al.* 2010). However, in some studies of mesenteric and renal arteries there was a rightward shift in the stress–strain relationship (increase compliance) with pregnancy in rats (Mackey *et al.* 1992; Gandley *et al.* 1997). It is likely that methodological differences may account for the conflicting results. In our study, passive mechanical wall properties were determined in the presence of solution containing zero Ca^{2+} and 1 mM EGTA (in both the lumen and bathing solutions) to remove any active influence of the smooth muscle. In the previous studies, mechanical wall properties were determined in the presence of papaverine, a smooth

muscle relaxant, but in a Ca^{2+} containing solution, and thus may not reflect truly passive properties. Our study demonstrates that there is a marked increase in uterine artery diameter accompanied by enhanced arterial wall stiffness in late pregnancy.

Consistent with previous findings (Osol & Mandala, 2009), pregnancy was associated with increases in both OD and ID in uterine arteries by ~43% and in mesenteric arteries by ~12%. The hypertrophic growth occurred without a change in WT. Importantly, blood vessels of growth restricted females adapted normally to these structural changes during late pregnancy. The increase in ID and OD in uterine and mesenteric arteries observed during pregnancy was associated with reduced

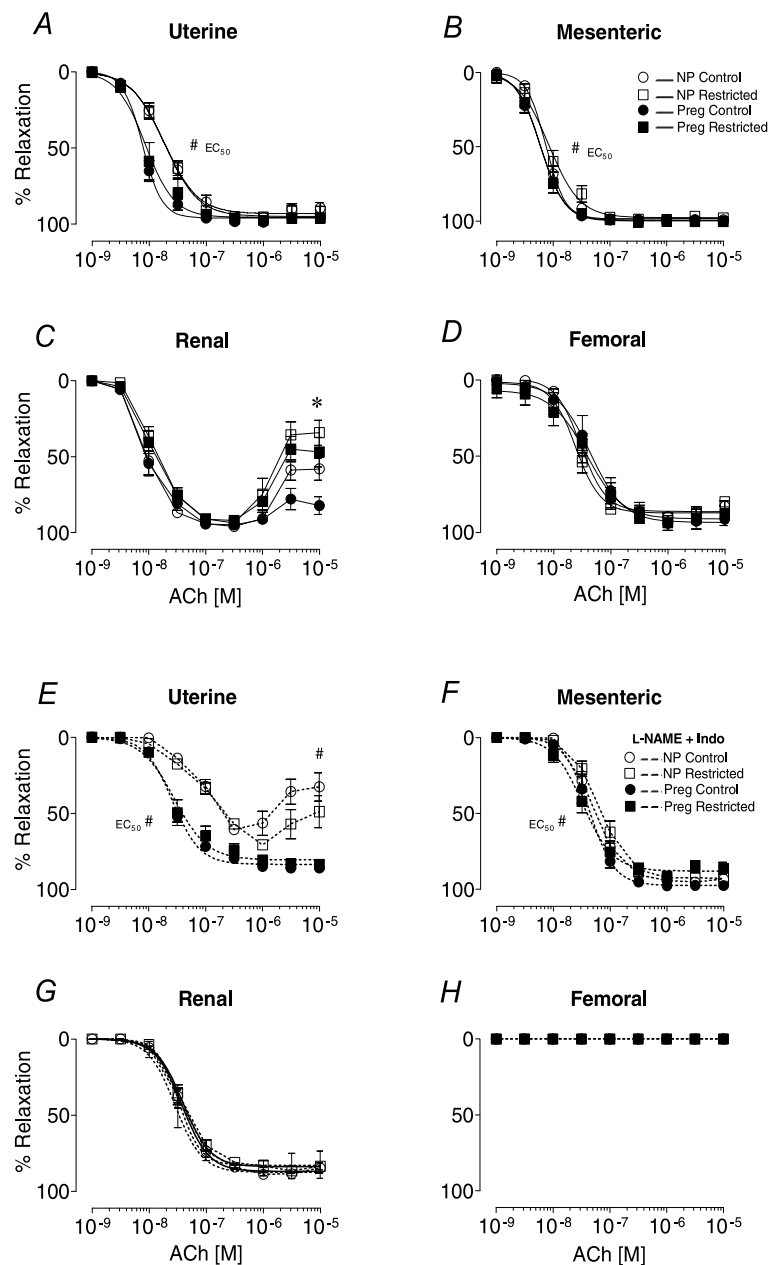


Figure 2. Total endothelium-dependent and EDHF-mediated relaxation in arteries
 Concentration–relaxation curves to ACh in the absence or presence L-NAME and Indo in uterine (A and E), mesenteric (B and F), renal (C and G) and femoral (D and H) arteries from non-pregnant and pregnant (E20) animals. Values are expressed as means \pm SEM; uterine ($n = 8–10$ /group); mesenteric ($n = 6–10$ /group); renal ($n = 8–10$ /group); femoral ($n = 6–10$ /group). * $P < 0.05$ vs. Control (main effect); # $P < 0.05$ vs. non-pregnant (main effect).

distensibility (increased arterial stiffness), which may reflect the fact that the arteries were already maximally distended and had reached their physiological limits of relaxation. The increased uterine and mesenteric arterial stiffness, as indicated by a leftward shift in the stress–strain relationship during pregnancy may not be detrimental in a physiological sense, because both diameter and endothelial vasodilator function were enhanced to accommodate the expected increase in uteroplacental blood flow to the fetal placental unit.

The underlying mechanisms responsible for changes in the structural composition of the artery wall during pregnancy likely involve alterations in extracellular matrix

proteins, including collagen and/or elastin (Osol & Mandala, 2009). Although this study failed to show differences in the relative mRNA expression of collagen type I(α 1), III(α 1), IV(α 1), elastin, and fibronectin in uterine and renal arteries between Control and Restricted groups, protein levels may be different. Unfortunately, due to the limited amount of vascular tissue available at postmortem, protein expression could not be measured. Studies have shown that collagen was reduced in main uterine arteries of pregnant sheep and pigs, whilst elastin remained unchanged, reducing the collagen to elastin ratio (Griendling *et al.* 1985). In our study, total adventitial collagen and elastin content in the uterine arteries

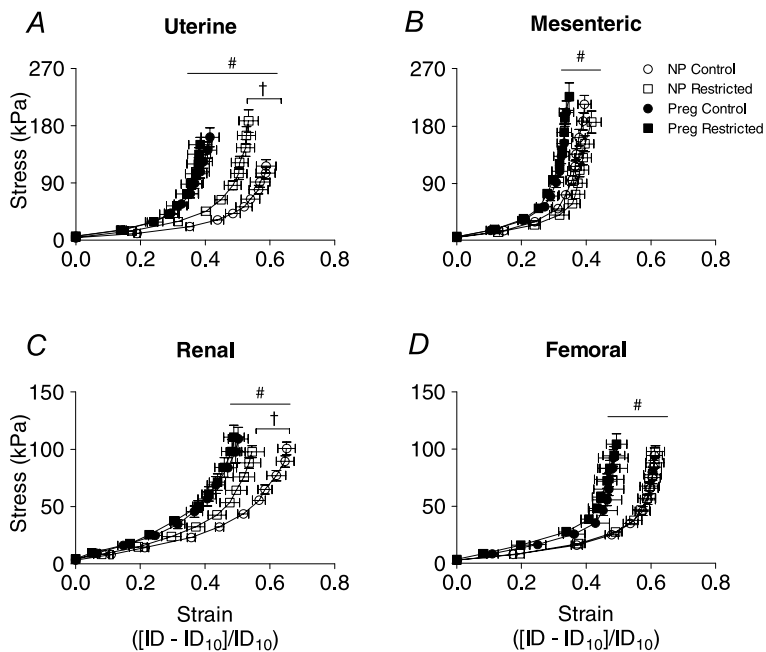


Figure 3. Passive mechanical wall properties in arteries

Stress–strain relationships for uterine (A), mesenteric (B), renal (C) and femoral (D) arteries from non-pregnant and pregnant (E20) animals. Values are expressed as means \pm SEM; uterine ($n = 8–10$ /group); mesenteric ($n = 6–10$ /group); renal ($n = 8–10$ /group); femoral ($n = 6–10$ /group). # $P < 0.05$ vs. non-pregnant (main effect). † $P < 0.05$ vs. non-pregnant Control (following significant interaction).

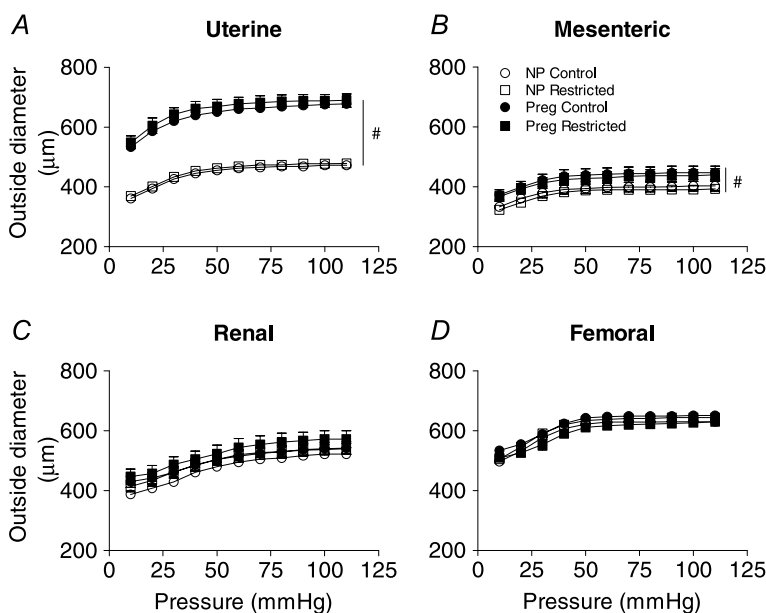


Figure 4. Outside diameters in arteries

Absolute ODs for uterine (A), mesenteric (B), renal (C) and femoral (D) arteries from non-pregnant and pregnant (E20) animals. Values are expressed as means \pm SEM; uterine ($n = 8–10$ /group); mesenteric ($n = 6–10$ /group); renal ($n = 8–10$ /group); femoral ($n = 6–10$ /group). # $P < 0.05$ vs. non-pregnant (main effect).

Table 4. Passive mechanical wall properties in arteries at 100 mmHg

Arteries	Non-pregnant control				Non-pregnant restricted			
	Uterine	Mesenteric	Renal	Femoral	Uterine	Mesenteric	Renal	Femoral
OD (μm)	473 \pm 9	403 \pm 15	522 \pm 12	627 \pm 5	479 \pm 6	394 \pm 6	540 \pm 18	644 \pm 7
ID (μm)	417 \pm 11	377 \pm 15	455 \pm 12	539 \pm 7	442 \pm 7	364 \pm 6	469 \pm 17	559 \pm 8
WT (μm)	28 \pm 2	14 \pm 1	34 \pm 1	44 \pm 3	19 \pm 1†	16 \pm 1	36 \pm 2	43 \pm 2
M-L ratio	0.068 \pm 0.007	0.035 \pm 0.002	0.075 \pm 0.005	0.081 \pm 0.006	0.042 \pm 0.004†	0.041 \pm 0.003	0.077 \pm 0.005	0.077 \pm 0.004
Arteries	Pregnant control				Pregnant restricted			
	Uterine	Mesenteric	Renal	Femoral	Uterine	Mesenteric	Renal	Femoral
OD (μm)	678 \pm 10#	447 \pm 23#	543 \pm 21	652 \pm 6	689 \pm 22#	441 \pm 20#	573 \pm 28	631 \pm 12
ID (μm)	617 \pm 11#	416 \pm 23#	474 \pm 17	560 \pm 8	626 \pm 21#	414 \pm 21#	504 \pm 27	551 \pm 11
WT (μm)	31 \pm 3	17 \pm 1	35 \pm 3	47 \pm 3	32 \pm 2§	14 \pm 1	36 \pm 3	40 \pm 3
M-L ratio	0.050 \pm 0.005‡	0.039 \pm 0.005	0.073 \pm 0.006	0.083 \pm 0.006	0.050 \pm 0.004	0.034 \pm 0.003	0.070 \pm 0.006	0.073 \pm 0.006

Outside diameter (OD), internal diameter (ID), wall thickness (WT) and media-to-lumen (M-L) ratio in arteries from non-pregnant and pregnant (E20) animals. Values are expressed as means \pm SEM; uterine ($n = 8-10/\text{group}$); mesenteric ($n = 6-10/\text{group}$); renal ($n = 8-10/\text{group}$); femoral ($n = 6-10/\text{group}$). # $P < 0.05$ vs. non-pregnant (main effect). † $P < 0.05$ vs. non-pregnant Control (following significant interaction). § $P < 0.05$ vs. non-pregnant Restricted (following significant interaction). ‡ $P < 0.05$ vs. non-pregnant Control (following significant interaction). — indicates no response.

was reduced in pregnant compared with non-pregnant animals and this correlated with increased OD and ID and increased stiffness during pregnancy. Using circular crossed-polarised light microscopy we demonstrated, for the first time, that pregnancy was associated with a ‘switch’ in the proportion of thick and thin collagen fibres, such that there was a reduction in thick (stiffer collagen) and an increase in thin (resilient collagen) collagen fibres in pregnant uterine arteries. Importantly, being born growth restricted did not alter collagen and elastin composition at 4 months of age.

Smooth muscle contraction

Growth restricted females had normal Ang II and PE-mediated contractions in both the non-pregnant or

pregnant state and this, may in part, explain the lack of elevated blood pressure at 4 months of age (Gallo *et al.* 2012). Absolute maximal contractile responses to K^+ -PSS, PE or Ang II (mN mm^{-1}) were also not different in any of the arteries of Restricted females. These data confirm our previous observations in our growth restricted 18-month-old female offspring (Mazzuca *et al.* 2010). Importantly, these data suggest that contractility *via* the adrenergic or Ang II receptor stimulation pathways or Ca^{2+} influx through voltage operated Ca^{2+} channels are intact in these arteries of Restricted female offspring. Although there were no differences between Control and Restricted groups, there was an effect with pregnancy. This study showed that in late pregnancy, uterine arteries of both Control and Restricted groups had reduced sensitivity to Ang II, increased sensitivity to PE, and

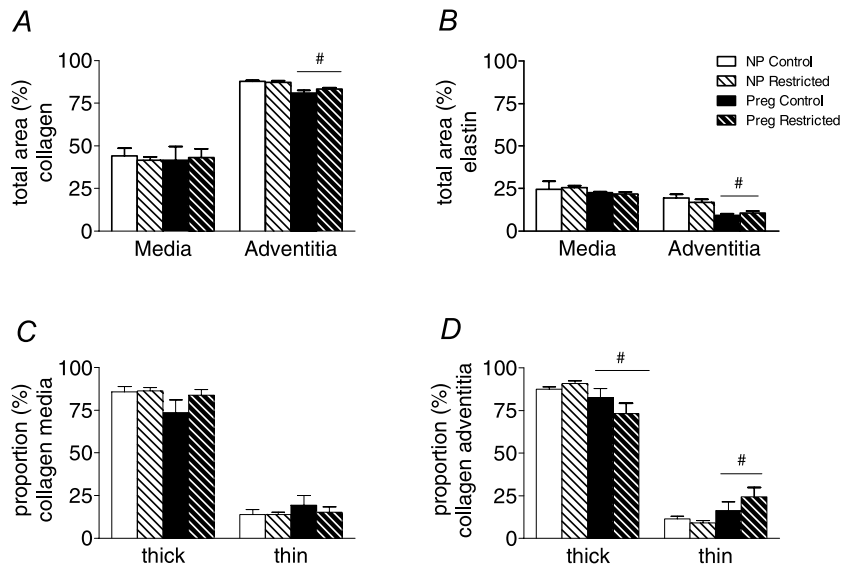


Figure 5. Collagen and elastin content in the uterine artery
Total collagen (A) and elastin (B) content, and thick and thin collagen content in media (C) and adventitia (D) in uterine arteries from non-pregnant and pregnant (E20) animals. Values are expressed as means \pm SEM; uterine ($n = 6-8/\text{group}$). # $P < 0.05$ vs. non-pregnant (main effect).

increased efficacy of both constrictors when compared with non-pregnant groups, and these findings are in accordance with previous studies (Annibale *et al.* 1989; D'Angelo & Osol, 1993; St Louis *et al.* 1997, 2001; Zwart *et al.* 1998; Xiao & Zhang, 2005). Alternatively, pregnancy had no effect on the sensitivity to Ang II and PE in mesenteric, renal and femoral arteries in both groups, highlighting the region-specific vascular effects that occur during pregnancy.

Endothelium-dependent and -independent relaxation

In the absence of NOS and COX inhibitors, total endothelium-dependent relaxation to ACh was unaltered in all arteries of non-pregnant or pregnant Restricted females when compared with Controls. These observations confirm our previous finding in Restricted females (Mazzuca *et al.* 2010). These data suggest that NO synthesis/bioavailability and/or release from endothelial cells were unaltered in Restricted females. This was further supported with our mRNA results showing no differences in eNOS expression in uterine and renal arteries of Restricted females when compared with Controls. In pregnant growth restricted offspring exposed to a low protein diet during F0 gestation, endothelium-dependent relaxation was impaired in mesenteric arteries (Torrens *et al.* 2003) but preserved in uterine arteries (Musha *et al.* 2011). Discrepancies in the results may be related to the nature and timing of the prenatal insult, growth profile trajectories, the location of the vascular bed used and the animal model studied. In our study, high concentrations of ACh caused contraction in renal arteries that were larger in arteries of Restricted females. This could be due to increased production of vasoconstrictor prostanoids, which stimulate thromboxane-prostanoid and/or prostaglandin E₂ receptors in the underlying smooth muscle or an interaction resulting from enhanced superoxide anion generation (Vanhoutte & Tang, 2008). Whether the ACh-induced vasoconstriction in the renal artery is endothelium dependent and/or due to a constrictor prostanoid would need to be explored in future studies by denuding the endothelium and/or adding a specific prostanoid receptor antagonists.

Pregnant Restricted females also had normal upregulation of total endothelium-dependent relaxation responses in mesenteric and uterine arteries. Sensitivity to ACh in the uterine arteries was ~2.5-fold greater in both pregnant groups, when compared with non-pregnant, and these findings agree with previous reports in uterine arteries from late pregnant rat and human (Ni *et al.* 1997; Nelson *et al.* 1998, 2000; Dalle Lucca *et al.* 2000*b*). This may be due to the greater synthesis and bioavailability of NO released from endothelial cells. This conclusion is supported by considerable work showing that eNOS

mRNA expression and protein activity are greater in uterine arteries of pregnant *vs.* non-pregnant animals (Bird *et al.* 2003). The increased sensitivity to ACh observed in the mesenteric arteries (by ~1.3-fold) but not in renal and femoral arteries of pregnant Control and Restricted offspring (~1.3-fold) also agree with earlier reports (Davidge & McLaughlin, 1992; Kim *et al.* 1994; Yamasaki *et al.* 1996; Gerber *et al.* 1998; Cooke & Davidge, 2003). In the systemic circulation, NO metabolites nitrite and nitrate are known to be upregulated during pregnancy (Conrad *et al.* 1993) and this may in part explain the increase in sensitivity to endothelial stimulation in the mesenteric artery. Taken together, these data suggest that pregnancy enhances total endothelium-dependent relaxation in a region-specific manner and that growth restriction did not alter this process.

EDHF-mediated relaxation plays an important role in small resistance vessels, which are important for vascular resistance and organ blood flow (Edwards *et al.* 2010). EDHF generation is underpinned by the activation of endothelial K_{Ca}2.3 and K_{Ca}3.1, leading to hyperpolarisation of the endothelium and relaxation of vascular smooth muscle. In this study, sensitivity and maximal EDHF-mediated relaxation were also unaltered in all arteries of non-pregnant or pregnant Restricted females compared with their Control counterparts. This is supported by the lack of difference in mRNA expression of K_{Ca}2.3 and K_{Ca}3.1 in uterine and renal arteries. Pregnancy potentiated EDHF-mediated relaxation in uterine and mesenteric arteries but not in renal arteries in both Control and Restricted. In fact, in late pregnancy we observed ~3.4-fold rightward shift in sensitivity to ACh in the presence L-NAME and Indo when compared to no blockers in the uterine artery, suggesting that most of the ACh-induced relaxation is largely EDHF mediated. However, we cannot exclude the possibility that PGI₂ synthesis may also have been upregulated in the pregnant uterine artery (Poston *et al.* 1995). Indeed, EDHF-mediated relaxation was increased in uterine and mesenteric resistance arteries of the pregnant rat (Gerber *et al.* 1998; Dalle Lucca *et al.* 2000*a, b*) and subcutaneous, myometrial and omental arteries of pregnant humans (Pascoal & Umans, 1996; Kenny *et al.* 2002; Gillham *et al.* 2003; Luksha *et al.* 2004; Lang *et al.* 2007). These data suggest that pregnancy enhances EDHF mediated relaxation in a region-specific manner and that growth restriction did not alter this process.

Lastly, relaxations to SNP or K_{ATP} channel opener LEV were not different between non-pregnant or pregnant Control and Restricted groups for all arteries tested. These results suggest that the soluble guanylyl cyclase signalling pathways and K_{ATP}-dependent membrane hyperpolarisation and relaxation pathways in the smooth muscle are intact in Restricted females and unaffected with pregnancy.

Summary

Late gestation uteroplacental insufficiency does not, by and large, impair maternal vascular adaptations when growth restricted females become pregnant. The major resistance arteries of these females essentially had normal smooth muscle and endothelial function, and the enhanced uterine and renal artery stiffness in non-pregnant growth restricted females was completely resolved during late pregnancy. Although the vascular adaptations during late pregnancy were preserved in pregnant growth restricted offspring, we have recently reported that these females have fetuses (F2) that are 6% lighter at day 20 of gestation when compared to fetuses of F1 control mothers (Gallo *et al.* 2012). It is likely that other factors including programmed maternal endocrine factors and physiological stressors or epigenetic modifications of the F1 gametes may also be involved in the programming of fetal weight across subsequent generations.

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Author contributions

All authors contributed to the conception and design, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and all authors gave final approval of the version to be published. This study was conducted at The University of Melbourne and Monash University.

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