*Journal of Developmental Origins of Health and Disease* (2012), **3**(5), 333–341.

doi:10.1017/S2040174412000268

ORIGINAL ARTICLE

# The effect of hypoxia-induced intrauterine growth restriction on renal artery function

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The risk of developing cardiovascular diseases is known to begin before birth and the impact of the intrauterine environment on subsequent adult health is currently being investigated from many quarters. Following our studies demonstrating the impact of hypoxia *in utero* and consequent intrauterine growth restriction (IUGR) on the rat cardiovascular system, we hypothesized that changes extend throughout the vasculature and alter function of the renal artery. In addition, we hypothesized that hypoxia induces renal senescence as a potential mediator of altered vascular function. We demonstrated that IUGR females had decreased responses to the adrenergic agonist phenylephrine (PE; $pEC_{50}$ $6.50 \pm 0.05$ control *v.* $6.17 \pm 0.09$ IUGR, $P < 0.05$) and the endothelium-dependent vasodilator methylcholine (MCh; $E_{max}$ $89.8 \pm 7.0\%$ control *v.* $41.0 \pm 6.5\%$ IUGR, $P < 0.001$). In IUGR females, this was characterised by increased basal nitric oxide (NO) modulation of vasoconstriction (PE $pEC_{50}$ $6.17 \pm 0.09$ IUGR *v.* $6.42 \pm 0.08$ in the presence of the NO synthase inhibitor *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME; $P < 0.01$) but decreased activated NO modulation (no change in MCh responses in the presence of L-NAME), respectively. In contrast, IUGR males had no changes in PE or MCh responses but demonstrated increased basal NO (PE $pEC_{50}$ $6.29 \pm 0.06$ IUGR *v.* $6.42 \pm 0.12$ plus L-NAME, $P < 0.01$) and activated NO ($E_{max}$ $37.8 \pm 9.4\%$ control *v.* $-0.8 \pm 13.0\%$ plus L-NAME, $P < 0.05$) modulation. No significant changes were found in gross kidney morphology, proteinuria or markers of cellular senescence in either sex. In summary, renal vascular function was altered by hypoxia *in utero* in a sex-dependent manner but was unlikely to be mediated by premature renal senescence.

*Received 22 July 2011; Revised 9 March 2012; Accepted 30 March 2012; First published online 25 April 2012*

**Key words:** intrauterine growth restriction, renal, senescence, vascular function

## Introduction

Cardiovascular diseases are a major health problem and are the leading cause of death worldwide. It is becoming increasingly clear that the risk of developing cardiovascular diseases in adult life is determined, in part, before birth (reviewed by Barker[1]). In compensation for a poor intrauterine environment, the fetus develops long-lasting adaptations in cardiovascular, metabolic and endocrine function, which can lead to an enhanced susceptibility to cardiovascular disease in adult life.[2–4] There is a growing body of evidence suggesting that a sub-optimal *in utero* environment can cause conditions such as endothelial dysfunction,[5–8] hypertension,[9] coronary artery disease,[10] obesity[11] and diabetes[12] later in life.

Intrauterine growth restriction (IUGR) has many different causes but is most commonly due to a placental insufficiency leading to an inadequate nutrient and oxygen supply to the fetus. Animal models developed to investigate this area have utilized a decrease in the supply of nutrients, oxygen or both to the fetus; however, each model has its limitations. The fetus has some recourse to adjust for a limited nutrient supply,[13] however, there are few mechanisms to compensate for a limited oxygen supply; which is a common occurrence in several obstetric pathological conditions that may lead to IUGR such as pre-eclampsia, placenta previa, maternal anemia and smoking. We, and others, have previously shown that hypoxia *in utero*, leading to growth restricted offspring, causes endothelial dysfunction in systemic resistance arteries.[5–8,14–16] Modifications in vascular function, particularly in resistance arteries, contribute to the acute regulation of blood pressure while chronic regulation of blood pressure is primarily mediated by the kidney. The early development of the kidney can be greatly affected by an adverse perinatal environment.[17] For example, IUGR infants display disproportionately reduced fetal kidney growth and have fewer nephrons at birth compared with normal birth weight infants.[18,19] Similarly, in animal models, uteroplacental insufficiency, prenatal dexamethasone or alterations in maternal nutrient intake have been shown to reduce nephron number in offspring, which is often associated with

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later development of spontaneous hypertension.[20–27] In addition, adverse intrauterine conditions have been shown to cause decreased glomerular filtration rate, altered expression of renal sodium transporters,[28–31] disturbances of the renin-angiotensin-aldosterone system (RAAS),[32–35] decreased levels of 11β-hydroxysteroid dehydrogenase[36,37] and increased nephrosclerosis[38] leading, eventually, to hypertension, renal disease and other cardiovascular diseases.[39] The early post-natal environment has been shown to further impact developmental programming. Rapid catch-up growth, for example, has been associated with premature vascular, renal and cardiac senescence, all of which likely contribute to vascular dysfunction and hypertension.[40,41] The role of cellular senescence in the effects of intrauterine hypoxia on renal vascular function is, however, unknown.

In IUGR rodent offspring, bilateral renal denervation has been found to reverse or prevent elevated blood pressure.[42,43] In addition, circulating catecholamines have been found to be increased in growth restricted girls but not boys[35] and in IUGR rats,[44] and increased sympathetic nervous system activity was found in female, but not male, IUGR rats.[45] These results suggest that growth restriction causes increased sympathetic innervation leading to enhanced vascular constriction and hypertension. More interestingly, there is a strong suggestion that these effects of IUGR may be sex-dependent. To date, few studies have investigated vascular function of renal arteries of offspring born from complicated pregnancies.[46–48] In one of these studies, intrauterine stress was induced by ligation of the uterine arteries at day 13 of pregnancy in Wistar Kyoto rats and changes in adrenoreceptor-mediated responses were found in male offspring exposed to intrauterine stress; the renal artery showed enhanced vasoconstrictor ($\alpha_1$ and $\alpha_2$) and vasodilator ($\beta_1$ and $\beta_2$) responses.[46,48] Although many systemic vascular effects of hypoxia-induced IUGR have been determined,[14–16,49–51] the specific effects on renal vascular function are not known. Our own lab has shown changes in endothelium-dependent vasodilator function in mesenteric arteries from a hypoxia-induced IUGR model in response to both pharmacological agonists and the physiological stimuli of flow,[14,52] changes which were sex-dependent. We hypothesized that these changes may extend throughout the vasculature and may alter vascular function of the renal artery following growth restriction *in utero*, potentially in a sex-dependent manner. In addition, we hypothesized that exposure to hypoxia may induce renal senescence as a potential mediator of altered vascular function.

## Materials and methods

### *Animals and study groups*

All protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines. As previously described,[14,49,52,53] female Sprague Dawley rats, 3 months old, were acclimatised in-house before breeding and maintained on a standard diet and tap water in a 12:12 h light:dark cycle. Pregnant dams were randomly allocated to control or hypoxia groups on day 15 of gestation. Maternal hypoxia was induced by placing rats into a Plexiglas chamber (A-Chamber, BioSpherix, NY, USA) where oxygen supply was reduced to 11.5% using a ProOx Oxygen Controller (BioSpherix, NY, USA) supplying a continuous infusion of an air/nitrogen mixture. Control animals were kept in a room air (21% oxygen) environment. On day 21 of pregnancy the rats were removed from the chamber and allowed to give birth in a normal oxygen environment. Litters were reduced to eight pups, four males and four females, to avoid the impact of competition for milk on the subsequent growth of the offspring. Pups were weaned at 3 weeks into standard cages and housed under standard conditions until the experimental day at 4 months of age. A total of 22 control offspring (from 10 litters) and 18 offspring from dams exposed to a hypoxic environment (IUGR, from 10 litters) were used for experimental procedures. Offspring were chosen for each experimental procedure such that no two animals in one experiment came from the same litter; hence the dam was the experimental unit.

### *Pressure myography*

Pressure myography was used to measure physiological vascular function of renal arteries. Animals were killed by exsanguination via excision of the superior vena cava under inhaled isoflurane anesthesia and the left kidney, including the renal artery and a section of the abdominal aorta, was excised. For consistency, the left artery was always chosen for study. The tissue was placed in ice-cold physiological saline solution (PSS, composition: 10 mM HEPES, 5.5 mM glucose, 1.56 mM $CaCl_2$, 4.7 mM KCl, 142 mM NaCl, 1.17 mM $MgSO_4$, 1.18 mM $KH_2PO_4$, pH 7.5). The renal artery and branches were dissected free of renal, adipose and connective tissues under a stereo-microscope. Taking into account the length, diameter and branching of the vessels, third-order arteries were found to be most suitable for vascular studies. Two arteries were mounted and tied to two glass cannulae in 2.5 ml PSS-containing chambers of a pressure myograph (Living Systems, Burlington, VT, USA). Intraluminal pressure was controlled by a peristaltic pump and set to 100 mmHg to mimic the physiological *in vivo* pressure of this artery. The temperature of the baths was maintained at 37°C. To measure arterial lumen diameter, the myograph was placed under a microscope connected to a video dimension analyser and video monitor.

All vessels were equilibrated for 1 h with multiple changes in PSS during which time pressure was increased to 140 mmHg for 10 min and then reset to 100 mmHg. The responses (change in lumen diameter) of arteries to the adrenergic vasoconstrictor phenylephrine (PE; 0.01 nM to 1 mM) or the endothelium-dependent vasodilator methylcholine (MCh; 0.01 nM to 10 μM) were observed in the presence or

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absence of the nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μM). All drugs were administered extralumenally into the bathing solution.

### *Nephron structure*

At the time of euthanasia, right kidneys were excised and a portion fixed in 10% formalin. Histopathological preparations and hematoxylin/eosin staining were performed at the Alberta Diabetes Institute Histology Core (Edmonton, Canada). Tissue sections were examined by light microscopy.

### *Proteinuria*

At the time of euthanasia, urine samples were aspirated from the bladder. Urine was snap frozen in liquid nitrogen and stored at −80°C until the time of analysis. Total urine albumin concentrations were measured using the AssayMax Rat Albumin ELISA Kit (AssayPro, catalog number ERA3201-1). Urine creatinine was measured using the Creatinine (Urinary) Assay Kit (Cayman Chemical Company, catalog number 500701). Proteinuria was quantified and expressed as the albumin to creatinine ratio.

### *Senescence markers*

At the time of euthanasia, right kidneys were excised and a portion was snap frozen in liquid nitrogen and stored at −80°C until the time of analysis. Total RNA was extracted from whole kidney samples using the RNeasy Minikit (Qiagen, Mississauga, ON, Canada). Expression of senescence markers p16, p21, p53 and the mitochondrial stress marker p66shc were analyzed by real time RT-PCR as previously described.[41] Gene transcript levels were calculated by the ΔCT method and expressed as percent hypoxanthine-guanine phosphoribosyltransferase (HPRT).

### *Data collection and statistical analysis*

Data were presented as mean ± S.E. Vasodilator responses were calculated as a percentage of constrictor tone. All data were normally distributed as tested using the Kolmogorov–Smirnov test for Gaussian distribution. Sources of variability considered in the model include sex (male, female) and prenatal treatment (control, IUGR), leading to four study groups. The significance of the difference in mean values of continuous variables between groups was determined by a two-way analysis of variance (ANOVA), with Bonferroni post-test for multiple comparisons. Differences between groups in physical characteristics were analyzed by Students' *t*-test.

## Results

### *Body weight*

Despite a significant reduction in body weight at birth in this model (data previously shown[14]), at 4 months of age hypoxia *in utero* had no effect on body weight in males (492 ± 17 g controls *v.* 480 ± 17 g IUGR), or females (298 ± 12 g controls *v.* 275 ± 11 g IUGR).

### *Vascular function*

Maximal responses to PE in the left renal artery were unaltered by sex or hypoxia *in utero*, however, there was a significant effect of IUGR on sensitivity to PE in females but not males (Table 1).

Vasodilation to MCh in the renal arteries of male offspring was unaltered by hypoxia *in utero*; no significant change in maximal vasodilation or sensitivity between IUGR and control offspring was observed (Fig. 1a). In the control male group, there was no effect of L-NAME on vasodilation ($P > 0.05$, Fig. 1b), however, the IUGR male group showed a significant reduction in MCh-induced vasodilation in the presence of L-NAME ($P = 0.013$, Fig. 1c).

In females, however, renal arteries from IUGR animals demonstrated a significant reduction in vasodilation to MCh compared with controls ($P = 0.0005$, Fig. 2a). Further, L-NAME significantly inhibited vasodilation in controls ($P = 0.031$, Fig. 2b), but did not inhibit vasodilation to MCh in offspring exposed to hypoxia *in utero* ($P > 0.05$, Fig. 2c).

### *Morphology*

During vascular function experiments, we found no differences in renal artery diameters in either males (control 613 ± 33 μm *v.* IUGR 611 ± 54 μm) or females (control

**Table 1.** *Sensitivity ($pEC_{50}$) of renal arteries from 4-month-old rat offspring to PE*

| | Control | | IUGR | | Two-way ANOVA significance | | |
|---|---|---|---|---|---|---|---|
| | w/o Inhibitor | L-NAME | w/o Inhibitor | L-NAME | IUGR | L-NAME | Int. |
| Male | 6.28 ± 0.14 (15) | 6.39 ± 0.13 (12) | 6.29 ± 0.06 (10) | 6.42 ± 0.12 (9) | | | |
| Female | 6.50 ± 0.05 (7) | 6.55 ± 0.10 (6) | 6.17 ± 0.09 (8) | 6.42 ± 0.08 (6) | * | | |

PE, phenylephrine; IUGR, intrauterine growth restriction; ANOVA, analysis of variance; L-NAME, *N*-nitro-L-arginine methyl ester hydrochloride; Int., interaction effect of two-way ANOVA. *$p < 0.05$, no value; $p > 0.05$.

In females, but not males, there was a significant effect of IUGR on the sensitivity of PE responses; data presented as mean ± S.E. (*n*).

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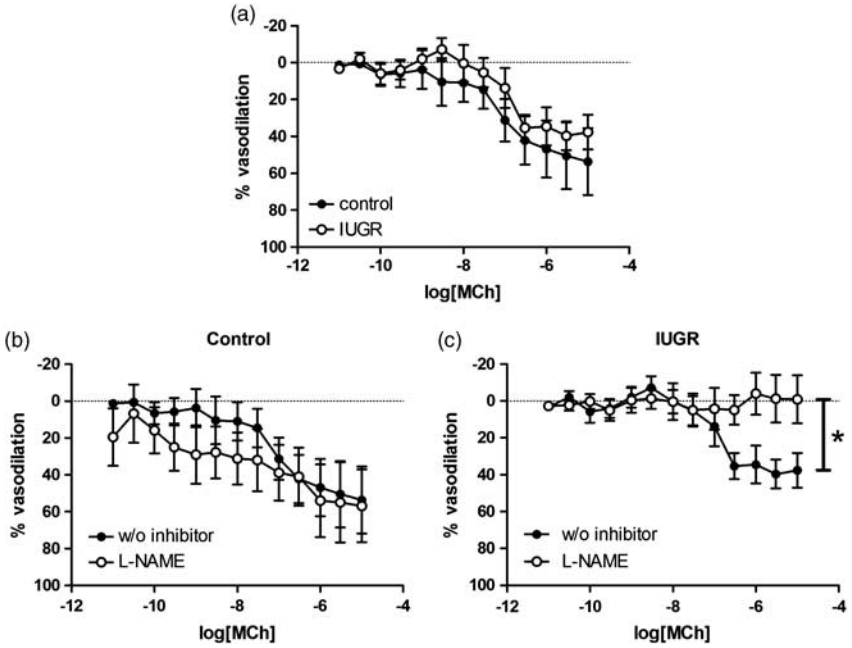


**Fig. 1.** Vasodilator responses to methylcholine (MCh) in the renal artery of 4-month-old male offspring exposed to hypoxia *in utero* (intrauterine growth restriction; IUGR) and controls (*a*, $n = 8$–10). Vasodilator responses to MCh in controls (*b*, $n = 6$–7) and IUGR offspring (*c*, $n = 8$–10) in the presence or absence (w/o inhibitor) of the nitric oxide synthase inhibitor, *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME; $^{*}P < 0.05$, two-way analysis of variance with a Bonferroni post-test).

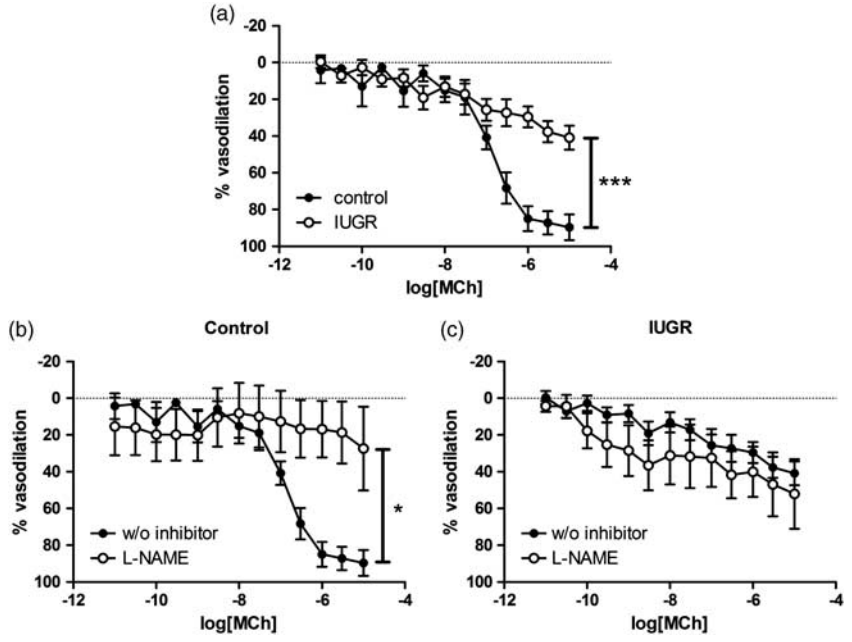


**Fig. 2.** Vasodilator responses to methylcholine (MCh) in the renal artery of 4-month-old female offspring exposed to hypoxia *in utero* (intrauterine growth restriction; IUGR) and controls (*a*, $n = 5$–7). Vasodilator responses to MCh in controls (*b*, $n = 5$) and IUGR offspring (*c*, $n = 5$–7) in the presence or absence of the nitric oxide synthase inhibitor, *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME; $^{*}P < 0.05$, $^{***}P < 0.001$, two-way analysis of variance with a Bonferroni post-test).

549 ± 24 μm *v.* IUGR 476 ± 28 μm; $P > 0.05$). Given the observed differences in vascular function, which may lead to differences in renal perfusion, we investigated renal morphology by histopathological techniques. Examination of the gross morphology of the kidneys did not uncover any differences in renal structures (Fig. 3).

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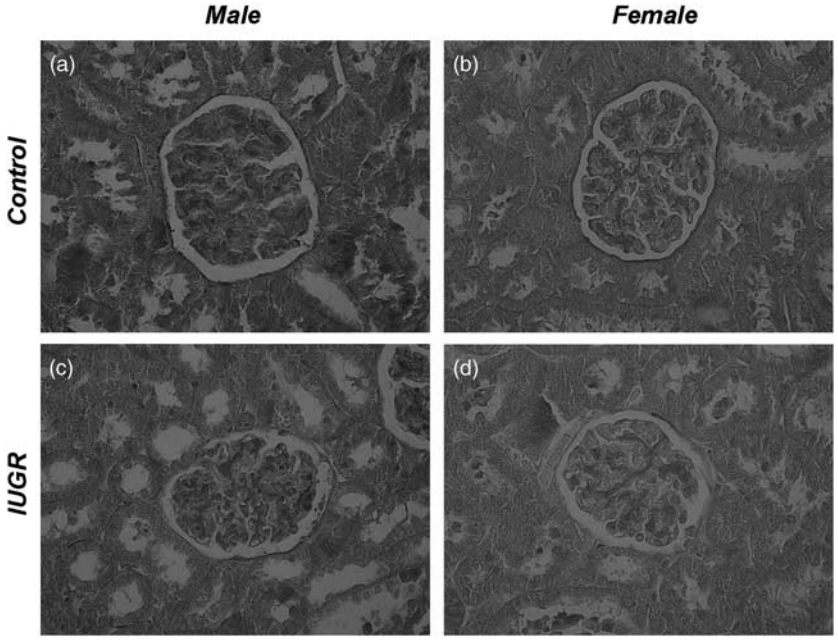


**Fig. 3.** Kidney sections from male control (*a*), male intrauterine growth restriction (IUGR) (*c*), female control (*b*) and female IUGR (*d*) rats. Representative images of kidney sections stained with hemotoxylin and eosin to investigate morphological characteristics.

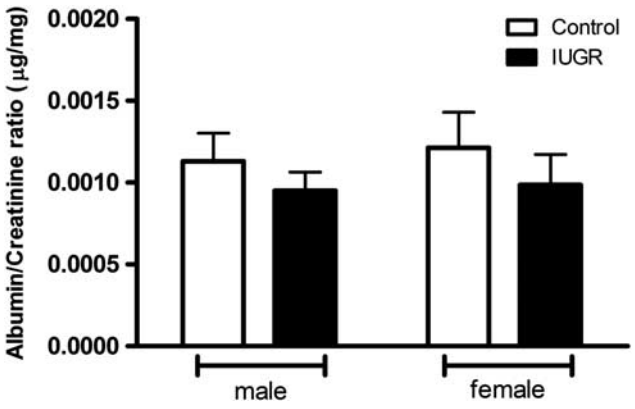


**Fig. 4.** Urine albumin/creatinine ratios in 4-month-old male and female, control and intrauterine growth restriction (IUGR) offspring ($P > 0.05$, two-way analysis of variance, $n = 5$–6 per group).

### *Proteinuria*

Urinary albumin excretion was examined as an early marker of glomerular hypertension and renal dysfunction. Adjusted for urine creatinine levels, there was no change in the albumin–creatinine ratio in IUGR males or females (Fig. 4).

### *Senescence markers*

Expression levels of the senescence markers p53, p21 and p16 and the mitochondrial stress marker p66shc in whole kidney tissue were not significantly different in IUGR males and females (Fig. 5a–5d, $P > 0.05$). By two-way ANOVA analysis, expression of p16 demonstrated a non-significant tendency to be increased in both IUGR males and females compared with their respective controls (Fig. 5c, $P = 0.089$).

## Discussion

Despite the well-recognised impact of growth restriction on the cardiovascular system, this study is one of the first to address the long-term impact of IUGR caused by reduced oxygen *in utero* on the function of the renal vasculature, an important organ in long-term blood pressure regulation. We have demonstrated that MCh-induced renal vasodilation in females at 4 months of age was reduced by hypoxia exposure *in utero,* while this parameter was unaffected in 4-month-old males.

Interestingly, in only male offspring, we observed a greater contribution of the nitric oxide (NO) pathway to MCh-induced vasodilation in IUGR *v.* controls. These data would suggest an upregulation of the NO pathway in stimulated conditions that may have been programmed by a compromised intrauterine environment and, further, may explain the unaltered vasodilation to MCh in the male IUGR group. Interestingly, contrary to the findings of previous studies performed in the renal arteries of rat models including hypertensive SHRs, diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) and Sprague Dawley rats, an NO contribution to vasodilation was absent in the male control group. In these studies renal arteries from both control and pathologic animals exhibited a dependence on both NO and endothelial derived hyperpolarizing factors (EDHF) for vasodilation.[54–59] Our data suggest that, in control males, pathways other than NO, for example, prostacyclin ($PGI_2$) or EDHF may be more important in vasodilation. A PE-induced increase in

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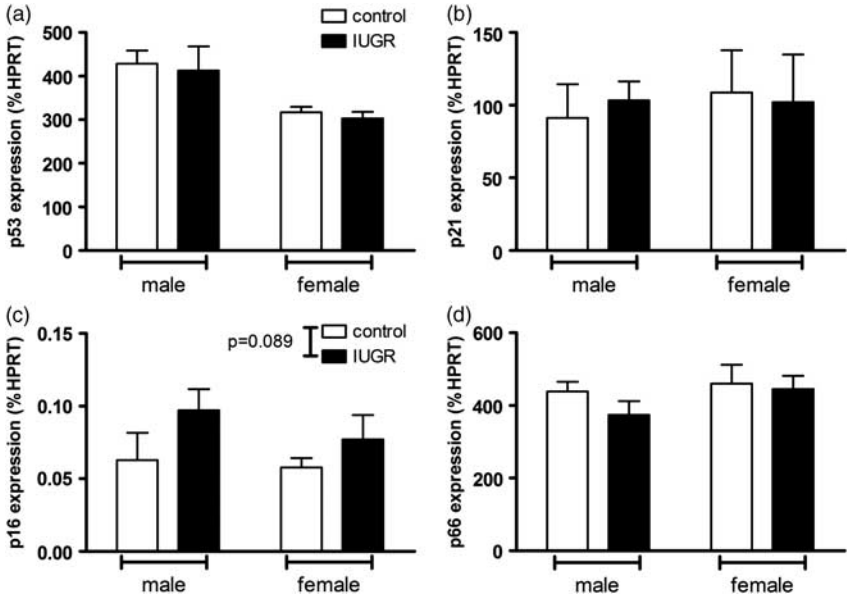


**Fig. 5.** Expression levels of the senescence markers p53 (*a*), p21 (*b*) and p16 (*c*) and the mitochondrial stress marker p66shc (*d*) in the kidneys of 4-month-old male and female, control and intrauterine growth restriction (IUGR) offspring ($P > 0.05$, two-way analysis of variance, $n = 6$ per group). HPRT, hypoxanthine-guanine phosphoribosyltransferase.

endothelial calcium may also be responsible for a greater release of EDHF and this may represent a compensatory mechanism in male offspring exposed to hypoxia *in utero*. Further studies would need to be performed to investigate the endothelium-dependent mechanisms responsible for renal artery vasodilation in this group.

Conversely, in females we found that hypoxia *in utero* led to a significant loss of MCh-induced vasodilation in the renal arteries. In addition, in females but not in males, there was a significant effect of IUGR on the sensitivity of renal arteries to PE. Contrary to males, a MCh-induced NO component was present in control females. These data suggest that, in female offspring, hypoxia *in utero* results in a downregulation of normal NO-mediated vasodilator mechanisms, which have an impact on the overall ability of the vessel to dilate. In this case, females appear to be affected by growth restriction to a greater extent than males. These findings are consistent with our previous work in this model where NO-induced vasodilation was also reduced in mesenteric arteries.[14] Given our observations of changes in NO-mediated vasodilation, it is possible that sex-specific changes in underlying NOS activity may be an explanatory factor in the different responses to the insult of IUGR. Although Franco *et al.*[60] have previously found there to be sex-specific changes in eNOS expression and activity in the aortae from a rat model of intrauterine undernutrition; these data would suggest a greater impact of growth restriction on the male offspring. This discrepancy may reflect vascular-bed specific alterations in NO pathways.

Interestingly, changes in the vascular function of the renal artery were not found to be associated with any changes in gross morphology or with proteinuria. The changes previously observed in the mesenteric arcade were reminiscent of an 'early aging' phenotype.[14,52] A study of the messenger ribonucleic acid (mRNA) expression of senescence markers did not show any significant differences between control and IUGR whole kidneys from either males or females. Analysis of whole kidney RNA may have missed a localized increase in vascular senescence. The marker, p16 represents one factor in a distinct pathway leading from environmental stressors to hypophosphorylated retinoblast protein, an indicator of accelerated or earlier senescence.[41] In our study we noted that p16 may be increased by IUGR and this was mirrored in both males and females. This may represent a very early indication of accelerated aging due to IUGR in these groups; however, since the animals were only studied at 4 months of age, representing young adults, these changes may be further amplified with increased age and would need to be studied at older age points to confirm or refute this observation. In addition, the levels of protein expression could also be verified to further delineate any potential changes in renal senescence.

The major strength of this study is that it is the first to describe renal artery function in this model of IUGR. To our knowledge, only three studies have previously investigated vascular function of renal arteries following a compromised pregnancy.[46–48] Sanders *et al.*, studied renal arteries from 21-day-old males with IUGR induced by ligation of the uterine arteries at day 13 of pregnancy in Wistar Kyoto rats. The renal artery showed enhanced vasoconstrictor ($\alpha_1$ and $\alpha_2$) and vasodilator ($\beta_1$ and $\beta_2$) responses in these rats, but no change in adrenergic responsiveness was found in mesenteric, femoral or saphenous arteries.[46] These authors reported similar findings in male offspring at 21 days, where IUGR

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was induced by maternal uterine artery ligation on day 17 of gestation.[46] Intriguingly in this study, enhanced vasodilation was observed in the right, but not the left renal artery of IUGR offspring, accompanied by an upregulation of $\alpha_2$-adrenoreceptor expression in the right kidney only.[48] The cause of the asymmetry of renal artery responsiveness is not known, but sympathetic nerve fiber density was found to be greater in left renal arteries in this study. Maximal contractile responses to PE were not affected by IUGR or laterality. Vascular reactivity of the renal, uterine, mesenteric and femoral arteries was also studied in 18-month-old female rats with IUGR induced by ligation of uterine vessels at 18 days of gestation.[47] These females were found to have selective uterine artery endothelial dysfunction, with reduced relaxation in response to EDHF, and increased stiffness. Vascular structure and function were largely preserved in the other arteries studied, although a small decrease in renal artery stiffness was noted. The authors do not mention whether left or right arteries were studied. The variability of results between these and our study likely reflects different animal models of IUGR, ages, gender, vascular bed and size and laterality of the vessel studied. Each of the prior studies has utilized first order renal arteries, which are conduit arteries, whereas we have studied 3rd order arteries, which are resistance vessels. In addition, all three studies utilized uteroplacental insufficiency as a programming model, which in addition to inducing fetal hypoxia, also results in nutrient restriction. In our model, hypoxia may have been more severe and, therefore, differs significantly from these published studies and adds new information to the field. Future studies should include investigation of the sympathetic innervation of the kidneys in our model. In general, most studies describe a relative loss of vasodilation in the IUGR offspring, which conceivably does participate in programmed susceptibility to premature hypertension, cardiovascular and renal disease.

In summary, this study demonstrated that IUGR females had decreased sensitivity to both PE and MCh that was characterised by increased basal but decreased activated NO. In contrast, IUGR males had no changes in PE or MCh responses but demonstrated increased basal and activated NO, which may have compensated for decreased endothelial function. No changes were found in gross kidney morphology, proteinuria or markers of cellular senescence in either sex although there was a suggestion of increased expression of the senescence marker p16. In summary, renal vascular function was altered by growth restriction *in utero* in a sex-dependent manner but these changes were unlikely to be mediated by renal senescence.

## Acknowledgments

The study was supported by grants from the Canadian Institute of Health Research. S.T. Davidge is funded by Alberta Innovates Health Solutions as an Alberta Heritage Foundation for Medical Research Scientist and is a Canada Research Chair (CRC) in Women's Cardiovascular Health. V. Luyckx is funded by the University of Alberta Hospital Foundation, Amgen Senior Research Fellowship, Western Canada.

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