Renal Dysfunction Is Associated With a Reduced Contribution of Nitric Oxide and Enhanced Vasoconstriction After a Congenital Renal Mass Reduction in Sheep

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Background—Children born with reduced congenital renal mass have an increased risk of hypertension and chronic kidney disease in adulthood, although the mechanisms are poorly understood. Similar sequelae occur after fetal uninephrectomy (uni-x) in sheep, leading to a 30% nephron deficit. We hypothesized that renal dysfunction is underpinned by a reduced contribution of nitric oxide (NO) and vascular dysfunction in uni-x sheep.

Methods and Results—In 5-year-old female uni-x and sham sheep, mean arterial pressure, glomerular filtration rate, and renal blood flow were measured before and during NO inhibition (Nω-nitro-L-arginine methyl ester [L-NAME]). Reactivity was assessed in resistance arteries, including renal lobar and arcuate arteries. Basal mean arterial pressure was 15 mm Hg higher and glomerular filtration rate and renal blood flow were ~30% lower (P<0.001) in uni-x animals. L-NAME increased mean arterial pressure by ~17 mm Hg in both groups, whereas glomerular filtration rate and renal blood flow were decreased less in uni-x sheep (P<0.01). Endothelial NO synthase and Ser-1177–phosphorylated endothelial NO synthase protein levels were upregulated in renal cortex of uni-x sheep (P<0.05). Lobar arteries of uni-x sheep had enhanced responsiveness to phenylephrine and nitrotyrosine staining and reduced sensitivity to endothelial stimulation. Vasodilator prostanoid contribution to endothelium-dependent relaxation was reduced in lobar arteries of uni-x sheep, accompanied by reduced cyclooxygenase-1 and -2 gene expression (P<0.05). Neurovascular constriction was enhanced ~1.5-fold in renal arteries of uni-x sheep (P<0.05).

Conclusions—Renal dysfunction after congenital renal mass reduction is associated with impaired regulation of renal hemodynamics by NO. Reductions in renal blood flow and glomerular filtration rate are underpinned by impaired basal NO contribution, endothelial dysfunction, and enhanced vascular responsiveness to sympathetic nerve stimulation. (Circulation. 2015;131:280-288. DOI: 10.1161/CIRCULATIONAHA.114.013930.)

Key Words: arteries ■ endothelium-derived relaxing factors ■ hypertension ■ kidney ■ nitric oxide

Epidemiological studies reveal that children born with a single functioning kidney have a predisposition to developing renal insufficiency and hypertension in adult life. A congenital reduction in renal mass is present in patients with unilateral renal agenesis (≈1 in 500 births) or unilateral multicystic kidney (≈1 in 4300 births). A reduced congenital renal mass results in impaired renal function, with 50% of patients developing hypertension before 18 years of age and a 50% probability of requiring dialysis by 30 years of age. Furthermore, individuals born with low birth weight have smaller kidneys and lower estimated glomerular filtration rate (GFR), with an increased risk of developing cardiovascular diseases. Many animal models of early life insults have a similar relationship between low birth weight and the development of hypertension in adulthood, with this link appearing to be mediated, at least in part, by an associated congenital nephron deficit. Substantial evidence suggests that developmental perturbations that alter kidney development play a critical role in the pathogenesis of hypertension. However, the mechanisms underlying the alterations in renal function are poorly understood, especially in more clinically relevant animal models of advanced age.

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We have established an ovine model of fetal uninephrectomy (uni-x) that replicates the consequences of a congenital nephron deficit in programming hypertension without the confounding effects of low birth weight often linked with other models. In this model, renal dysfunction precedes the onset of hypertension in female uni-x sheep, with a decline

Received June 28, 2013; accepted October 24, 2014.
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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.114.013930/-/DC1.
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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.114.013930.
in GFR observed at 1 year of age, before the development of hypertension, that is associated with elevations in blood volume and cardiac output at 2 years of age. Furthermore, in this model, a greater reduction in renal blood flow (RBF), associated with increases in renal and total peripheral resistance, was observed between 2 and 5 years of age, indicative of disease progression with age. Similarly, uni-x in rats on postnatal day 1 (a period of active nephrogenesis in rodents) results in age-dependent reductions in GFR and hypertension. Nitric oxide (NO) produced within the kidney plays an important role in the regulation of renal hemodynamics and sodium excretion, thus maintaining systemic vascular volume and blood pressure. Vascular endothelial and smooth muscle cells and the extracellular matrix are also important determinants of vascular homeostasis and blood pressure regulation. Deficiency in NO production/bioavailability has been reported in different animal models of chronic kidney disease associated with renal mass reduction and aging, contributing to a progressive loss of renal function. Moreover, vascular dysfunction in adulthood is a common legacy of exposure to early life insults and may contribute to the development of hypertension.

The aim of the present study was to investigate the cardiovascular and renal responses to in vivo NO synthase (NOS) inhibition via the administration of L-Nω-nitro-l-arginine methyl ester (L-NAME) in conscious female uni-x and sham sheep at 5 years of age. The effects of fetal uni-x on smooth muscle and endothelial function, passive wall stiffness, nerve-mediated vasoconstriction, and oxidative stress were assessed in isolated renal arteries. To examine the effects of uni-x over the wider circulation, resistance arteries from 3 other vascular beds (mesenteric, femoral, and coronary) were also studied. We hypothesized that NO-mediated control of renal and vascular function is impaired in animals born with a congenital reduction in renal mass.

Methods

Please refer to the online-only Data Supplement for an expanded Methods section.

Animals

All experiments were approved by the Monash University, School of Biomedical Sciences Animal Ethics Committee and were in agreement with the guidelines of the National Health and Medical Research Council of Australia. Merino ewes carrying female fetuses of known gestational age underwent surgery at 100 days after conception, as described previously. The left renal artery, vein, and ureter were ligated, and the kidney was removed in the uni-x group. The kidney was cleared of surrounding fat but left intact in the sham group. After natural birth, lambs were kept with their mothers until weaned at 4 months of age and had carotid arterial loops prepared at 5 months of age. At 5 years of age, uni-x and sham animals were placed in individual metabolic cages, and fitted with a carotid catheter for measurement of arterial pressure, and the left and right jugular veins were catheterized for drug infusions. Animals were fitted with a removable Foley bladder catheter for urine collection and measurement of renal function.

Blood Pressure and Renal Function

Mean arterial pressure (MAP) and heart rate were recorded continuously via the carotid arterial loop. GFR was determined via the clearance of 51chromium-ethylenediamine-tetra-acetic acid (51Cr EDTA); RBF was determined via the clearance of para-aminohippuric acid; and filtration fraction was calculated. Para-aminohippuric acid concentrations were assessed with a rapid microplate assay.

Cardiovascular and Renal Responses to NO Inhibition

Cardiovascular function and renal function were monitored during a 60-minute basal period, after which animals were infused with L-NAME (40 mg/kg bolus plus 20 mg/kg·h−1 IV; Sapphire Biosciences) for 60 minutes. Urine samples were collected at 30-minute intervals, with an arterial blood sample (5 mL) collected at the midpoint of each urine sample collection during the first basal hour and 1 hour after L-NAME infusion. Seven days after the in vivo experiments, sheep were euthanized (overdose of pentobarbitone [Lethabarb]; 325 mg/mL), and renal lobar and arcuate arteries and mesenteric, femoral, and coronary arteries were isolated for assessment of vascular function and passive mechanical wall properties.

Smooth Muscle, Endothelial, and Nerve-Mediated Responses in the Vasculature

Rings of renal, mesenteric, femoral, and coronary arteries 1 to 2 mm in length were mounted on a 4-channel myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) for measurement of isometric tension, as previously described. Contractile properties were assessed by cumulative additions of either the α1-adrenoceptor agonist phenylephrine (10−6–10−4 mol/L, renal, mesenteric and femoral arteries) or the thromboxane analog U46619 (U4; 10−5–3×10−6 mol/L, coronary artery). Contractions were expressed as a percentage of contraction evoked by high K+ physiological saline solution (isotonic replacement of Na+ with 100 mmol/L K+). In arteries that were submaximally constricted, endothelium-dependent relaxation was tested with either bradykinin (10−10–10−6 mol/L, renal, mesenteric, and coronary arteries) or acetylcholine (10−6–10−5 mol/L, femoral artery). Responses were obtained before and after sequential blockade of NOS with L-NAME (2×10−4 mol/L) and cyclooxygenase with indomethacin (10−6 mol/L). Endothelium-independent relaxation was tested with the NO donor sodium nitroprusside (10−5–10−4 mol/L). For perivascular nerve stimulation, segments of renal lobar arteries were mounted on a single-channel wire myograph, and the smaller renal arcuate arteries were mounted on a pressure myograph (Living Systems Instrumentation, Burlington, VT). Platinum electrodes positioned on either side of the artery were used to stimulate the perivascular nerves along the artery segment, as previously described.

Passive mechanical wall properties were determined in leak-free segments of arteries mounted on a pressure myograph with no luminal flow and bathed in zero-Ca2+ physiological saline solution containing 2 mmol/L EGTA at ≈36°C, as previously described.

Markers of Endothelial Function and Oxidative Stress

At postmortem, a 0.5-cm slice in the transverse plane was taken from the right kidney, the cortex, and medulla divided; and snap-frozen for molecular studies. Renal lobar arteries were isolated and snap-frozen for gene and protein expression and 3-nitrotyrosine immunohistochemistry.

Statistical Analysis

Data are reported as mean±SEM, and n represents the number of animals studied. Renal function variables were corrected for body weight. Data were analyzed with repeated-measures ANOVA or an unpaired Student t test. For endothelium-dependent relaxation, the contribution of each vasodilator was determined with an area under the curve (AUC) analysis. The response evoked by NO was determined by subtracting the AUC in the presence of L-NAME from that obtained in normal physiological saline solution. The relaxation attributed to the vasodilator prostanooids was calculated by subtracting the AUC in the
Results

Reduced Role for NO in the Modulation of Renal Hemodynamic Function in Uni-x Sheep

Body weight and kidney weight were not significantly different between the uni-x and sham groups at 5 years of age, as previously reported for this cohort. Basal MAP (±15 mm Hg) and renal vascular resistance (RVR; ±55%) were greater and RBF (±42%), GFR (±38%), and urinary sodium excretion (±46%) were lower in the uni-x compared with the sham group (all P<0.01; Table I in the online-only Data Supplement). In response to L-NNAME infusion, MAP increased by 16±3 mm Hg in uni-x and 18±3 mm Hg in sham sheep, with the response not significantly different between groups (PInteraction=0.3; Figure 1A). This increase in MAP was associated with a similar reduction in heart rate in both groups (PInteraction=0.2; Figure 1B). The reduction in RBF in response to L-NNAME was blunted in the uni-x compared with the sham sheep (13±5% versus 60±8%, respectively; PInteraction<0.001; Figure 1C). In response to L-NNAME infusion, the increase in RVR was modest in uni-x compared with sham sheep (20±10% versus 160±31%, respectively; PInteraction=0.04; Figure 1D).

L-NNAME infusion caused a reduction in GFR, with the change attenuated in uni-x compared with sham animals (27±5% versus 48±6%, respectively; PInteraction=0.002; Figure 1E). Filtration fraction did not significantly change in response to L-NNAME infusion in either group (PTreatment=0.9; Figure 1F). In response to L-NNAME infusion, there was no significant difference in urine flow between the 2 groups (PInteraction=0.6; Figure 1H). However, urinary sodium excretion was markedly decreased in sham but not in uni-x animals (PInteraction=0.002; Figure 1H).

Vascular Function

Endothelial Dysfunction Localized Predominantly to the Renal Arteries of Uni-x Sheep

Stimulation of the endothelium with bradykinin or acetylcholine evoked concentration-dependent relaxation in all arteries (Figure 1 in online-only Data Supplement). Renal arteries from uni-x animals were less sensitive to bradykinin compared with those from sham sheep (pD2 [negative logarithm of the concentration of agonist producing half maximal response], P<0.01; Table II in the online-only Data Supplement), and the AUC for endothelium-dependent relaxation was significantly reduced in uni-x sheep (P=0.04; Figure 2A). In contrast, the AUC for endothelium-dependent relaxation was not different between treatment groups for mesenteric, coronary, or femoral arteries (Figure 2B–2D). In the presence of L-NNAME, the renal arteries of uni-x sheep developed markedly less spontaneous tone compared with those from sham sheep (P=0.04; Figure 2E). In the presence of L-NNAME, the concentration-relaxation curve to bradykinin was shifted to the right to a significantly greater extent in renal lobar arteries of uni-x (4-fold) compared with sham sheep (2.5-fold; Figure 1A and IB in the online-only Data Supplement). In the presence of L-NNAME and indomethacin, the rightward shift of the concentration-relaxation curve to bradykinin was markedly greater in renal arteries of sham (16-fold) compared with uni-x (3-fold) sheep (Figure 1A and IB in the online-only Data Supplement). AUC analysis revealed that endothelium-dependent relaxation attributed to prostanoids was significantly (±46%) reduced in renal arteries of uni-x compared with sham sheep (P<0.05; Figure 2A). The contribution of NO and EDHF to bradykinin-induced endothelium-dependent relaxation was unaltered between the sham and uni-x groups.

Sensitivity to bradykinin or acetylcholine was not different in mesenteric, coronary, and femoral arteries between the sham and uni-x sheep (Figure 1C–1H in the online-only Data Supplement). The AUC for endothelium-dependent relaxation in these arteries was also not different between the sham and
Phenylephrine evoked concentration-dependent contractions, with renal lobar, mesenteric, and femoral arteries from uni-x sheep demonstrating enhanced sensitivity to the \( \alpha \)-adrenoceptor agonist compared with arteries from sham sheep \( (P_{\text{Group}}=0.04 \text{ [lobar]} \text{ and } P_{\text{Group}}=0.02 \text{ [arcuate]; Figure 4A and 4C}). These contractions were markedly attenuated by the \( \alpha \)-adrenoceptor blocker prazosin and all but abolished in the presence of tetrodotoxin (Figure III in the online-only Data Supplement). For any given stimulus frequency, increasing stimulus voltage increased contraction amplitude, and to a greater extent in renal lobar arteries from uni-x sheep compared with sham sheep \( (P_{\text{Group}}<0.05 \text{ for all; Figure IV in the online-only Data Supplement})\). The enhanced responses to nerve stimulation in renal lobar arteries of uni-x sheep were associated with an augmented sensitivity of the smooth muscle to exogenous \( \alpha \)-adrenoceptor stimulation \( (P_{\text{Group}}=0.001; \text{ Figure 4B})\). In contrast, enhanced neurovascular constriction in renal arcuate arteries from uni-x sheep was not accompanied by changes in smooth muscle sensitivity to \( \alpha \)-adrenoceptor stimulation (Figure 4D).

Figure 2. Contribution of endothelial vasodilators to agonist-evoked relaxation in isolated arteries and spontaneous tone generation in renal lobar arteries of uninephrectomy (uni-x) and sham sheep at 5 years of age. The contribution of each vasodilator (nitric oxide [NO], prostanoids [PG], and endothelium-derived hyperpolarizing factor [EDHF]) to the total endothelium-dependent relaxation evoked by bradykinin or acetylcholine was determined using an area under the curve (AUC) analysis for (A) renal lobar, (B) mesenteric, (C) coronary, and (D) femoral arteries in sham \((n=9)\) and uni-x sheep \((n=8)\). E, Spontaneous tone generation in renal lobar arteries after endothelial nitric oxide synthase inhibition. Values are mean±SEM. *\( P<0.05 \) for sham vs uni-x sheep, 2-tailed unpaired Student t test.

Enhanced Vasosconstriction to Sympathetic Nerve Activation

Stimulation of the renal lobar and arcuate arteries with pulses of increasing frequency evoked contractions of increasing amplitude \( (P_{\text{Frequency}}<0.001; \text{ Figure 4A and 4C})\). Neurovascular constriction was enhanced in both the renal lobar and arcuate arteries of uni-x compared with sham sheep \( (P_{\text{Group}}=0.04 \text{ [lobar]} \text{ and } P_{\text{Group}}=0.02 \text{ [arcuate]; Figure 4A and 4C}). These contractions were markedly attenuated by the \( \alpha \)-adrenoceptor blocker prazosin and all but abolished in the presence of tetrodotoxin (Figure III in the online-only Data Supplement). For any given stimulus frequency, increasing stimulus voltage increased contraction amplitude, and to a greater extent in renal lobar arteries from uni-x sheep compared with sham sheep \( (P_{\text{Group}}<0.05 \text{ for all; Figure IV in the online-only Data Supplement})\). The enhanced responses to nerve stimulation in renal lobar arteries of uni-x sheep were associated with an augmented sensitivity of the smooth muscle to exogenous \( \alpha \)-adrenoceptor stimulation \( (P_{\text{Group}}=0.001; \text{ Figure 4B})\). In contrast, enhanced neurovascular constriction in renal arcuate arteries from uni-x sheep was not accompanied by changes in smooth muscle sensitivity to \( \alpha \)-adrenoceptor stimulation (Figure 4D).

Preservation of Arterial Passive Mechanical Wall Properties

Arterial wall stiffness, as assessed from stress-strain relationships, was not different between uni-x and sham animals for any of the arteries tested (Figure VA–VD in the online-only Data Supplement). The ratio of media thickness to lumen diameter at 100 mmHg was not different between the uni-x and sham groups for any of the arteries tested (Table II in the online-only Data Supplement).

Enhanced Endothelial NOS Protein Expression and Oxidative Stress in Renal Arteries of Uni-x Sheep

At 5 years of age, endothelial NOS (eNOS) mRNA expression was significantly upregulated in the renal cortex of uni-x sheep compared with sham sheep \( (P<0.01; \text{ Figure VIA in the online-only Data Supplement})\). Although expression of eNOS was greater within the medulla compared with the cortex \( (P<0.002; \text{ Figure VIA in the online-only Data Supplement})\), there was no difference in eNOS expression in the medulla between sham and uni-x sheep. Similar to renal cortex, eNOS mRNA expression was significantly greater in isolated renal lobar arteries of uni-x sheep \( (P=0.03; \text{ Figure VIB in the online-only Data Supplement})\). Renal lobar arteries of uni-x sheep had significantly lower expression of both cyclooxygenase (COX)-1 \( (P=0.03; \text{ Figure VIC in the online-only Data Supplement})\) and COX-2 \( (P=0.026; \text{ Figure VIB in the online-only Data Supplement})\) mRNA compared with those from sham animals. Consistent with the increase in renal eNOS gene expression in the uni-x sheep, eNOS protein expression was also increased in the renal cortex of uni-x sheep compared with the sham sheep \( (P=0.02; \text{ Figure 5A})\). Furthermore, the Ser-1177–phosphorylated eNOS protein level in the renal...
The present study provides compelling in vivo and in vitro evidence of the deleterious effects of being born with a reduction in congenital renal mass on the regulation of renal and vascular function. Our study provides the first report that renal dysfunction in adult sheep after fetal uni-x is associated with impaired modulation of renal hemodynamics and sodium excretory function by NO. We also report renal vascular dysfunction with impaired contribution of basal NO, endothelial dysfunction, increased oxidative stress, and enhanced smooth muscle responsiveness to vasoconstrictors and sympathetic nerve stimulation in sheep born with a reduction in congenital renal mass. Dysfunction was also observed in blood vessels from other regions of the body, but this was less extensive than in the renal vasculature.

In the present study, sham sheep had significant increases in MAP and RVR within 60 minutes of L-NAME infusion. This finding is consistent with studies in several species, including sheep, and confirms the important contribution of NO to the cardiovascular system. The striking increase in RVR in sham sheep was associated with reductions in both RBF (≈60%) and GFR (≈48%), suggesting that NO tonically generated within the kidney lowers resting RVR and directly modulates glomerular hemodynamics. Systemic NO blockade in rodents produces increases in both afferent and efferent arteriolar resistances, leading to reductions in renal plasma flow. There is also evidence for a role for NO in facilitating sodium excretion by blocking sodium transporters in proximal tubules and collecting ducts and in mediating pressure natriuresis via increases in renal medullary blood flow and interstitial hydrostatic pressure. Sham sheep exhibited significant reductions in urinary sodium excretion (≈50%) after L-NAME infusion, consistent with studies in anesthetized dogs in which significant reductions in natriuresis occurred. These results highlight the importance of endogenous NO within the kidney in the regulation of renal hemodynamics and sodium excretory function under normal physiological conditions.

In contrast to sham animals, the reductions in GFR (27%) and RBF (13%) in uni-x sheep in response to L-NAME infusion were modest and occurred despite a similar rise in MAP (≈17%) in both groups. The minimal renal hemodynamic responses to NO inhibition suggest a deficit in basal NO generation/bioavailability within the vasculature of the uni-x kidney in female sheep at 5 years of age. A role of endothelial NO deficiency has been implicated in mediating hypertension in several animal models of fetal programming in which the maternal environment has been adversely affected. Moreover, there have been reports of reductions in neuronal NOS expression and activity within the renal cortex and medulla in a chronic kidney disease model produced by renal mass reduction after 5/6 nephrectomy in adult rats. The increase in RVR in response to L-NAME was far greater in sham (=160%) compared with uni-x (=20%) sheep, and this was associated with a reduction in sodium excretion in sham but not uni-x animals. A reduction in basal NO generation within the kidneys has been shown to decrease renal sodium excretory function directly by inhibiting tubular reabsorption or indirectly by increasing basal RVR or enhancing renal vascular responsiveness to vasoconstrictors such as angiotensin II.

**Discussion**

**Figure 3.** Smooth muscle contraction and relaxation in isolated arteries from uninephrectomy (uni-x) and sham sheep at 5 years of age. Concentration-contraction curves for phenylephrine (PE) or U46619 in (A) renal lobar, (B) mesenteric, (C) coronary, and (D) femoral arteries of uni-x (●, n=8) and sham (○, n=9) sheep. Concentration-relaxation curves for sodium nitroprusside (SNP) in (E) renal, (F) mesenteric, (G) coronary, and (H) femoral arteries of uni-x (●, n=8) and sham (○, n=9) sheep. P values represent the results from a repeated-measures ANOVA, with factors group (G; sham, uni-x), concentration (C), and their interaction (I). Values are mean±SEM. *P<0.05, **P<0.01, and ***P<0.001, pD2 or maximum contraction to phenylephrine (Emax) between sham and uni-x sheep, 2-tailed Student t test.
II and renal sympathetic nerve activity.\(^\text{11}\) Previously, we have shown that these 5-year-old uni-x sheep have a reduced ability to excrete a saline load; thus, reduced NO production may be a contributing factor.\(^\text{24}\) In the present study, the eNOS gene and eNOS and phosphorylated eNOS protein expression were upregulated in the renal cortex of uni-x sheep, although the ratio of phosphorylated eNOS to eNOS protein was unchanged. Furthermore, 3-nitrotyrosine staining, indicative of oxidative stress, was increased in the renal vasculature. Under conditions of increased oxidative stress, for example, in diseases such as hypertension and diabetes mellitus, an upregulation of eNOS gene and protein expression is generally observed with eNOS uncoupling, resulting in decreased NO bioavailability, increased superoxide formation, and disrupted eNOS dimer formation.\(^\text{31,32}\) Collectively, our in vivo data suggest that the contribution of NO to the regulation of renal hemodynamics and sodium excretion is markedly blunted in uni-x sheep (See the online-only Data Supplement for further discussion of the timing of these changes).

Endothelium-dependent vasodilation in vivo is elicited by shear stress and paracrine and circulating agents.\(^\text{33}\) The reduction in basal RBF and increased RVR in uni-x animals, together with the blunted renal hemodynamic responses to NO inhibition, may point to dysfunction in NO release or bioavailability in these animals. Indeed, a decrease in flow/shear stress–induced dilation has commonly been reported in humans with chronic hypertension, and this is underpinned by reductions in NO-mediated vasodilation.\(^\text{34}\) Studies in spontaneously hypertensive rats have demonstrated impairments in flow-dependent dilation of small arteries in which endothelial NO activity in response to shear stress was impaired\(^\text{35}\) or preserved,\(^\text{36}\) depending on the region of the vascular bed under investigation. In the present study, basal tone generation after NOS inhibition in isolated renal lobar arteries was markedly blunted in uni-x sheep (See the online-only Data Supplement for further discussion of the timing of these changes).

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### Figure 4

**Neurovascular constriction and smooth muscle contraction to phenylephrine (PE)** in isolated renal lobar and arcuate arteries in uninephrectomy (uni-x) and sham sheep at 5 years of age. Contraction evoked by perivascular nerve stimulation, 5-second trains of pulses of increasing frequency at 150 V (dial setting), in (A) renal lobar arteries and (C) renal arcuate arteries. Concentration-contraction curves for PE in (B) renal lobar (graph reproduced from Figure 3A to aid in the interpretation of the data) and (D) renal arcuate arteries of sham (○, n=9) and uni-x (●, n=8) sheep. \(P\) values represent the results from a repeated-measures ANOVA, with factors group (G; sham, uni-x), frequency (F), concentration (C), and their interaction (I). Values are means±SEM. **\(P<0.01\) and ***\(P<0.001\), \(pD_2\) or maximum contraction to phenylephrine (\(E_{\text{max}}\)) between sham and uni-x sheep, 2-tailed Student \(t\) test.

### Figure 5

**Renal cortical endothelial nitric oxide synthase (eNOS) protein expression.** Protein expression of (A) eNOS (140 kDa) and (B) Ser-1177–phosphorylated eNOS (Phospho-eNOS; 140 kDa) and (C) ratio of eNOS to phosphorylated eNOS protein (densitometry units) in the kidney cortex of 5-year-old female sheep having undergone either uninephrectomy (uni-x; black bars; \(n=4\)) or sham surgery (white bars; \(n=4\)) at gestational age of 100 days. Representative blots are shown (S, sham; U, uni-x). Protein expression is relative to the expression of \(\beta\)-actin (42 kDa). Values are mean±SEM. *\(P<0.05\) vs sham, unpaired Student \(t\) test.
sodium nitroprusside was unaltered, indicating that the guanylate cyclase–cGMP pathway is intact in arteries from uni-x sheep. Collectively, our results indicate the possibility of a reduction in NO production or bioavailability. The observation that renal arteries had significantly greater 3-nitrotyrosine staining in the aged uni-x sheep suggests that a reduction in NO bioavailability may be occurring.

In isolated arteries, agonist stimulation of the endothelium evoked the release of NO, vasodilator prostanoids, and EDHF. Whereas the contribution of basal NO to renal artery tone suppression was reduced in arteries of uni-x sheep, the role of NO in agonist-induced endothelium-dependent relaxation was unaltered. However, endothelium-dependent relaxation was impaired in renal lobar arteries of uni-x animals and attributable to the reduced contribution of vasodilator prostanoids. The expression and location of COX-1 and COX-2 enzymes appear to be the major rate-limiting factors in the synthesis of prostanoids. Both COX-1 and COX-2 are expressed in the vascular endothelium, with COX-2 proposed to be the major mediator of vasodilator prostanoid synthesis, especially during the evolution of compensatory renal functional changes after renal ablation. In the present study, the reduced contribution of vasodilator prostanoids to endothelium-dependent relaxation in renal lobar arteries of uni-x sheep was associated with reductions in both COX-1 and COX-2 gene expression. Endothelial dysfunction in programming models has been attributed to reductions in NO-mediated or EDHF-mediated relaxation, whereas the involvement of vasodilator prostanoids in the regulation of vascular tone in programming models is less widely reported. Reduced prostanoid production underpins impaired coronary artery vasodilation in adolescent lambs exposed to dexamethasone in utero. Interestingly, the contribution of vasodilator prostanoids was also reduced in coronary arteries of uni-x animals, but not in the mesenteric and femoral beds. Within the renal vasculature, vasodilator prostanoids have myriad functions such as modulating RBF and GFR and promoting natriuresis via their vasodilator actions. Thus, the reduced vasodilator prostanoid contribution to endothelial vasodilation within the renal vasculature of uni-x animals may also contribute to reductions in RBF and GFR and subsequently to the lower basal urinary sodium excretion. Endothelial vasodilator dysfunction has been reported in patients with chronic renal failure, attributed mainly to a reduction in NO activity; however, alterations in other vasodilators (eg, prostanoids and EDHF) are also implicated. Despite the hypertension associated with fetal uni-x, endothelial dysfunction was not generalized across all vascular beds. Our findings highlight the heterogeneity across the vasculature in response to fetal uni-x, rendering certain vascular beds more vulnerable than others. Similarly, when arteries from different vascular beds were tested in other fetal programming models, there appeared to be regional variations in the extent and nature of dysfunction.

Enhanced vasoconstrictor responses have been reported for some fetal programming models. Enhanced responsiveness to \( \alpha_1 \)-adrenoreceptor agonists occurs in femoral and renal arteries of adult rats exposed to nutrition deprivation in utero. We demonstrated enhanced smooth muscle responsiveness to \( \alpha_1 \)-adrenoreceptor agonists in the renal lobar, mesenteric, and femoral arteries in uni-x sheep. The female uni-x sheep used in the present study had higher total peripheral resistance at 2 years of age. Furthermore, there was an exacerbation in total peripheral resistance with aging between 2 and 5 years, which was greater in uni-x compared with sham sheep. Thus,
generalized increased responsiveness to vasoconstrictors may contribute to the physiological maintenance and progression of the higher baseline peripheral resistance in uni-x sheep. Neurovascular constriction was upregulated in renal vessels of uni-x sheep. In the renal lobar arteries, this increase in responsiveness to neurovascular constriction could be explained by the enhanced sensitivity and maximal response of the smooth muscle to α1-adrenoreceptor stimulation. In contrast, in the smaller arcuate arteries, enhanced neurovascular constriction occurred in the absence of any changes in α1-adrenoreceptor responsiveness of the smooth muscle. Thus, in the arcuate arteries of uni-x sheep, there may be augmentation in the density of the perivascular nerve network or alteration adrenoceptor responsiveness of the smooth muscle. Thus, in abolishing hypertension.

Conclusions
The present study demonstrates that renal dysfunction and hypertension after congenital nephron deficit are underpinned by a reduced contribution of NO to the modulation of renal hemodynamics and function in association with enhanced oxidative stress. This is further exacerbated by a proconstrictor profile in the renal vasculature characterized by endothelial vasodilator dysfunction, enhanced sensitivity to vasoconstrictors, and sympathetic nerve stimulation. Identification of these pivotal mechanisms may have potential implications for improving prognosis and treatment for children born with 1 kidney. For instance, recent studies have suggested that catheter ablation of the renal nerves may be effective in the treatment of hypertension and chronic renal disease. Our data suggest that such a strategy might slow the progression of renal dysfunction in patients with renal agenesis because it would remove the enhanced response to sympathetic activation and improve endothelial function. This warrants further investigation.

Sources of Funding
This study was funded by a National Heart Foundation grant (G 05M 2110), National Health and Medical Research council (SRF 1041844, GNT 0546087), Australia and New Zealand Trustees, The University of Queensland Research Fund, and the Monash University Research Fund.

Disclosures
None.

References
Clinical Perspective

Children born with a reduced renal mass have an increased risk of chronic kidney disease and hypertension in adulthood. To understand the mechanisms underlying the early development of renal insufficiency in children born with a solitary functioning kidney, we established a model of fetal uninephrectomy (uni-x) in sheep, which, similar to humans, completes nephrogenesis before birth. This model results in a 30% reduction in nephron number rather than 50% as a result of compensatory nephrogenesis in the remaining kidney. Similar to children with a congenital solitary functioning kidney, uni-x sheep demonstrate a progressive increase in arterial pressure and loss of renal function with aging. The present study, using a combination of in vivo studies in conscious sheep and in vitro functional and molecular studies in isolated arteries, defines the mechanisms responsible for impairments in renal function in uni-x sheep at an advanced age. We report that renal dysfunction in conscious 5-year-old female sheep after fetal uni-x is associated with impaired modulation of renal hemodynamics and sodium excretion by nitric oxide. Furthermore, marked vascular dysfunction, predominantly in the renal vasculature, was observed in uni-x sheep that was underpinned by endothelial vasodilator dysfunction involving nitric oxide and prostanooid deficiencies, enhanced oxidative stress, and augmented smooth muscle responsiveness to vasoconstrictors and sympathetic nerve stimulation. Identification of these pivotal mechanisms may have potential implications for improving the prognosis and treatment of children born with a solitary functioning kidney.
Renal Dysfunction Is Associated With a Reduced Contribution of Nitric Oxide and Enhanced Vasoconstriction After a Congenital Renal Mass Reduction in Sheep
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_Circulation_. 2015;131:280-288; originally published online November 4, 2014;
doi: 10.1161/CIRCULATIONAHA.114.013930
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/131/3/280

Data Supplement (unedited) at:
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SUPPLEMENTAL MATERIAL
METHODS

Animals

All experiments were approved by the Monash University, School of Biomedical Sciences Animal Ethics Committee, and were in agreement with the guidelines of the National Health and Medical Research Council of Australia. Merino ewes carrying female fetuses of known gestational age underwent surgery at 100 days post-conception, as described previously. The left renal artery, vein and ureter were ligated and the kidney was removed in the uni-x group. In the sham group, the kidney was cleared of the surrounding fat but left intact. Following surgery, ewes were housed in large pens with free access to food and water for 2 weeks before being returned to the farm. After natural birth, lambs were kept with their mothers until weaned at 4 months of age. At 5 months of age, the right carotid artery was exteriorized into a skin fold to form a carotid arterial loop as previously described. At 5 years of age, female uni-x and sham animals were brought into the laboratory and placed in individual metabolic cages. The following week all animals were instrumented with a carotid catheter for measurement of arterial pressure and the left and right jugular veins were catheterized for the infusion purposes. A removable Foley bladder catheter was inserted (size 12, 30cc, Euromedical, Malaysia) for urine collection and measurement of renal function. The bladder catheters were flushed with antibiotics (Neomycin (Jurox, 4.5 ml (200 mg/ml)) to minimize the risk of bladder infections. Animals were fed a diet of lucerne chaff (1kg) and allowed access to 5 litres of water at 17.00h daily. Experiments commenced following a week of acclimatization.
**In vivo protocols**

**Blood pressure and renal function**

For direct measurements of arterial pressure, a tygon catheter was inserted into the carotid arterial loop under local anaesthesia. The tygon catheter was then connected to a pressure transducer (TD XIII; Cobe) for continuous measurement of mean arterial pressure (MAP) and heart rate (HR). Glomerular filtration rate (GFR) was determined via the clearance of $^{51}$Chromium-ethylenediamine-tetra-acetic acid ($^{51}$Cr EDTA, 15 μCi bolus + 15 μCi/h, i.v) and effective renal plasma flow (ERPF) and hence renal blood flow (RBF; ERPF/ (1-hematocrit)) was determined via clearance of para-aminohippuric acid (PAH, 4.8 mg/kg bolus + 750 mg/h, i.v). These drugs were infused at a combined rate of 12 ml/h. Plasma and urine PAH concentrations were assessed using a rapid microplate assay and $^{51}$Cr EDTA levels were determined using a gamma counter (PerkinElmer Wizard 1470). Urinary sodium concentrations were measured using the RapidChem 744 Electrolyte analyser. Filtration fraction (FF) was determined as GFR/ERPF. Renal vascular resistance (RVR) was determined as MAP/RBF.

Urinary sodium excretion ($U_{Na^+}V$) was calculated as (Urinary $[Na^+] \times$ urine flow (UF)).

**Cardiovascular and renal responses to L-NAME administration**

Initially, cardiovascular (MAP, HR) and renal function (UF, GFR, RBF, RVR, FF, $U_{Na^+}V$) were monitored during a 60 minute basal period. Following which animals were infused with of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 40 mg/kg bolus + 20 mg/kg/h i.v. Sapphire Biosciences) for 60 minutes. Urine samples were collected 2 x 30 minute intervals, with arterial blood samples (5ml) collected at the mid-point of each urine collection prior to and following L-NAME infusion. When determining GFR and RBF the first 30 minute L-NAME treatment
period was discarded to allow for steady state to be obtained. This dose of L-NAME has previously been shown to inhibit intra-renal and systemic NOS in conscious sheep.\(^5\) Seven days following \textit{in vivo} experiments, sheep were euthanized (overdose of pentobarbitone, Lethabarb\(^\circledR\), 325 mg/ml) and renal lobar and arcuate arteries, as well as, mesenteric, femoral and coronary arteries were isolated for the assessment of vascular function and passive mechanical wall properties. Body, kidney and heart weights for this cohort of animals have been previously reported.\(^6\),\(^7\)

\textit{In vitro} protocols

\textit{Vascular reactivity}

Rings of renal (outside diameter (OD ~ 400 \(\mu\)m)), mesenteric (OD ~ 300 \(\mu\)m), femoral (OD ~ 350 \(\mu\)m), and coronary (OD ~ 350 \(\mu\)m) artery ~1-2 mm in length, were mounted on a four channel myograph (Model 610M, Danish Myo Technology, Aarhus, Denmark) for measurement of isometric tension, as previously described.\(^8\),\(^9\) Arteries were bathed in warm (36°C) physiological saline solution (PSS (mM): 120 NaCl, 5 KCl, 2.5 CaCl\(_2\), 25 NaHCO\(_3\), 11 glucose, 1 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\)), and bubbled with 95% O\(_2\) and 5% CO\(_2\). Endothelial function was assessed in arteries sub-maximally constricted (60 – 70% of maximum) with the \(\alpha_1\)-adrenoceptor agonist phenylephrine (PE; for mesenteric, renal and femoral) or the thromboxane analogue U46619 (for coronary).

\textit{Smooth muscle and endothelial function}

Contractile properties were assessed by exposing arteries to cumulative additions of either PE (\(10^{-9} – 10^{-4}\) M) or U46619 (\(10^{-9} – 3 \times 10^{-6}\) M). Contractions were expressed as a percentage of contraction evoked by high K\(^+\) PSS (isotonic replacement of Na\(^+\) with 100 mM K\(^+\)). In arteries
that were sub-maximally constricted (60-70% of contraction evoked by high K⁺ PSS),
endothelium-dependent relaxation was tested via cumulative addition of increasing
concentrations of either bradykinin (BK, 10⁻¹⁰–10⁻⁶ M) or acetylcholine (ACh, 10⁻⁹–10⁻⁵ M).
Responses were obtained before, and after sequential blockade of endothelial nitric oxide
synthase (eNOS) with L-NAME (2 ×10⁻⁴ M) and cyclooxygenase with indomethacin (Indo; 10⁻⁶
M). Blockers were added to the arteries 30 minutes prior to and during endothelium
stimulation. Endothelium-independent relaxation was tested using the NO donor sodium
nitroprusside (SNP, 10⁻⁹–10⁻⁵ M), as previously described.

Nerve stimulation

Renal lobar arteries were mounted on single channel wire myograph (Monash University,
Clayton, Australia) and the smaller renal arcuate arteries were mounted on a pressure myograph
(Living Systems Instrumentation, Burlington, VT, USA) and continuously superfused with PSS
at 36°C and bubbled with 95% O₂ and 5% CO₂. Platinum electrodes positioned on either side of
the artery were used to stimulate the perivascular nerves along the entire segment as previously
described. The arteries were stimulated transmurally with continuous trains (1 – 8 Hz
(lobar); 1 – 50 Hz (arcuate)) over 5 seconds at increasing intensities (80 – 150 V) dial settings,
with a pulse duration of 0.8 ms (lobar) and 0.2 ms (arcuate) using a Grass S88 stimulator. Nerve
stimulation was repeated following the blockade of α₁- adrenoceptors with prazosin (10⁻⁶ M)
and the neurogenicity of the responses was confirmed using tetrodotoxin (TTX, 10⁻⁷ M). All
responses to perivascular nerve stimulation were expressed as a percentage of contraction
evoked by high K⁺ PSS.
Arterial passive mechanical wall properties

Blood vessels were mounted on a pressure myograph with no luminal flow as previously described.\textsuperscript{8, 10} Arteries were superfused at \(~15\) ml/min in zero-Ca\textsuperscript{2+} PSS containing 2mM EGTA at \(\sim 36^\circ\)C. Vessels were pressurized from 0 – 200 mmHg in 10 mmHg increments and their length, outside diameter and wall thickness were measured at each increment. Wall stress and strain were calculated using the following derivatives: Wall stress (kPa) = \([\text{pressure} \times \text{internal diameter (ID)}] / [2 \times \text{wall thickness (WT)}]\); Wall strain = \((\text{ID} @ \text{incremental pressure} – \text{ID} @ 5\text{mmHg}) / \text{ID} @ 5\text{mmHg}\)\textsuperscript{8, 10, 13}

Quantitative real-time polymerase chain reaction

At post-mortem, a 0.5cm slice in transverse plane was taken from the right kidney, the cortex and medulla divided and snap frozen for molecular studies. Renal lobar arteries were isolated and snap frozen for gene expression studies. 30 mg of cortex and medulla tissue and isolated renal lobar arteries were homogenized for RNA extraction and 1\(\mu\)g RNA was transcribed into cDNA using the BIO-RAD iScript\textsuperscript{TM} Reverse Transcription Supermix (Bio-Rad Laboratories, California, USA). Gene expression for eNOS, in the renal cortex and medulla, as well as, eNOS, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) expression in renal lobar arteries were assessed using SYBR-GREEN I chemistry and 18S was used as the housekeeping gene (Applied Biosystems, Scoresby, VIC, Australia) using an Eppendorf RealPlex Cycler real-time PCR machine. A comparative cycle of \(C_T\) (threshold fluorescence) was used to quantify gene expression, as previously described.\textsuperscript{14}
**Protein Expression**

Western blot analysis was performed on renal cortical tissue. Protein was extracted from 100mg of renal cortex using RIPA buffer in the presence of a protease inhibitor cocktail (Sigma, Australia). Protein content was determined using the BCA assay with bovine serum albumin (BSA) as standard (Pierce Biotechnology Inc, Rockford, Illinois). 20µg of protein was resolved by SDS polyacrylamide gel electrophoresis using representative samples from each treatment group (Sham; \( n=4 \), uni-x; \( n=4 \)) and proteins from gel transferred onto nitrocellulose membrane. Blots for eNOS and ser-1177 phosphorylated eNOS (phospho-eNOS) were placed in blocking agent containing 5% BSA, 0.1% TBST (10mM Tris HCL, 150mM NaCl and 0.1% Tween 20) for 30 mins. β-actin was blocked in 5% milk 0.1% PBST (10mM PBS, 150mM NaCl and 0.1% Tween 20) for 30 mins. Following this, blots were incubated overnight at 4°C with anti-β-actin (Sigma, Australia, 1:3000), with anti-eNOS (Cat#610297 (polyclonal) BD Bioscience, San Jose, California, 1:750 dilution) and with anti-phospho-eNOS (SER1177; Cat#9571 (monoclonal), Cell Signaling Danvers Massachusetts, 1:750 dilution). The next day, all bots were washed in 0.1% TBST (3 x 10 mins washes) and incubated blots for β-actin and eNOS were incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary anti-body (Millipore, Australia; 1:3000 dilution) and for phospho-eNOS the blots were incubated with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, 1:2000 dilution). Bands were visualised by enhanced chemiluminescence (ECL, Pierce Biotechnology Inc, Rockford, Illinois) and protein densitometry calculated using the Chemi-doc Imaging software (Bio-rad Laboratories Inc, Australia). Protein expression for eNOS and phospho-eNOS were quantified after normalising to β-actin protein levels.
3-Nitrotyrosine immunohistochemistry

Detection of 3-nitrotyrosine was performed on frozen sections of renal lobar arteries (3µm) from sham and uni-x sheep (n=5 per group). Briefly, mounted sections were washed in 0.01M phosphate buffered saline (PBS), incubated in 10% goat serum in 0.01M PBS containing 0.3% triton X 100 at room temperature for 60 minutes and then incubated overnight at room temperature in monoclonal mouse anti-3-nitrotyrosine (Abcam, Sapphire Bioscience, Waterloo, NSW, Australia; final dilution 1:30 in 10% goat serum). Antibody specificity was tested by not incubating in primary antibody (negative control). Following washes in 0.01M PBS (3 × 10mins) all sections were incubated in Goat-polyclonal secondary anti-mouse Alexa Fluor®488 (Abcam, Sapphire Bioscience, Waterloo, NSW, Australia; final dilution 1:500 in 0.01M PBS) for 45 mins at room temperature, washed in 0.01M PBS (3 × 10mins) and then incubated in 4’, 6-diamidino-2-phenylindole (DAPI, Molecular probes; final dilution, 1:10,000, 10 mins at room temperature) to detect cell nuclei. Sections were washed briefly in distilled water, cover slipped and mounted with a fluorescent mounting medium and photographed with The Aperio Scanscope Fluorescent scanner (Leica Biosystems). The intensity of 3-nitrotyrosine in the vessel was quantified on one section per animal at 5 randomly selected areas of uniform size of the vessel and subtracted from negative using ImageJ analysis software (ImageJ; imagej.nih.gov/ij/).

Statistical analysis

All data are reported as mean ± SEM, where n represents the number of animals. In vivo data:
All renal function variables were corrected for body weight (bw). Analysis of variables in response to L-NAME infusion was assessed using repeated measures ANOVA with factors
group (sham or uni-x), treatment and their interaction. In vitro data: Responses of arteries to constrictors and dilators were analysed as previously described.\textsuperscript{9, 10} Contraction-response curves were calculated and a sigmoidal curve was fitted to data for each artery using the least squares method. These curves were analysed in the first instance using repeated measures ANOVA. Responses to PE and U46619 were expressed as a percentage of contraction elicited by the high K\textsuperscript{+} PSS. Relaxations evoked by BK, ACh and SNP were expressed as percentage of the level of pre-contraction evoked by either PE or U46619. The concentration of the agonist which was effective in producing a half-maximal response (EC\textsubscript{50}) was determined for each curve and the $pD_2$ (-logEC\textsubscript{50}) and maximum response (E\textsubscript{max} contraction or relaxation) were compared between the sham and uni-x groups. For endothelium-dependent relaxation the contribution of each vasodilator was determined using an area under the curve (AUC) analysis.\textsuperscript{15} The response evoked by NO was determined by subtracting the AUC in presence of L-NAME from that obtained in control PSS. The relaxation attributed to the vasodilator prostanoids (likely prostacyclin, PG) was calculated by subtracting the AUC in the presence of L-NAME + Indo from that obtained in L-NAME alone. Responses remaining in the presence of both blockers were attributed to endothelium-derived hyperpolarizing factor (EDHF). Stress-strain relationships were analysed as previously described\textsuperscript{13} and responses to perivascular nerve stimulation were assessed using repeated measures ANOVA with factors group (sham or uni-x), frequency/voltage and their interaction. Gene and protein expression was analysed by unpaired t-test. Statistical analysis was performed using GraphPAD PRISM 5.03 for Windows.
RESULTS

*Endothelium-dependent relaxation*

Stimulation of the endothelium with BK or ACh evoked concentration-dependent relaxation in all arteries (Fig 1A-H). In the presence of L-NAME, the concentration-relaxation curve to BK or ACh was shifted to the right in all arteries, with this shift being significantly greater in renal lobar arteries of uni-x (4-fold) compared with the sham sheep (2.5-fold) (P < 0.01; Fig 1A & B). In the presence of L-NAME and Indo, the rightward shift in the concentration-relaxation curves to BK was markedly greater in the renal arteries of the sham (16-fold) compared with the uni-x (3-fold) sheep (P < 0.001; Fig 1A & B).

*Spontaneous tone generation following eNOS & cyclooxygenase inhibition*

In the presence of L-NAME, the renal lobar arteries of the uni-x sheep developed markedly less spontaneous tone compared with those from the sham sheep (P = 0.042, Fig 2A). Tone development following combined NOS and COX blockade was not different in the renal lobar arteries between groups (Fig 2B). Basal tone generation following NOS or NOS + COX inhibition was similar in all other arteries tested (Fig 2).

*Perivascular nerve stimulation*

Stimulation of the renal lobar arteries with 5 second trains of pulses of increasing frequency evoked contractions of increasing amplitude. Neurovascular constriction was enhanced in the renal lobar arteries of uni-x compared with the sham sheep in the absence of blockers (P_{Group} = 0.044, Fig 3) The constrictor responses to perivascular nerve stimulation in renal lobar arteries were markedly attenuated by α1-adrenoceptor blocker prazosin, and all but abolished in the presence of TTX (Fig 3). For any given stimulus frequency, increasing stimulus voltage
increased contraction amplitude, as more nerves were recruited, and contractions were greater in renal lobar arteries from uni-x compared with sham sheep ($P_{\text{Group}} < 0.05$, for all; Fig 4).

**Arterial passive mechanical wall properties**

Passive arterial wall stiffness, as assessed using stress-strain relationships, was not different between uni-x and sham animals for any of the arteries tested ($P_{\text{Group}} > 0.05$ for all, Fig 5A-D).

**Renal gene expression**

At 5 years of age, eNOS mRNA expression was significantly up-regulated in renal cortex of uni-x versus sham sheep ($P < 0.001$, Fig 6A). Whilst expression of eNOS was greater within the medulla compared to cortex ($P = 0.002$; Fig 6A), there was no difference in eNOS expression in the medulla between sham and uni-x sheep. Similar to renal cortex, eNOS mRNA expression was significantly greater in isolated renal lobar arteries of uni-x sheep ($P = 0.03$; Fig 6B). Renal lobar arteries of uni-x sheep had significantly lower expression of both COX-1 ($P = 0.03$; Fig 6C) and COX-2 ($P = 0.026$; Fig 6D) mRNA compared with those from sham animals.

**DISCUSSION**

Smooth muscle responsiveness to SNP was preserved in arteries from across the circulation in uni-x animals indicating that the guanylate cyclase/cGMP pathway is preserved in our uni-x model. In agreement with our findings, vascular SNP responses have been demonstrated to be essentially unaltered in patients with hypertension\textsuperscript{16} and/or chronic renal failure.\textsuperscript{17,18}

Interestingly, despite the uni-x female sheep developing sustained elevations in arterial pressure from 2 years of age and renal and cardiac hypertrophy at 5 years of age\textsuperscript{7}, there was an absence of any generalized stiffening in the arterial vasculature of uni-x sheep. This is in contrast to
studies demonstrating increased arterial stiffness in male pre-pubertal children born of low birth weight\textsuperscript{19} and growth restricted hypertensive male rat offspring born with a congenital nephron deficit.\textsuperscript{20, 21} This being said, female growth restricted rat offspring born with a congenital nephron deficit do not exhibit increased arterial stiffness of the mesenteric and femoral arteries, and moreover, displayed a decrease in arterial stiffness within their renal vasculature.\textsuperscript{8} These findings highlight the existence of sex-differences in fetal programming, where alterations in blood pressure or changes in vascular properties tend to be greater in males than in females of the same age.\textsuperscript{22} Consistent with this paradigm, we have previously demonstrated that female uni-x sheep with intact ovaries do not develop hypertension until 2 years of age\textsuperscript{7}, unlike males\textsuperscript{23} or ovariectomised females\textsuperscript{2}, which had elevated arterial pressure from as early as 6 months of age. Thus, consistent with the literature uni-x females are also relatively protected from renal and cardiovascular disease compared to age matched males due to the action of ovarian hormones.\textsuperscript{7} Moreover, the absence of generalized stiffening of the arterial vasculature may in part, explain why there was no overt age-dependent exacerbation in hypertension in these uni-x female sheep from 2-5 years of age.\textsuperscript{7}

The current study demonstrates significant renal abnormalities in uni-x sheep at 5 years of age, strongly suggesting that the future risk of renal dysfunction and cardiovascular disease will continue to advance with age. A pertinent question not addressed in the current study is at what age the changes in renal function start to become apparent and which changes occur first? Few studies have addressed these questions. In children born with a solitary functioning kidney, such studies are difficult to perform, but GFR has been reported to rapidly decline from the third decade of life, leading to significant albuminuria and elevation in blood pressure.\textsuperscript{24} In our ovine fetal uni-x model, we have evidence that function is altered very early in the remaining kidney.
For instance, in chronically catheterized uni-x fetuses, urine flow and sodium excretion were significantly reduced at ~125 days of gestation (term 150 days) compared to the sham group.\textsuperscript{25} Moreover, we have previously demonstrated that plasma creatinine levels are increased, indicative of a reduction in GFR, in conscious uni-x lambs from as early as 6 weeks of age.\textsuperscript{23} Collectively, these findings suggest that alterations in renal function in response to loss of a kidney during fetal life start to occur very early in life. In the current study alterations in the renal NO and sympathetic systems were demonstrated at 5 years of age in the uni-x sheep, although at what point these changes were initiated are currently unknown. However, in other models of low nephron endowment associated with adult hypertension (i.e. placental insufficiency, maternal nutrient restriction), altered NO and renal sympathetic nerve responses have been demonstrated in young adults.\textsuperscript{26, 27} Further mechanistic studies in the neonatal period in uni-x sheep are warranted, as the findings of such studies may indicate time-points when interventions might be most effective.
REFERENCES


Table 1: Basal cardiovascular and renal characteristics in uni-x and sham sheep at 5 years of age

Values are mean ± SEM. *P <0.05, **P<0.01 and ***P<0.001 comparing uni-x and sham groups using a two-tailed unpaired Student’s t test.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Uni-x</th>
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<tbody>
<tr>
<td></td>
<td>n = 7</td>
<td>n = 7</td>
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<tr>
<td>Body weight (kg)</td>
<td>56 ± 2</td>
<td>54 ± 6</td>
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<tr>
<td>Kidney weight (g)</td>
<td>121 ± 6</td>
<td>108 ± 6</td>
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<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>81 ± 1</td>
<td>95 ± 1***</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>85 ± 8</td>
<td>85 ± 8</td>
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<tr>
<td>Plasma sodium (mmol/l)</td>
<td>141 ± 1</td>
<td>141 ± 1</td>
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<tr>
<td>Plasma potassium (mmol/l)</td>
<td>4.5 ± 0.2</td>
<td>4.7 ± 0.1</td>
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<tr>
<td>Renal vascular resistance (mmHg/ml/min/bw)</td>
<td>4 ± 1</td>
<td>9 ± 1***</td>
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<tr>
<td>Renal blood flow (ml/min/bw)</td>
<td>19 ± 1</td>
<td>11 ± 1***</td>
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<tr>
<td>Glomerular filtration rate (ml/min/bw)</td>
<td>2.0 ± 0.15</td>
<td>1.2 ± 0.15**</td>
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<tr>
<td>Urine flow (ml/min/bw)</td>
<td>0.018 ± 0.003</td>
<td>0.015 ± 0.002</td>
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<tr>
<td>Sodium excretion (μmol/min/bw)</td>
<td>2.4 ± 0.2</td>
<td>1.7 ± 0.4**</td>
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<tr>
<td>Potassium excretion (μmol/min/bw)</td>
<td>3.0 ± 0.3</td>
<td>2.0 ± 0.3*</td>
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<tr>
<td>Chloride excretion (μmol/min/bw)</td>
<td>3.6 ± 0.3</td>
<td>3.4 ± 0.5</td>
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</table>
Table 2. Vascular function in uni-x and sham sheep at 5 years of age.

% K, contraction as a percentage of contraction evoked by 100 mmol$^{-1}$ K$^+$ PSS. BK/ACh indicates either BK or ACh. PE/U46619 indicates either PE or U46619. *P <0.05, **P<0.01 and ***P<0.001 comparing uni-x (n = 8) and sham (n = 9) using a two-tailed unpaired Student’s t test. Values are mean ± SEM.

<table>
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<tr>
<th>Smooth muscle function</th>
<th>Renal</th>
<th>Mesenteric</th>
<th>Femoral</th>
<th>Coronary</th>
<th>Renal</th>
<th>Mesenteric</th>
<th>Femoral</th>
<th>Coronary</th>
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<td>100 mmol $^{1}$ K (mN mm$^{-1}$)</td>
<td>11.2 ± 1</td>
<td>9.8 ± 0.9</td>
<td>12.9 ± 1</td>
<td>9.8 ± 0.9</td>
<td>11.1 ± 1</td>
<td>10.4 ± 0.6</td>
<td>12.9 ± 1</td>
<td>10.1± 1.1</td>
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<td>PE/U46619, $E_{max}$ (% K)</td>
<td>90.4 ± 3.6</td>
<td>75.1 ± 9.3</td>
<td>59.2 ± 6.3</td>
<td>69 ± 5.5</td>
<td>114.4 ± 4.8***</td>
<td>99.5 ± 8.4*</td>
<td>80.7 ± 5.7*</td>
<td>73.4 ± 8.4</td>
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<td>SNP $E_{max}$ (%)</td>
<td>2.5 ± 1.2</td>
<td>5.2 ± 2.7</td>
<td>7.9 ± 3.2</td>
<td>11.8 ± 4.0</td>
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<td>Endothelium-dependent relaxation ($E_{max}$ %)</td>
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<td>BK/ACh</td>
<td>1.7 ± 0.7</td>
<td>0.3 ± 0.1</td>
<td>1.7 ± 1</td>
<td>0.09 ± 0.01</td>
<td>6.4 ± 3.4</td>
<td>1.3 ± 0.12</td>
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<td>BK/ACh + L-NAME</td>
<td>7.9 ± 1.8</td>
<td>31 ± 67</td>
<td>15.3 ± 5.8</td>
<td>15.3 ± 8.1</td>
<td>6.4 ± 1.3</td>
<td>19.7 ± 6.6</td>
<td>9.8 ± 5.4</td>
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<td>BK/ACh + L-NAME+Indo</td>
<td>9.7 ± 2.2</td>
<td>57.6 ± 7</td>
<td>57.3 ± 11.7</td>
<td>36.6 ± 8.8</td>
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<td>PE/U46619</td>
<td>5.7 ± 0.04</td>
<td>5.5 ± 0.1</td>
<td>5.2 ± 0.2</td>
<td>6.5 ± 0.1</td>
<td>6.0 ± 0.1**</td>
<td>5.8 ± 0.2*</td>
<td>5.6 ± 0.1**</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>SNP</td>
<td>7.2 ± 0.2</td>
<td>7.1± 0.1</td>
<td>7.2 ± 0.3</td>
<td>6.6 ± 0.1</td>
<td>7.1 ± 0.3</td>
<td>7.1 ± 0.1</td>
<td>7.1 ± 0.2</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>BK/ACh</td>
<td>9.2 ± 0.1</td>
<td>8.8 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>8.8 ± 0.1</td>
<td>8.8 ± 0.1**</td>
<td>8.9 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>8.8 ± 0.1</td>
</tr>
<tr>
<td>BK/ACh + L-NAME</td>
<td>8.8 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>7.1 ± 0.3</td>
<td>8.5 ± 0.2</td>
<td>8.2 ± 0.1***</td>
<td>8.1 ± 0.1</td>
<td>7.1 ± 0.2</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>BK/ACh + L-NAME+Indo</td>
<td>7.6 ± 1.6</td>
<td>7.2 ± 0.2</td>
<td>7.0 ± 0.3</td>
<td>7.4 ± 0.2</td>
<td>7.7 ± 0.1</td>
<td>7.3 ± 0.2</td>
<td>7.0 ± 0.2</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Media thickness: lumen ratio (100 mmHg)</td>
<td>0.18 ± 0.03</td>
<td>0.17 ± 0.01</td>
<td>0.28 ± 0.04</td>
<td>0.14 ± 0.003</td>
<td>0.22 ± 0.04</td>
<td>0.17 ± 0.02</td>
<td>0.32 ± 0.1</td>
<td>0.15 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 1 - Endothelium-dependent relaxation in isolated arteries from sham and uni-x animals at 5 years of age

Concentration-relaxation curves for endothelium-dependent relaxation in isolated arteries. (A) renal lobar, (C) mesenteric, (E) coronary and (G) femoral arteries from sham sheep \((n = 9)\) and in (B) renal, (D) mesenteric, (F) coronary and (H) femoral arteries from uni-x sheep \((n=8)\) at 5 years of age. Solid lines indicate the responses in the absence of blockers, dashed lines in the presence of L-NAME, and dotted lines in the presence of L-NAME + indomethacin. Values are mean ± SEM.
Figure 2 - Spontaneous tone generation following eNOS and eNOS + cyclooxygenase inhibition in isolated arteries from sham and uni-x animals at 5 years of age

Tone generated following eNOS inhibition in (A) renal lobar, (C) mesenteric, (E) coronary and (G) femoral arteries from sham and uni-x sheep. Tone generated following eNOS + cyclooxygenase inhibition in (B) renal lobar, (D) mesenteric, (F) coronary and (H) femoral arteries from sham and uni-x sheep. All responses are expressed as a percentage of contraction evoked by high K⁺ PSS. *P<0.05 comparing basal tone generation between uni-x (n = 8) and sham (n = 9) using a two-tailed unpaired Student’s t test. Values are mean ± SEM.
Figure 3 - Constrictor responses evoked by neurovascular stimulation in the presence and absence of blockers

Contractions evoked by 5 second trains of stimuli at 150V at increasing frequencies in isolated renal lobar arteries of sham \((n = 9; \text{open circles})\) and uni-x sheep \((n = 8; \text{closed circles})\) at 5 years of age. Solid lines represent the responses in the absence of blockers. Dashed lines represent the responses in the presence \(\alpha_{1}\)-adrenoceptor antagonist prazosin \((10^{-6}\text{M})\). Dotted lines represent the responses in the presence of voltage-dependent \(\text{Na}^{+}\) channel blocker tetrodotoxin \((\text{TTX}, 10^{-7}\text{M})\). 

\(P\) values represent the results from a repeated measures ANOVA, with factors group \((\text{sham, uni-x})\), frequency and their interaction for constrictor responses in the absence of blockers. Values are mean ± SEM.
Figure 4 - Constrictor responses in renal lobar arteries to nerve stimulation at increasing voltages and frequencies

Contractions evoked by increasing frequencies at 80, 130 & 150V in isolated renal lobar arteries of sham (n = 9; open circles) and uni-x sheep (n = 8; closed circles) at 5 years of age. P values represent the results from a repeated measures ANOVA, with factors group (sham, uni-x), voltage and their interaction for constrictor responses in the absence of blockers. Values are mean ± SEM.
1Hz

\[ P_{\text{Group}} = 0.042 \]
\[ P_{\text{Voltage}} < 0.001 \]
\[ P_{\text{Interaction}} = 0.005 \]

2Hz

\[ P_{\text{Group}} = 0.047 \]
\[ P_{\text{Voltage}} < 0.001 \]
\[ P_{\text{Interaction}} = 0.04 \]

5Hz

\[ P_{\text{Group}} = 0.048 \]
\[ P_{\text{Voltage}} < 0.001 \]
\[ P_{\text{Interaction}} = 0.01 \]

8Hz

\[ P_{\text{Group}} = 0.045 \]
\[ P_{\text{Voltage}} < 0.001 \]
\[ P_{\text{Interaction}} = 0.037 \]
Figure 5 - Passive mechanical wall properties in isolated arteries in uni-x and sham sheep at 5 years of age

A-D, stress-strain relationships in (A) renal, (B) mesenteric, (C) coronary and (D) femoral arteries of uni-x \( (n=8) \) and sham \( (n=9) \) sheep. P values represent the results from a repeated measures ANOVA, with factors group (sham, uni-x), pressure and their interaction. Values are mean ± SEM.
Figure 6: Relative eNOS mRNA expression in the renal cortex and medulla and eNOS, COX-1 and COX-2 mRNA expression in isolated renal lobar arteries of uni-x and sham sheep at 5 years of age

(A) eNOS expression in renal cortical and medullary regions of kidneys taken from 5-year old female sheep. (B) eNOS, (C) COX-1 and (D) COX-2 expression in isolated renal lobar arteries from 5-year old female sham and uni-x sheep. All expression was normalised to a calibrator (sham kidney cortex for whole kidney expression and sham lobar expression for vascular expression) with 18S as the housekeeping gene in sham (n = 9; open bars) and uni-x (n = 8; closed bars) sheep. P values represent results from a two-tailed Student’s t test. *P <0.05 and ***P <0.001. Values are mean ± SEM.