Renal responses to furosemide are significantly attenuated in male sheep at 6 months of age following fetal uninephrectomy

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Submitted 13 October 2011; accepted in final form 6 February 2012

Singh RR, Moritz KM, Bertram JF, Denton KM. Renal responses to furosemide are significantly attenuated in male sheep at 6 months of age following fetal uninephrectomy. Am J Physiol Regul Integr Comp Physiol 302: R868–R875, 2012. First published February 8, 2012; doi:10.1152/ajpregu.00579.2011.—We have previously shown that fetal uninephrectomy (uni-x) at 100 days of gestation (term = 150 days) in male sheep results in a 30% nephron deficit, reduction in glomerular filtration rate (GFR) and renal blood flow, and elevation in arterial pressure at 6 mo of age. Furthermore, in response to an acute 0.9% saline load, sodium excretion was significantly delayed in uni-x animals leading us to speculate that tubuloglomerular feedback (TGF) activity was reset in uni-x animals. In the present study, we induced TGF blockade by furosemide administration (1.5 mg/kg iv over 90 min) and determined GFR, effective renal plasma flow, and urine and sodium excretion responses in 6-mo-old male sheep. In response to furosemide, a significant diuresis and natriuresis was observed in the sham group; however, the response was significantly delayed and reduced in uni-x animals (both, P<0.001). Cumulative urinary and sodium output was significantly less in the uni-x compared with the sham sheep (both, P<0.001). GFR was increased in the sham but not the uni-x sheep (P<0.0001). In conclusion, the excretory response to furosemide was attenuated in the uni-x sheep, and this suggests a rightward resetting of the TGF operating point. The TGF mechanism is important in the fine tuning of sodium homeostasis and is likely a contributing factor for the dysfunction in sodium regulation we have previously observed in the uni-x animals.

REDCUED NEPHRON ENDOWMENT is associated with an increased risk of developing chronic kidney and cardiovascular diseases, particularly hypertension in adulthood (6). To investigate the direct association between a congenital nephron deficit and development of hypertension, our group has established a model of fetal uninephrectomy (uni-x) in sheep (15, 21). Development of the permanent (metanephric) kidney in sheep (20) is very similar to that in humans (45) with both species completing nephrogenesis before birth. This is different from the rat where nephrogenesis is ongoing after birth and final nephron complement can be altered by the postnatal environment (46). We have previously reported that fetal uni-x in male sheep results in an approximate 30% reduction in nephron number at the completion of nephrogenesis due to compensatory nephrogenesis in the remaining kidney (15). Male uni-x sheep have low plasma renin levels, elevated arterial pressure, and reduced glomerular filtration rate (GFR) and renal blood flow at 6 mo of age (15, 31–33).

Recently, we reported, that in response to an acute period of volume expansion (0.9% saline at 50 ml·kg−1·30 min−1), the onset of sodium and water excretion was significantly delayed in the uni-x animals (32). Furthermore, the delay in onset of sodium and water excretion in response to acute volume expansion, both diuresis and natriuresis were significantly exaggerated in the uni-x animals resulting in greater water and sodium loss than control animals (32) further indicating that the tight regulation of sodium homeostasis is impaired in the uni-x sheep, which could indicate a rightward resetting of the tubuloglomerular feedback (TGF) response. Very recently we have shown that renal responses to exogenous angiotensin II (ANG II) infusion are significantly altered in the uni-x animals, with a reduction in GFR occurring at low-dose ANG II but an increase in GFR occurring at higher doses of ANG II when maximal increases in arterial pressure were observed, suggesting an impairment in renal autoregulation (33). The response to ANG II in conjunction with the response to volume expansion in the uni-x sheep suggested to us that the supposed impairment in renal autoregulation in response to ANG II may be associated with a rightward resetting of the TGF response.

Loop diuretics such as furosemide cause significant diuresis and natriuresis by inhibiting the actions of the Na-K-2Cl cotransporter NKCC2 in the thick ascending limb, including the macula densa and cause vasodilation by inhibiting the TGF mechanism (47). Therefore in the present study we used furosemide to induce TGF blockade and hypothesized that the TGF responses to furosemide in the uni-x sheep would be significantly diminished. Direct measurements of TGF are only possible at the single nephron level through micropuncture studies. This study, therefore, assessed TGF sensitivity on a whole kidney level based on the presumption that whole kidney function would reflect that of the single nephron.

MATERIALS AND METHODS

Animals. All experiments were approved and performed in accordance with the guidelines of the National Health and Medical Research Council of Australia. Merino ewes carrying a male fetus of known gestational age underwent surgery at 100 days postconception. Anesthesia was induced in ewes and fetus’ with Pentothal (thiopental sodium 1 g iv) and maintained with halothane (1.5–2% in O2). The fetal left kidney was cleared from surrounding fat, and the left renal artery, left renal vein, and ureter were ligated (uni-x group = 6), and the kidney was excised. In six fetuses, the kidney was cleared from the surrounding fat but was not excised (sham-operated group = 6). Postsurgery, ewes were housed in large pens in the animal house for 2 wk, before being transported to a farm for the remainder of pregnancy. After birth, lambs remained with their mothers on pasture.
until 16 wk of age when they were weaned. As fully developed rams are aggressive in behavior and difficult to house, all animals underwent rubber-ring castration at 12 wk of age. At 5 mo of age the lambs underwent surgery, and the right carotid artery was surgically exteriorized into a skinfold to form a carotid arterial loop (14) to enable insertion of a carotid arterial catheter for long-term blood pressure measurements at later time points. At 6 mo of age, animals underwent surgery for insertion of chronic bladder catheters as previously described (32), and after appropriate recovery (4–5 days), they were brought into the laboratory, housed in individual metabolic cages, and allowed a week to acclimatize to laboratory conditions. The following week a Tygon cannula was inserted into the carotid artery and connected to a pressure transducer for blood pressure measurements. Catheters were also inserted into the right and left jugular veins for infusion purposes. Basal mean arterial pressure (MAP) and heart rate (HR) were measured in conscious animals, continuously for 72 h (data for this have been previously reported (32)). On a separate day, starting at 0730 h, time-control measurements of renal function were made where urine flow rate (41) was recorded, and plasma and urine samples were collected to determine sodium and osmolality levels. GFR and effective renal plasma flow (ERPF) were determined by clearance methods where GFR was determined via clearance of 51chromium EDTA (10 ml bolus of 15 μCi, followed by intravenous infusion at 15 μCi/h), and ERPF was determined via clearance of p-aminohippurate (PAH, 4.8 mg·kg⁻¹·h⁻¹ in 10-ml bolus, followed by intravenous infusion at 750 mg/h); these were infused at a combined rate of 12 ml/h. These data have also been previously reported (32). All animals were fed a diet of hay and oaten chaff at 1700 h each evening from the same feed batch and given water ad libitum, while in the laboratory thus sodium intake was similar in both groups as animals were all given the same quantity of food, which was eaten in its entirety.

Renal function in response to furosemide. Two days after the 7-h time-control measurements of renal and cardiovascular function were performed, responses to furosemide were determined. Similarly, experiments commenced at 0730 h and were performed in conscious animals during which MAP (mmHg), HR (beats/min), GFR (ml·min⁻¹·g kidney wt⁻¹), ERPF (ml·min⁻¹·g kidney wt⁻¹), and urine flow rate (UFR, ml·min⁻¹·g kidney wt⁻¹) were measured. Urine and plasma sodium concentrations were determined (Beckman Instruments). Sodium excretion (U Na⁺V, μmol·min⁻¹·g kidney wt⁻¹) was calculated as UFR × urine sodium concentration, filtered load of sodium (μmol·min⁻¹·g kidney wt⁻¹) was calculated as plasma sodium concentration × GFR, and fractional sodium excretion (FE Na⁺ %) was calculated as (U Na⁺V/filtered load of sodium) × 100. Plasma and urine osmolalities (freezing point depression, Advanced Instruments, Norwood, MA) were determined; and osmolar excretion (μosM·min⁻¹·g kidney wt⁻¹) was calculated as UFR × urine osmolality; and free water clearance (Ch₂O, ml·min⁻¹·g kidney wt⁻¹) was calculated as UFR × (osmolar excretion/plasma osmolality). After a 2-h basal period all animals were administered 1.5 mg/kg furosemide via the right jugular vein (1 mg·kg⁻¹·h⁻¹ iv; Sigma-Aldrich, St. Louis, MO) at a constant infusion of 1 ml/min over 90 min. Urine samples were collected at 20-min intervals during the control period and 30-min intervals during infusion of furosemide and the recovery period. At every collection point, the animal was flushed to ensure that the bladder was completely emptied. This dose of furosemide is within the range previously described to inhibit the TGF mechanism (39).

Statistical analysis. Values are means ± SE. Hypothesis testing was performed using the software package SYSTAT (Version 11; SPSS, Chicago, IL). Two-sided P = 0.05 was considered statistically significant. Comparisons of individual variables between uni-x and sham sheep were performed using Student’s unpaired t-test. To determine whether responses to furosemide or vehicle differed in uni-x compared with sham sheep, we used repeated measures analysis of variance (ANOVA) to test effects of the within subjects factor “time,” the between-subjects factor “treatment” (sham or uni-x) and their interaction (18). Post hoc analysis was performed to determine the time points, compared with control with-in a group, that furosemide caused a significant effect using a Student’s paired t-test with a Bonferroni correction for multiple comparisons.

RESULTS

Gestation length and birth weight were not different between the groups. As reported previously (32), at the time of cull at 6 mo of age body weight [body weight (kg): sham, 39 ± 3; uni-x, 35 ± 3] and total kidney weight [total kidney weight (g): sham (two kidneys), 73 ± 6; uni-x (1 kidney), 79 ± 6] between the treatment groups were similar.

Seven-hour time control (vehicle infusion) studies were performed on a separate day in these sheep, and the results have been previously reported (32). No changes in arterial pressure or renal function were observed across time, and the basal cardiovascular and renal values were not statistically different from those observed during the basal period in these animals in the current study (32). Basal cardiovascular and renal variables obtained over 2 h before furosemide infusion are shown in Table 1. Uni-x animals had significantly elevated basal MAP and renal vascular resistance (RVR) and reduced GFR and reduced (ERPF) (P < 0.001 for all) compared with the sham animals. HR, UFR, and filtration fraction were similar between the groups, whereas basal UNa⁺V, filtered load of sodium, and FE Na⁺ were significantly reduced in the uni-x group compared with the sham (P < 0.001 for all Table 1). Plasma sodium and osmolality were not different between the treatment groups. In response to furosemide, plasma sodium and osmolality were also not different between the groups (data not shown).

Table 1. Basal cardiovascular and renal variables monitored over 2 h before furosemide infusion

<table>
<thead>
<tr>
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<th>Basal Variables</th>
<th>Sham</th>
<th>Uni-x</th>
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<tbody>
<tr>
<td>Plasma</td>
<td></td>
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<tr>
<td>Sodium, mmol/l</td>
<td>142 ± 3</td>
<td>143  ± 4</td>
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<tr>
<td>Osmolality, mosmol/kgH₂O</td>
<td>295 ± 5</td>
<td>300 ± 4</td>
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<td>Hematocrit, %</td>
<td>28.2 ± 1.1</td>
<td>27.8 ± 0.9</td>
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<tr>
<td>Cardiovascular</td>
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<tr>
<td>MAP, mmHg</td>
<td>78 ± 1</td>
<td>90   ± 1*</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>80 ± 2</td>
<td>79   ± 1</td>
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<td>Renal</td>
<td></td>
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<tr>
<td>GFR, ml·min⁻¹·g kidney wt⁻¹</td>
<td>1.01 ± 0.03</td>
<td>0.71 ± 0.03*</td>
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<tr>
<td>ERPF, ml·min⁻¹·g kidney wt⁻¹</td>
<td>6.24 ± 0.17</td>
<td>4.89 ± 0.23*</td>
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<tr>
<td>RVR, mmHg·ml·min⁻¹·g kidney wt⁻¹</td>
<td>14.5 ± 0.4</td>
<td>18.5 ± 0.9*</td>
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<tr>
<td>Filtration fraction</td>
<td>0.15 ± 0.01</td>
<td>0.18 ± 0.01</td>
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<tr>
<td>UFR, ml·min⁻¹·g kidney wt⁻¹</td>
<td>0.014 ± 0.002</td>
<td>0.013 ± 0.001</td>
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<tr>
<td>UNa⁺V, μmol·min⁻¹·g kidney wt⁻¹</td>
<td>1.35 ± 0.06</td>
<td>0.65 ± 0.02*</td>
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<tr>
<td>Filtered load sodium, μmol·min⁻¹·g kidney wt⁻¹</td>
<td>142.7 ± 4.3</td>
<td>100.4 ± 4.3*</td>
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<tr>
<td>Fractional excretion sodium, %</td>
<td>0.95 ± 0.01</td>
<td>0.68 ± 0.06*</td>
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<tr>
<td>Osmolar excretion, μosM·min⁻¹·g kidney wt⁻¹</td>
<td>9.13 ± 0.67</td>
<td>9.09 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Free water clearance, ml·min⁻¹·g kidney wt⁻¹</td>
<td>-0.019 ± 0.001</td>
<td>-0.018 ± 0.001</td>
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Values are means ± SE; n = 6. Basal cardiovascular and renal function variables measured over 2 h before furosemide infusion in male sheep at 6 mo of age following either sham or uninephrectomy (uni-x) at 100 g DA. MAP, mean arterial pressure; GFR, glomerular filtration rate; ERPF, effective renal plasma flow; RVR, renal vascular resistance; UFR, urine flow rate, UNa⁺V, sodium excretion. All renal variables were corrected for kidney weight. P values are from two-tailed Student’s unpaired t-test; *P < 0.001.
Cardiovascular and renal responses to furosemide infusion. Furosemide infusion caused a minor decrease in MAP in both treatment groups. The extent of this decrease was similar in both treatment groups (P(treatment×time) = 0.47, Fig. 1A). The decrease in arterial pressure was observed at 90 min of furosemide infusion in both groups (% decrease in MAP compared with basal levels at 90 min; sham: -1.8 ± 0.3, P = 0.001; uni-x: -1.1 ± 0.3, P = 0.01, P values from a paired t-test comparing 90 min to basal levels in both groups).

UFR (per gram of kidney weight) increased in both groups following furosemide; however, this increase was reduced and delayed in the uni-x animals compared with the sham group (P(treatment = 0.13, P(time < 0.0001, P(treatment×time < 0.0001, Fig. 2A). Urine output started to increase in the sham animals at 30 min of furosemide infusion, whereas in the uni-x animals, urine output increased at 60 min of infusion with cumulative urine output from the commencement of furosemide infusion till the end of the recovery period being significantly less in the uni-x animals compared with the sham group (P(treatment = 0.04, P(time < 0.001, P(treatment×time < 0.0001, Fig. 3A).

UNaV increased in both groups following furosemide infusion; however, this increase was more moderate in the uni-x animals (P(treatment = 0.003, P(time < 0.0001, P(treatment×time < 0.0001, Fig. 2B). Cumulative sodium output increased significantly during furosemide infusion and through the recovery period (P(time < 0.001); however, uni-x animals had a significantly lower cumulative sodium output compared with sham animal (P(treatment×time = 0.02, Fig. 3B).

Similarly, osmolar excretion increased in both groups following furosemide infusion, but the increase was more moderate and delayed in the uni-x group compared with the sham animals (P(treatment = 0.11, P(time < 0.0001, P(treatment×time < 0.0001, Fig. 2E). CH2O decreased in the sham animals in the first 30 min of furosemide infusion; however, uni-x animals had an increase in CH2O during this period (CH2O; sham: 63% decrease, P < 0.001, uni-x: 71% increase, P < 0.001) following which it declined to basal levels in both groups (P(treatment = 0.04, P(time = 0.03, P(treatment×time < 0.0001, Fig. 2F).

GFR (per gram of kidney weight) was estimated via EDTA clearance measurements. The GFR response to furosemide was markedly different in the sham and uni-x groups (P(treatment = 0.0003, P(time < 0.0001, P(treatment×time < 0.0001, Fig. 1B). In the uni-x sheep GFR did not differ significantly from the control value at any time point during the furosemide infusion or the recovery period (Fig. 1B). Whereas GFR in the sham animals, as estimated via EDTA clearance, was increased compared with the control value at the 30-min (126 ± 19%, P < 0.001) and 60-min (28 ± 2%, P < 0.05) time points and had decreased below control values from the 90-min period to the end of the study (all, P < 0.001). Thus the filtered load of sodium increased significantly in the sham animals but remained unchanged in the uni-x group in response to furo-

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Fig. 1. Cardiovascular and renal response to furosemide infusion. Furosemide was infused intravenously for 90 min at 1 mg·kg⁻¹·h⁻¹ (hatched bars). The recovery period commenced immediately following the end of the 90 min of furosemide infusion. A: mean arterial pressure (MAP); B: glomerular filtration rate (GFR); C: effective renal plasma flow (ERPF); D: renal vascular resistance (RVR); E: filtration fraction. All renal variables were corrected for kidney weight (kw). P values are from a repeated measures ANOVA with factors treatment (uni-x, sham), time, and their interaction. Values are means ± SE. Sham (n = 6): open bars or white-hatched bars depicting period of furosemide administration, uni-x (n = 6): dark bars or dark-hatched bars depicting period of furosemide administration. #P < 0.05, ##P < 0.01, ###P < 0.001, compared with baseline values for sham group and *P < 0.05, **P < 0.01, ***P < 0.001, compared with baseline values for uni-x group from post hoc analysis with a Student’s paired t-test with Bonferroni correction for multiple comparisons.
semide (P_treatment < 0.0001, P_time < 0.0001, P_treatment x time < 0.0001, Fig. 2C). FENa increased in both groups in response to furosemide; however, while FENa returned to basal levels in the sham animals, it remained elevated in the uni-x animals for a longer duration (P_treatment < 0.0001, P_time < 0.0001, P_treatment x time < 0.0001, Fig. 2D). However, it should be noted that since UFR increased markedly in the sham, GFR was likely overestimated during the first 30-min period of the furosemide. This same limitation also applies to ERPF measurements and those variables that use GFR or ERPF in their calculation during the 30-min period in the sham group (see DISCUSSION).

ERPF (per gram of kidney weight) increased in both groups during furosemide infusion; however, this increase was less in the uni-x animals compared with the sham group (P_treatment < 0.0001, P_time < 0.0001, P_treatment x time < 0.0001, Fig. 1C). The peak increase in ERPF occurred at 30 min of furosemide infusion in both treatment groups with the percentage increase in ERPF after 30 min of furosemide infusion being ~200% in the sham animals compared with ~80% in the uni-x sheep (P < 0.001, t-test). Both treatment groups had an initial decrease in RVR in response to furosemide with levels returning to baseline by the end of the study (P_treatment < 0.0001, P_time < 0.0001, P_treatment x time < 0.0001, Fig. 1D). This decrease in RVR was less in the uni-x animals compared with the sham group (% decrease in RVR at 30 min; sham: 68 ± 2, uni-x: 44 ± 1, P < 0.001). Whereas RVR returned to basal levels at 90 min in the sham animals, it remained lower in the uni-x animals for a longer duration. Filtration fraction decreased in both groups following furosemide administration. However, whereas filtration fraction increased gradually in the sham animals following furosemide infusion, it remained re-

Fig. 2. Urinary excretion variables in response to 90 min of furosemide (1 mg·kg\(^{-1}\)·h\(^{-1}\) iv) infusion. Urine flow rate (UFR, A), urinary sodium excretion (U\(_{Na}\)V, B), filtered load of sodium (Na) (C), fractional excretion of sodium (FENa, D), osmolar excretion (E), and free water clearance (CH\(_{2}O\), F) in response to furosemide infusion. All variables were corrected for kidney weight. P values are from a repeated measures ANOVA with factors treatment (uni-x, sham), time, and their interaction. Values are means ± SE. Sham (n = 6): open bars or white-hatched bars depicting period of furosemide administration, uni-x (n = 6): dark bars or dark-hatched bars depicting period of furosemide administration. Values are means ± SE. #P < 0.05, ##P < 0.001 compared with baseline values for sham group and *P < 0.05, **P < 0.01, ***P < 0.001 compared with baseline values for uni-x group from post hoc analysis with a Student’s paired t-test with Bonferroni correction for multiple comparisons.
Changes in concentration of Na responses observed during TGF blockade. TGF is initiated by nephron level, our data in response to furosemide reflect while the present study did not measure TGF at the single animal. This led us to speculate that the delayed responses were markedly reduced and/or delayed in the uni-x animals compared with the control animals (32), whereas in the sham animals furosemide administration resulted in significant changes in renal hemodynamic responses and tubular sodium and water excretion, these responses were markedly reduced and/or delayed in the uni-x animals. In a previous study in the same animals, we investigated the renal responses to an acute period of saline loading and observed that sodium and water excretion was significantly delayed in the uni-x animals compared with the control animals (32). This led us to speculate that the delayed responses indicated resetting of the TGF mechanism in the uni-x animals.

In a previous study in the same animals, we investigated the renal responses to acute saline loading and observed that sodium and water excretion was significantly delayed in the uni-x animals compared with the control animals (32). This led us to speculate that the delayed responses indicated resetting of the TGF mechanism in the uni-x animals. While the present study did not measure TGF at the single nephron level, our data in response to furosemide reflect responses observed during TGF blockade. TGF is initiated by changes in concentration of Na, Cl, and K in tubular fluid delivered to the distal tubules (4). Furosemide is a loop diuretic that blocks NKCC2 cotransporters in the thick ascending limb of Henle that reduces salt and water reabsorption in this segment and increases diuresis and natriuresis and blockade of TGF can cause vasodilation resulting in increases in renal blood flow as well (11, 23, 24).

Consistent with other studies (35, 36), furosemide administration in the present study increased urine flow, UNaE, GFR, and ERPF in the sham animals. Using a similar furosemide dosing regimen (2 mg/kg over 120 min), Smith and colleagues (36, 39) have reported a similar increase in urine flow and UNaE and increases in ERPF in sheep. In the current study in response to furosemide, GFR (EDTA clearance) increased rapidly in the sham animals, such that at 30 min, GFR had increased by ~120% compared with basal levels. The increase in ERPF (PAH clearance) in the sham animals at this time was greater relative to the increase in GFR, hence, filtration fraction decreased following furosemide infusion. This suggests that the vasodilation in response to furosemide was predominantly preglomerular in the sham animals. Thus the observed increase in GFR and ERPF in the sham animals is compatible with TGF blockade in response to furosemide, since previous studies have demonstrated that TGF predominantly modulates afferent arteriolar tone, an effect inhibited by furosemide (4, 9, 43). A caveat to this conclusion is that the EDTA and PAH may not have been in steady state (though plasma levels of both varied by <10% during the experiment), and while the bladder was flushed to ensure complete urine collection for each time point, it is possible that there is inaccuracy in the GFR and ERPF measurements due to dead-space error. Thus the magnitude of increase in GFR and ERPF in the sham animals at 30 min of furosemide infusion may not be as great as estimated and should be interpreted with caution. However, undoubtedly the clearance of both these substances was markedly different between the sham and uni-x sheep. We conservatively estimate urinary dead space in a 30-kg sheep to be ~30 ml based on evidence in the literature (5, 10). Taking this into account, we estimate that the increase in GFR in response to furosemide was likely in the order of ~60% in the sham sheep. Urinary dead space would need to be 50 ml in the sheep for this level of EDTA clearance to indicate no change in GFR under the present conditions; this is unlikely given that urine volume is ~2–3 ml (5) and the total renal mass is only 73 g. Moreover, at 60 min of furosemide infusion, when there was no difference in UFR between the groups, the increase in GFR in the sham animals was ~30%. This measurement period is less likely influenced by dead-space error issues and is similar to the increase in GFR observed in a previous study in rats (27), thus may represent a reasonable increase in GFR during furosemide in our study.

In contrast to the sham animals, supporting our hypothesis that there is a right and upward shift in TGF, GFR was not altered by furosemide treatment in the uni-x sheep. Previously, we reported that the renal responses, specifically the increases in ERPF, GFR, urine flow, and UNaE were significantly delayed in the uni-x animals in response to saline loading (32), suggesting that TGF was reset. In adult nephrectomy models in the rat, the TGF operating point has been shown to be reset to function at a lower single nephron GFR and higher solute delivery (1, 3, 13). However, it has also been shown that due to this resetting, the reactivity of the TGF is attenuated following nephrectomy (22). However, we believe we have to be cautious when extrapolating findings from adult nephrectomy.

**DISCUSSION**

The major finding of this study was that renal responses to furosemide infusion were significantly attenuated in the uni-x animals. Whereas in the sham animals furosemide administration resulted in significant changes in renal hemodynamic responses and tubular sodium and water excretion, these responses were markedly reduced and/or delayed in the uni-x animals.

In a previous study in the same animals, we investigated the renal responses to an acute period of saline loading and observed that sodium and water excretion was significantly delayed in the uni-x animals compared with the control animals (32). This led us to speculate that the delayed responses indicated resetting of the TGF mechanism in the uni-x animals. While the present study did not measure TGF at the single nephron level, our data in response to furosemide reflect responses observed during TGF blockade. TGF is initiated by changes in concentration of Na, Cl, and K in tubular fluid delivered to the distal tubules (4). Furosemide is a loop diuretic that blocks NKCC2 cotransporters in the thick ascending limb of Henle that reduces salt and water reabsorption in this segment and increases diuresis and natriuresis and blockade of TGF can cause vasodilation resulting in increases in renal blood flow as well (11, 23, 24).

Consistent with other studies (35, 36), furosemide administration in the present study increased urine flow, UNaE, GFR, and ERPF in the sham animals. Using a similar furosemide dosing regimen (2 mg/kg over 120 min), Smith and colleagues (36, 39) have reported a similar increase in urine flow and UNaE and increases in ERPF in sheep. In the current study in response to furosemide, GFR (EDTA clearance) increased rapidly in the sham animals, such that at 30 min, GFR had increased by ~120% compared with basal levels. The increase in ERPF (PAH clearance) in the sham animals at this time was greater relative to the increase in GFR, hence, filtration fraction decreased following furosemide infusion. This suggests that the vasodilation in response to furosemide was predominantly preglomerular in the sham animals. Thus the observed increase in GFR and ERPF in the sham animals is compatible with TGF blockade in response to furosemide, since previous studies have demonstrated that TGF predominantly modulates afferent arteriolar tone, an effect inhibited by furosemide (4, 9, 43). A caveat to this conclusion is that the EDTA and PAH may not have been in steady state (though plasma levels of both varied by <10% during the experiment), and while the bladder was flushed to ensure complete urine collection for each time point, it is possible that there is inaccuracy in the GFR and ERPF measurements due to dead-space error. Thus the magnitude of increase in GFR and ERPF in the sham animals at 30 min of furosemide infusion may not be as great as estimated and should be interpreted with caution. However, undoubtedly the clearance of both these substances was markedly different between the sham and uni-x sheep. We conservatively estimate urinary dead space in a 30-kg sheep to be ~30 ml based on evidence in the literature (5, 10). Taking this into account, we estimate that the increase in GFR in response to furosemide was likely in the order of ~60% in the sham sheep. Urinary dead space would need to be 50 ml in the sheep for this level of EDTA clearance to indicate no change in GFR under the present conditions; this is unlikely given that urine volume is ~2–3 ml (5) and the total renal mass is only 73 g. Moreover, at 60 min of furosemide infusion, when there was no difference in UFR between the groups, the increase in GFR in the sham animals was ~30%. This measurement period is less likely influenced by dead-space error issues and is similar to the increase in GFR observed in a previous study in rats (27), thus may represent a reasonable increase in GFR during furosemide in our study.

In contrast to the sham animals, supporting our hypothesis that there is a right and upward shift in TGF, GFR was not altered by furosemide treatment in the uni-x sheep. Previously, we reported that the renal responses, specifically the increases in ERPF, GFR, urine flow, and UNaE were significantly delayed in the uni-x animals in response to saline loading (32), suggesting that TGF was reset. In adult nephrectomy models in the rat, the TGF operating point has been shown to be reset to function at a lower single nephron GFR and higher solute delivery (1, 3, 13). However, it has also been shown that due to this resetting, the reactivity of the TGF is attenuated following nephrectomy (22). However, we believe we have to be cautious when extrapolating findings from adult nephrectomy.
models to that of the fetus as regulation of renal function is distinct in the fetus compared with the adult (8).

We have also recently reported that basal plasma renin and ANG II and renal tissue renin and ANG II levels are significantly reduced in 6-mo-old uni-x male sheep as are levels of both ANG II receptors (AT1R and AT2R) (33). Additionally, we showed that in response to increases in arterial pressure induced by exogenous ANG II infusion, uni-x male sheep exhibited a paradoxical increase in GFR, suggesting the renal autoregulatory mechanisms that usually buffer the kidney against fluctuations in arterial pressure were perturbed in these uni-x animals (33). TGF responses are markedly reduced in rats following administration of angiotensin-converting enzyme (ACE) (13) or ANG II receptor blockers (26, 28, 38), and AT1R- or ACE-deficient mice have minimal TGF activity (30, 40) suggesting that an active renin-angiotensin system (RAS) is important in modulating TGF. Therefore, suppression of the RAS in the uni-x animals may be a contributing factor to the resetting of TGF. Together, our studies support a role for a rightward shift in TGF in uni-x sheep at 6 mo of age. A preliminary report in a dexamethasone model of fetal programming observed increased TGF sensitivity at 3 wk of age in lambs (7). Thus temporal changes in TGF may occur in response to a congenital nephron deficit. Further studies are warranted to substantiate this hypothesis.

Furosemide, by blocking TGF, caused a reduction in RVR, an effect that was attenuated in the uni-x sheep. We have no direct evidence as to the mechanism for the resetting of TGF in the uni-x sheep. However, the TGF signal is mediated by adenosine and modulated by nitric oxide, ANG II, and renal prostaglandins (29, 42). It is possible that altered activity of each or all of these aforementioned factors may be contributing to the reduced response to furosemide in the uni-x animals. As previously discussed, basal plasma and renal levels of ANG II and renin are significantly reduced in these uni-x sheep (33), and while furosemide has been shown to stimulate renin release in the dog (2), we have no information on the activity of the RAS during furosemide infusion in the uni-x animals. It is possible that the blunted basal RAS is contributing to the modest decrease in RVR in the uni-x animals. Furosemide administration has also previously been shown to increase renal prostaglandin synthesis (16, 24), and an increase in these vasodilators may also account for an increase in renal blood flow in the uni-x animals. Furthermore, levels of adenosine and nitric oxide, other important mediators and modulators of TGF, may be different in the uni-x animals and may account for the renal hemodynamic responses observed. Further studies need to be undertaken to investigate the roles/contributions of the mediators and modulators of TGF in animals with a reduced nephron endowment.

While UFR in response to furosemide did not increase in the uni-x animals to the same extent as control animals, CH2O was greater in the uni-x animals compared with control animals, indicating excretion of more dilute urine. Studies in isolated dog kidneys have reported that furosemide administration in the presence of the anti-diuretic hormone arginine vasopressin (AVP) increases CH2O, which is associated with reduced water reabsorption in the collecting ducts, indicating that the collecting ducts may be desensitized to AVP in the presence of a diuretic (44). Recently, we have shown that in response to both AVP infusion and to a 30-h period of dehydration, these uni-x animals have a significantly reduced urine concentrating ability (34). This defect in concentrating ability was observed despite plasma AVP levels being similar between the treatment groups (34). AVP regulates water transport by acting on the aquaporin 2 (AQP-2) channels in the collecting duct segments, and the expression of AQP-2 is significantly reduced in the uni-x animals at 6 mo of age (34); therefore, it is possible that the increase in CH2O in response to furosemide in the current study is due to the decrease in the AQP-2 channels in these animals.

As reported in previous studies in this cohort (32), basal sodium excretion was significantly lower in the uni-x compared with the sham group. This may reflect sodium retention as we have previously reported that these 6-mo-old uni-x male sheep do have greater plasma and blood volumes compared with the sham group (32). However, given the degree of disparity in sodium excretion between the groups, which one would predict would cause marked increases in extracellular fluid volume and arterial pressure, it is unlikely to simply reflect sodium retention. It is possible that reabsorption via the gut is altered or influenced by circadian rhythm in the uni-x animals (37), more sodium is being sequestered in the sheep rumen, or fecal sodium excretion (19) is greater in the uni-x animals. It is also possible that the diurnal regulation of renal function is altered in the uni-x animals. Indeed in children with reduced renal function a significant diurnal variation in sodium excretion has been reported (25). The circadian regulation of sodium needs to be further investigated in this model to establish the basis of discrepancy in sodium output in the uni-x animals.

There is significant evidence to suggest that sex differences exist in the developmental programming of renal and cardiovascular dysfunction, with a tendency for male disadvantage (12); therefore, findings in female uni-x sheep could be different from what has been observed in this study in male sheep. However, it should be noted that the male offspring in the present study were castrated early in life to make them easier to handle and safe to work with. Interestingly, a recent study by Woods et al. (34) has shown that castration of male offspring did not prevent the increase in arterial pressure in the maternal low-protein model of reduced nephron endowment and developmental programming of hypertension. The role of sex hormones on renal and cardiovascular dysfunction needs to be further elucidated in this model.

**Perspectives and Significance**

Previous observations of impaired sodium handling (32) and reduced renal autoregulatory (33) efficiency in the uni-x animals led us to speculate that the TGF responses of the remnant kidney were likely reset to a higher operating point. The responses to furosemide were attenuated in the present study and provide evidence that TGF activity in the uni-x animals was reduced and this may be due to a resetting of this mechanism. TGF is reset as a compensatory adaptation to reduction in renal mass (1); however, findings from the present study indicate that these adaptations may be beneficial in the short term but maladaptive with time, resulting in renal dysfunction and the onset of hypertension in adulthood. Although TGF resetting can be partly associated with a suppression of the RAS, other factors such as nitric oxide, may also play a
role. Future studies will need to examine the role of these factors to elucidate the compensatory adaptations that occur in the remnant kidney following a reduction in renal mass. This may help us understand mechanisms pertaining to renal insufficiency in this model and provide avenues for intervention in individuals born with a nephron deficit.

ACKNOWLEDGMENTS

The authors thank Alex Satragno and Alan McDonald for assistance in surgical preparation of the animals and Andrew Jefferies for technical assistance.

GRANTS

This project was supported by a National Heart Foundation Grant (G 05M 2110) and Monash University Research Fund. R. Singh was supported by an Australian Postgraduate Award. K. Moritz was supported by an NH&MRC Career Development Award and by an NH&MRC Senior Research Fellowship, and K. Denton was supported by an NH&MRC Senior Research Fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.R.S., K.M.M., J.F.B., and K.M.D. conceived and designed the research; R.R.S. and K.M.D. interpreted results of experiments; R.R.S. prepared figures; R.R.S. and K.M.D. performed experiments; R.R.S. analyzed data; R.R.S. and K.M.D. wrote the manuscript; R.R.S., K.M.M., J.F.B., and K.M.D. approved final version of manuscript.

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