Nitric Oxide Deficiency and Increased Adenosine Response of Afferent Arterioles in Hydronephrotic Mice With Hypertension

Mattias Carlström, En Yin Lai, Andreas Steege, Mauricio Sendeski, Zufu Ma, Sheller Zabihi, Ulf J. Eriksson, Andreas Patzkz, A. Erik G. Persson

Abstract—Afferent arterioles were used to investigate the role of adenosine, angiotensin II, NO, and reactive oxygen species in the pathogenesis of increased tubuloglomerular feedback response in hydrenephrosis. Hydrenephrosis was induced in wild-type mice, superoxide dismutase-1 overexpressed mice (superoxide-dismutase-1 transgenic), and deficient mice (superoxide dismutase-1 knockout). Isotonic contractions in isolated perfused arterioles and mRNA expression of NO synthase isoforms, adenosine, and angiotensin II receptors were measured. In wild-type mice, $N^\text{G}$-nitro-$L$-arginine methyl ester (l-NAME) did not change the basal arteriolar diameter of hydrenephrotic kidneys ($\pm 6\%$) but reduced it in control ($\pm 12\%$) and contralateral arterioles ($\pm 43\%$). Angiotensin II mediated a weaker maximum contraction of hydrenephrotic arterioles ($\pm 18\%$) than in control ($\pm 42\%$) and contralateral arterioles ($\pm 49\%$). The maximum adenosine-induced constriction was stronger in hydrenephrotic ($\pm 19\%$) compared with control ($\pm 8\%$) and contralateral kidneys ($\pm 0\%$). The response to angiotensin II became stronger in the presence of adenosine in hydrenephrotic kidneys and attenuated in contralateral arterioles. L-NAME increased angiotensin II responses of all of the groups but less in hydrenephrotic kidneys. The mRNA expression of endothelial NO synthase and inducible NO synthase was upregulated in the hydrenephrotic arterioles. No differences were found for adenosine or angiotensin II receptors. In superoxide dismutase-1 transgenic mice, strong but similar L-NAME response ($\pm 40\%$) was observed for all of the groups. This response was totally abolished in arterioles of hydrenephrotic superoxide dismutase-1 knockout mice. In conclusion, hydrenephrosis is associated with changes in the arteriolar reactivity of both hydrenephrotic and contralateral kidneys. Increased oxidative stress, reduced NO availability, and stronger reactivity to adenosine of the hydrenephrotic kidney may contribute to the enhanced tubuloglomerular feedback responsiveness in hydrenephrosis and be involved in the development of hypertension. (Hypertension. 2007;51:1386-1392.)

Key Words: angiotensin II ■ l-NAME ■ oxidative stress ■ NO synthase ■ superoxide dismutase ■ tubuloglomerular feedback

Hydrenephrosis because of uretero-pelvic junction obstruction is a common condition in newborns, with an incidence of $\approx 1\%$. Both rats and mice with hydrenephrosis, because of chronic partial ureteral obstruction, develop renal injury$^1$ and salt-sensitive hypertension$^{2,3}$ that can be attenuated by relief of the obstruction.$^4$ The mechanisms are thought to be associated with increased activity of the renin-angiotensin system,$^2$ increased oxidative stress, and reduced NO availability in the diseased kidney.$^5$

In micropuncture studies of hydrenephrotic animals, the tubuloglomerular feedback (TGF) has a higher responsiveness in the hydrenephrotic kidney.$^{5,6}$ An increased TGF response, as seen in hydrenephrotic kidneys, has also been described in spontaneously hypertensive rats,$^7$ Milan hypertensive rats$^8$ during development of hypertension, and in animals subjected to neuronal NO synthase (NOS) inhibition.$^9$ These studies suggest an important role for changes of the TGF function in the pathophysiology of hypertension. Afferent arterioles (AAs) are effectors in the feedback loop and, therefore, contribute to the responsiveness of TGF. Mediation of the TGF includes the constrictor action of adenosine on AAs.$^{10-12}$ In addition, arteriolar tone and TGF responsiveness are further determined by several vasoactive compounds. Angiotensin (Ang) II mediates vasoconstriction through activation of Ang II type 1 receptor,$^{13}$ and this vasoconstrictor effect is counteracted by locally produced...
NO.\textsuperscript{14,15} It has been demonstrated that increased production of reactive oxygen species, primarily superoxide, can reduce the NO bioavailability and subsequently increase the TGF responsiveness.\textsuperscript{7,16} Finally, a complex interaction among the vascular effects of adenosine, Ang II, and NO has been described to determine the characteristics of the TGF mechanism.\textsuperscript{17–21} The present study was performed to investigate the mechanisms behind the increased TGF response in hydronephrosis that could contribute to the development of hypertension.

### Materials and Methods

#### Animals

Male homozygous superoxide dismutase-1 (SOD1) transgenic (Tg[SOD1]3Cje/J) or SOD1 knockout mice (Sod1tm1Leb) from the Jackson Laboratory (Bar Harbor, ME), were used. They were backcrossed with C57bl/6j mice, which served as wild-type controls (Muslegaard, Copenhagen, Denmark). All of the mice had body masses between 25 and 30 g. The animals were fed with standard mice chow and allowed free access to tap water. The experiments were performed in sham-operated controls and in hydronephrotic animals.

#### Creation of Partial Unilateral Ureteral Obstruction

Hydronephrosis was induced by partial unilateral ureteral obstruction, as described earlier.\textsuperscript{3} The animals underwent surgical obstruction at 3 weeks of age. Sham-operated animals were used as controls. All of the animals were then left to grow for 6 to 8 weeks with free access to standardized diet (0.7% NaCl, TD96329, Harland Scandinavia). In this study, only obstructed animals with significant hydronephrosis were used.\textsuperscript{3}

#### Contraction Measurements

**Dissection of AAs**

Dissection and perfusion procedures have been described before.\textsuperscript{22} In brief: AAs with their glomeruli were perfused in a thermostatically regulated chamber (37°C) by a perfusion system (Vestavia Scientific). The chamber and perfusion system were fixed to the stage of an inverted microscope (Nikon). The AAs were perfused with a pressure in the pressure head of 100 mm Hg, which corresponded with physiological pressure and flow (≈50 nL/min) in the connected AA. Criteria for using an AA were a satisfactory, remaining basal tone and no vasodilatation. Both criteria were tested by rapidly increasing the perfusion pressure and assessing the change in the luminal diameter, which corresponded with transient constriction. A further criterion was a fast and complete constriction in response to a KCl solution (100 mmol/L). Such tested AAs have also been demonstrated to possess good dilatory capabilities.\textsuperscript{23} Solutions used for dissection, perfusion, and bath were the same as described before.\textsuperscript{23}

**Measurements of Arteriolar Diameter**

The experiments were recorded by a video system, digitized offline, and luminal diameters were measured as described before.\textsuperscript{22} All in the series, the last 10 seconds of a control or treatment period were used for statistical analysis of steady-state responses. Each experiment in all of the series used a separate dissected AA, and only 1 AA was used per animal.

#### Isolation of Preglomerular Vessels

Preglomerular vessels (including mainly interlobular arteries and AAs) of mice were isolated with a modified iron oxide–sieving technique described previously\textsuperscript{24,25} and immediately stored at −80°C.

### Analysis of mRNA Expression

RNA was isolated with Trizol reagent and reverse transcribed with Superscript and random hexamers (Invitrogen) according to the manufacturer’s protocol. Quantitative PCR analysis was performed with GeneAmp 5700 (Applied Biosystems). Experiments were performed in triplicate with similar results. The expression levels of receptor mRNA were normalized to β-actin by the ΔΔCt method. Parallelism of standard curves of the test and control was confirmed. Please see the data supplement available online at http://hyper.ahajournals.org for more details.

#### Pharmacological Agents

All of the drugs, Ang II, adenosine, and N\textsuperscript{l}-nitro-l-arginine methyl ester (l-NAME; Sigma-Aldrich), were applied to the bath solution.

### Calculations and Statistics

Values are presented as means±SEMs. ANOVA for repeated measurements (nonparametric Brunner test) was used to test time-dependent changes in the arteriolar diameter and to check for differences between the groups (SAS system). Posthoc comparisons were performed by Tukey’s test. Wilcoxon tests were applied for analysis of treatment effects on basal diameter, for comparison of control diameters between groups, and for gene expression data. Differences were considered to be statistically different if $P<0.05$.

#### Ethics

The experiments were approved by the Uppsala Ethical Committee for Animal Experiments.

### Results

All of the animals were in good condition, and at the time of decapitation there were no differences in body weights between hydronephrotic and control animals.

#### Reactivity of AAs to Vasoactive Substances

**Ang II in Wild-Type Mice**

Basal diameters did not differ between groups. Concentration-response curves were measured by cumulative application of Ang II from $10^{-14}$ to $10^{-8}$ mol/L, 2 minutes of each concentration. Ang II constricted the AA concentration dependent with a maximum of −42% reduction of arteriolar diameters in the control group. AAs of hydronephrotic animals had smaller maximum responses (−18%). The contractile response of AAs from the contralateral kidney (−49%) did not differ from that of the control group (Figure 1).

**NOS Inhibition With l-NAME in Wild-Type Mice**

Basal diameters did not differ between groups in the beginning. l-NAME (10\textsuperscript{-4} mol/L; 15 minutes) constricted AAs of control mice by approximately −12%, the AA of the hydronephrotic kidney did not respond significantly (−6%), and AA of the contralateral kidney constricted strongly (−43%; Figure 2).

**Effect of l-NAME Treatment on Ang II–Induced Constrictions in Wild-Type Mice**

Vessels were pretreated with l-NAME (10\textsuperscript{-4} mol/L; 15 minutes). The NOS inhibitor was also present during the subsequent measurement of the concentration response to Ang II (10\textsuperscript{-14} to 10\textsuperscript{-8} mol/L), at 2 minutes of each concentration. l-NAME treatment, per se, influenced the diameter of AAs of control mice and the hydronephrotic and contralateral kidney similarly compared with data in Figure 2. Arteriolar diameter was reduced in control (−14%) and contralateral...
kidneys (−41%) but not in the hydronephrotic kidneys (−6%). As shown in Figure 3, the response to Ang II was significantly stronger in all 3 of the groups compared with Ang II administration without L-NAME (compare with Figure 1). Furthermore, AAs of contralateral kidneys and control mice constricted more strongly to Ang II than did AAs of hydronephrotic kidneys.

**Effect of Adenosine in Wild-Type Mice**

Adenosine was applied cumulatively from 10⁻¹¹ to 10⁻⁴ mol/L, at 2 minutes for each concentration. Control AA showed a moderate, biphasic response with a small constriction (−8%) at lower concentrations and opening of the vessel at higher concentrations (+9%) compared with basal diameter (Figure 4). AAs of hydronephrotic kidneys constricted stronger (−19%) at lower concentrations and reopened incompletely at higher concentrations of adenosine (−12%). In contrast, AA of the contralateral kidney did not show any contractile response at the lower concentrations, but there was a dilatory trend at higher adenosine concentrations (+8%).

**Adenosine-Ang II Interaction in Wild-Type Mice**

Both substances were added simultaneously to the bath solution. In the first series, a low dose of adenosine (10⁻⁸ mol/L) was combined with cumulative application of Ang II (10⁻¹⁴ to 10⁻⁶ mol/L, 2 minutes for each concentration). In a second series, adenosine was used at a dose of 10⁻⁵ mol/L instead of 10⁻⁸ mol/L.

In the control kidneys, the response to Ang II, together with adenosine at 10⁻⁸ mol/L, was stronger than without adenosine (compare with Figure 1). Adenosine at 10⁻⁵ mol/L...
weakened the response to Ang II in contralateral AA but strengthened the response in hydronephrotic AA compared with the control curve (Figure 5).

Adenosine $10^{-5}$ mol/L did not change the response to Ang II in control animals (Figure 6, compare with Figure 1). However, AAs of hydronephrotic kidneys had clearly stronger responses. In contrast, the response in AAs of the contralateral kidneys ($20\%$) was weaker than in control ($40\%$) and hydronephrotic kidneys ($49\%$) and in the dose response with only Ang II ($50\%$; compare with Figure 1).

**NOS Inhibition With L-NAME in SOD1 Transgenic Mice**

Basal diameters did not differ between groups in the beginning. A strong but similar contractile response was observed with L-NAME ($10^{-4}$ mol/L; 15 minutes) for the AAs of transgenic controls and hydronephrotic and contralateral kidneys, with a maximum response of approximately $-40\%$ (Figure 7).

**NOS Inhibition With L-NAME in SOD1 Knockout Mice**

Basal diameters did not differ among the 3 groups in the beginning but were significantly smaller compared with the wild-type and SOD1 transgenic mice. L-NAME ($10^{-4}$ mol/L; 15 minutes) constricted AAs of knockout controls by approximately $-14\%$ and AAs of the contralateral kidney by $-23\%$. The AAs of the hydronephrotic kidneys did not show any contractile response at all (Figure 8). For changes in the absolute diameters of the AAs during reactivity measurements please see the online data supplement.
Traditional vascular tone. Indeed, in the SOD1 transgenic mice, overexpressing CuZnSOD, a strong but similar L-NAME–induced response (approximately 40%) was observed for A1, A2a, and A2b or in the expression of Ang II type 1 receptor mRNA in preglomerular vessels (Table).

**Discussion**

The present study demonstrates that hydronephrosis induces a variety of functional changes in the AAs of the hydropnephrotic and contralateral kidneys. The most impressive observations are the increased vascular reactivity to adenosine and the reduced NO availability in the hydropnephrotic kidney, whereas there is a reduced reactivity to adenosine of the contralateral kidney along with an increased influence of NO. Data suggest that oxidative stress in the hydropnephrotic kidney will reduce the NO availability, whereas in the contralateral kidney there is an increased NO availability.

The responsiveness of the TGF mechanism involves the action of different vasoactive substances, of which NO is an important determinant. Hydrophoretic kidneys display reduced renal NO availability, which is associated with a sensitized TGF response. In the present study, unspecific NOS inhibition with L-NAME did not cause any significant constriction of AAs from the hydropnephrotic kidneys of wild-type mice, but the controls constricted 12% and the AAs of the contralateral kidney constricted as much as 43% after 15 minutes of treatment. Moreover, a stronger effect must be assumed in contralateral arterioles, because a steady state of arteriolar diameter was not reached at this time. The arteriolar responses indicate a low renal NO concentration in the AAs of hydropnephrotic kidneys and high renal NO concentration in the contralateral kidneys. This is further supported by the dose response of Ang II in combination with L-NAME, where contralateral AAs had a larger contraction than the AAs of the hydropnephrotic kidneys (Figure 3). The result suggest that the Ang II–induced NO release is increased in the contralateral side and reduced in the hydropnephrotic kidney. It is well known that reactive oxygen species, primarily superoxide, can interact with NO. Oxidative stress has been demonstrated in the hydropnephrotic kidney, and could consequently reduce the NO availability and increase the renal vascular tone. Indeed, in the SOD1 transgenic mice, overexpressing CuZnSOD, a strong but similar L-NAME–induced response (approximately 40%) was observed for all of the groups, whereas in the SOD1-knockouts, lacking CuZnSOD, there was an abolished L-NAME response in AAs from the hydropnephrotic kidney.

The mechanisms of the TGF are not yet fully understood, but recent evidence suggests that the TGF is mediated by the release of ATP from the macula densa cells, forming adenosine by 5′ nucleotidase, which causes vasoconstriction of the AA. Adenosine has modest contractile re-

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**Table. Expressions of mRNA for Adenosine and Ang II Receptors in Preglomerular Vessels**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Control</th>
<th>Hydropnephrotic</th>
<th>Contralateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>2.65±0.50 10⁻³</td>
<td>9.24±3.15 10⁻³</td>
<td>7.03±1.49 10⁻³</td>
</tr>
<tr>
<td>A2A</td>
<td>2.06±0.48 10⁻³</td>
<td>3.57±1.09 10⁻³</td>
<td>3.77±0.91 10⁻²</td>
</tr>
<tr>
<td>A2B</td>
<td>4.76±1.13 10⁻³</td>
<td>4.20±1.09 10⁻³</td>
<td>6.97±1.73 10⁻³</td>
</tr>
<tr>
<td>AT₁</td>
<td>26.38±4.43 10⁻³</td>
<td>21.30±5.19 10⁻³</td>
<td>17.72±3.41 10⁻³</td>
</tr>
</tbody>
</table>

Relative mRNA expression (arbitrary units) of adenosine type 1 receptor (A1), adenosine type 2A receptor (A2A), adenosine type 2B receptor (A2B), and Ang II type 1 (AT₁) for sham-operated controls and hydropnephrotic and contralateral kidneys in isolated preglomerular vessels of wild-type mice (normalized to β-actin) are shown.

**Figure 9.** Relative mRNA expression of endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) NOS for sham-operated control, hydropheoptic, and contralateral kidneys in isolated preglomerular vessels of wild-type mice. *Significant differences.
sponse (approximately −8%) in the lower concentration range, followed by vasodilatation in the higher concentration range in AAs of control mice. This agrees with results of a recent study. However, AAs from the hydronephrotic kidneys had a stronger contractile response (−19%) and no dilatory phase, whereas in the contralateral kidney, no contraction was observed, but at higher doses a stronger vasodilatation (+8%) occurred. These data indicate a reduced dilatory ability mediated by A_2 receptors in hydropnephrotic AAs and/or increased constrictor abilities via A_1 receptors. A contrary behavior has to be assumed in the AAs of the contralateral side. Differential expression of receptors should not play a role, because no differences were found among the AAs for mRNA expression of adenosine (A_1, A_2A, and A_2B) receptors. However, changes in the protein expression and/or signaling pathways cannot be excluded.

In the present study we found that the Ang II itself mediated a weaker contraction of the hydropnephrotic AAs than in both controls and contralateral kidneys (Figure 1). This was rather surprising, because there is an increased TGF responsiveness of the hydropnephrotic kidney. Expression of mRNA of the Ang II type 1 receptor was not changed in AAs, indicating that Ang II receptor expression does not play a role in this context. However, we cannot exclude changes in the receptor protein expression and/or in receptor signaling pathways. From this study, the mechanisms for the reduced Ang II reactivity in hydropnephrotic AAs are not clear. However, in combination with adenosine, a reversal of the contractile response by Ang II occurred (Figures 4 and 5). Both adenosine doses amplified the Ang II response in the hydropnephrotic kidney but reduced the AA response from the contralateral kidney (compare Figures 1 and 5). These findings demonstrate that adenosine caused a totally different vascular response in the kidneys of hydropnephrotic mice, which is in accordance with the vasodilatory behavior of the contralateral kidney and the vasoconstrictive behavior of the hydropnephrotic kidney and is in agreement with studies measuring renal blood flow, GFR, and TGF in hydropnephrotic animals with hypertension. Adenosine and Ang II interact on AAs via calcium-dependent and calcium-independent pathways. Low concentrations of adenosine increase the response of afferent AA to Ang II via predominant action on A_1 receptors. In the present study, even high concentrations of adenosine, also activating dilatory A_2 receptors, increased the Ang II response in the hydropnephrotic kidney. This indicates low dilatory capabilities to adenosine of AAs.

In the gene expression analysis, an elevation of endothelial NOS and inducible NOS, but not neuronal NOS mRNA, was observed for the hydropnephrotic animals. Despite the fact the NO availability was reduced in the AA from the obstructed kidney, the mRNA expression for all of the NOS isoforms was higher in the hydropnephrotic kidney than in the contralateral one. It has been demonstrated that reduced NO availability may increase the NOS transcription, and, therefore, one could speculate that the gene expressions of the different NOS isoforms are upregulated to compensate for low NO availability in hydropnephrosis. It is likely that oxidative stress can increase the elimination of NO, increase AA resistance, and contribute to hypertension. This idea was strengthened by the findings in the SOD1 mice. Reduced oxidative stress in the SOD1 transgenic mice increased the renal NO availability, whereas increased oxidative stress in the SOD1 knockout mice, particularly in the hydropnephrotic AA, resulted in NO deficiency.

**Perspectives**

From the present study, it was evident that the strong contractile response of the hydropnephrotic AA can explain the increased TGF responsiveness of the obstructed kidney, and, conversely, reduced contractile response of the contralateral AA is in accordance with low TGF responsiveness of the nonobstructed kidney. Apparently, most of the TGF resetting occurred in the smooth muscle cells of the AAs, and this important process occurred as a consequence of the interactive response of the different vasoactive substances, NO, adenosine, and Ang II. The increased TGF responsiveness in the hydropnephrotic kidney is associated with compensatory changes of the filtration properties of the nonobstructed contralateral kidney. If this compensation, in water and electrolyte excretion, is insufficient, it will lead to volume retention and consequently have an important role in the development of salt-sensitive hypertension.

**Acknowledgment**

We thank Dr Erdmann Seeliger for helpful discussions.

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**Disclosures**

None.

**References**


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Supplemental Material

**Analysis of mRNA expression**

SYBR Green was used for the fluorescent detection of DNA generated during PCR. The PCR reaction was performed in a total volume of 25µl with 0.4pmol/µl of each primer, and 2x SYBR Green master mix (Applied Biosystems); 2µl cDNA corresponding to 10ng RNA was used as template.

**S 1. Primer sequences used for the analysis of mRNA expression of angiotensin and adenosine receptors and for nitric oxide synthases.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ref.</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>AT₁A</td>
<td>NM_177322</td>
<td>AT₁A fw: GAT TGG TAT AAA ATG GCT GG&lt;br&gt;AT₁A rev: TCT GGG TTG AGT TGG TCT CA</td>
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<tr>
<td>AT₁B</td>
<td>NM_175086</td>
<td>AT₁B fw: CAC TGT AGA TGG GGA GCA GCC AA&lt;br&gt;AT₁B rev: GGG AGT AGG GAT CAT GAC AA</td>
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<tr>
<td>A₁</td>
<td>NM_009629</td>
<td>A₁ fw: GGA TCG GTA CCT CCG AGT CA&lt;br&gt;A₁ rev: AGG CCT ACC ACA AGG GAG AGA</td>
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<tr>
<td>A₂A</td>
<td>NM_009630</td>
<td>A₂A fw: GAA TTC CAC TCC GGT ACA ATG G&lt;br&gt;A₂A rev: TGA TGC CCT TCG CCT TCA</td>
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<tr>
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<td>NM_007413</td>
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<tr>
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<td>NM_010927</td>
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<td>nNOS fw: TCG GCT GTG CTT TGA TGG A&lt;br&gt;nNOS rv: TTG AAT CGG ACC TTG TAG CTC TTC</td>
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</table>
Angiotensin II (Ang II) concentration response curves in afferent arterioles of sham-operated control (n=6), hydronephrotic (n=6), and contralateral kidneys (n=6) of wild-type mice.

* indicates significant differences of concentration response curves.
Effect of L-NAME on afferent arteriolar diameter in sham-operated control (n=9), hydronephrotic (n=7), and contralateral kidneys (n=6) of wild-type mice.

* indicates significant differences between concentration response curves.
Effect of L-NAME on angiotensin II (Ang II) concentration response in afferent arterioles of sham-operated control (n=7), hydronephrotic (n=6), and contralateral kidneys (n=6) of wild-type mice.

* indicates significant differences of concentration response curves compared to control.
Adenosine concentration response in afferent arterioles of sham-operated control (n=9), hydrenephrotic (n=6), and contralateral kidneys (n=6) of wild-type mice.

* indicates significant differences between concentration response curves.
Effect of low dose adenosine (10^{-8} \text{ mol/l}) on angiotensin II (Ang II) concentration response in afferent arterioles of sham-operated control (n=9), hydronephrotic (n=6), and contralateral kidneys (n=8) of wild-type mice.

* indicates significant differences between concentration response curves.
Effect of high dose adenosine ($10^{-5}$ mol/l) on angiotensin II (Ang II) concentration response in afferent arterioles of sham-operated control mice (n=10), hydronephrotic (n=6), and contralateral kidneys (n=10) of wild-type mice.

* indicates significant differences between concentration response curves.
Effect of L-NAME on afferent arteriolar diameter in sham-operated control mice (n=7), hydronephrotic (n=5), and contralateral kidneys (n=6) of SOD1-transgenic mice.
Effect of L-NAME on afferent arteriolar diameter in sham-operated control mice (n=6), hydronephrotic (n=4), and contralateral kidneys (n=6) of SOD1-knock-out mice.

* indicates significant differences between concentration response curves.