AT1 receptor blockade prevents interstitial and glomerular apoptosis but not fibrosis in pigs with neonatal induced partial unilateral ureteral obstruction

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may be multifactorial. By inducing vasoconstriction via AT$_1$ receptors in presumably the efferent arterioles, consequential hypoxia of the tubulointerstitial compartment may stimulate fibroblast proliferation, extracellular matrix synthesis, and apoptosis (40). Furthermore, the AT$_1$ receptors play a role in the stimulation of a number of cytokines such as the profibrotic growth factor transforming growth factor-β (TGF-β) (16, 56). The stimulatory impact of ANG II on this multitude of components in the cascade of events leading to fibrosis has given hope for the development of novel therapeutic approaches aiming at inhibiting the effect of ANG II. In a recent study of complete ureteral obstruction in adult rats, the ANG II antagonist losartan appeared to attenuate apoptosis and fibrosis, leading the authors to conclude that ANG II blockade may be valuable in the antifibrotic armamentarium (33). Such a recommendation may, however, not necessarily be applicable to the situation of congenital partial obstruction, where ANG II is demonstrated to be of importance (40). Furthermore, the AT1 receptors play a role in the hypoxia of the tubulointerstitial compartment may stimulate fibroblast proliferation, extracellular matrix synthesis, and apoptosis.

The purpose of the present study was to characterize apoptosis and fibrosis formation in a well-established neonatal pig model with PUUO induced during ongoing nephrogenesis. The role of ANG II was investigated by blocking the AT$_1$ receptor with candesartan, (28), after the end of nephrogenesis. To quantitate apoptosis, we used a specific technique with immunohistochemical staining for active caspase 3 specifically identifying the effector caspases only found in apoptotic cells (43). By use of stereological techniques, the absolute numbers of apoptotic cells in the interstitial compartment, in the glomeruli, and in the different tubular segments were counted (18). Distinguishing between different tubular segments and intrarenal capillaries often proves problematic. We therefore introduce a new variant of the physical fractionator involving a third immunostained section to localize the apoptotic cells to specific tubular segments. The extent of interstitial collagen accumulation was quantified using stereological techniques after visualization by picro Sirus red staining and further by hydroxyproline quantification.

**MATERIALS AND METHODS**

**Experimental design.** The experiments were performed in 30-day-old female Danish Landrace pigs who at age 2 days were subjected to partial ureteral obstruction of the left ureter using the psoas implantation method introduced by Ulm and Miller (51) or sham operation (previously described by us in detail) (23). In brief, under isoflurane anesthesia a 3- to 3.5-cm flank incision was made on the left side using sterile techniques. The muscle layers were divided, and the anesthesia a 3- to 3.5-cm flank incision was made on the left previously described by us in detail) (23). In brief, under isoflurane.

Table 1. Kidney wet weight and kidney volume in the 4 experimental groups

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 9)</th>
<th>Sham + can (n = 9)</th>
<th>PUUO (n = 7)</th>
<th>PUUO + can (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, kg</td>
<td>4.86 (1.04, [5.66, 4.05])</td>
<td>4.86 (0.66, [5.47, 4.25])</td>
<td>4.89 (1.71, [6.46, 3.31])</td>
<td>4.67 (1.25, [5.82, 3.52])</td>
</tr>
<tr>
<td>Kidney wet weight, g</td>
<td>24.35 (5.57, [28.63, 20.06])</td>
<td>23.11 (5.46, [28.17, 18.06])</td>
<td>37.03* (10.71, [48.27, 25.78])</td>
<td>33.41 (10.98, [42.59, 24.23])</td>
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<tr>
<td>Kidney volume, mm$^3$</td>
<td>25.71 (7.77, [31.68, 19.73])</td>
<td>27.72 (9.04, [34.67, 20.77])</td>
<td>33.00 (5.08, [37.70, 28.29])</td>
<td>27.28 (7.36, [32.94, 21.62])</td>
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Values are means (SD, [95% confidence interval]). Sham, sham operated; can, Candesartan; PUUO, partial unilateral ureteral obstruction. *$P = 0.004$ PUUO vs. sham.
were added to 10 mg of dried and pulverized tissue and hydrolyzed at 118°C for 18 h. This was neutralized using 120
samples were collected and defatted in chloroform:methanol (2:1)
midsection of the kidneys in the four experimental groups. The
sections by picro Sirius red, which stains connective tissue fibers
showed no staining.
replacement of the primary antibodies with nonimmune sera, which
DakoCytomation, Glostrup, Denmark). The negative control included
von Willebrand factor after demasking with proteinase K (both from
principal cells (N-20, 1:50, Chemicon, AH Diagnostics, Aarhus,
staining with aquaporin-2 (AQP2) antibodies for collecting duct
purified biotinylated goat anti-rabbit IgG (Vector Laboratories, Bur-
(polyclonal antibody specific for the activated subunit of caspase 3
were identified with the active caspase 3 technique using a rabbit
the test frame,
where
is the number of cells,
randomly sampled sections by serial sectioning and weighted by the
number of cells, 
, counted in this tissue. The section number is denoted 
and 
(frame) is the area of the counting frame. Finally, 
 is the number of cells (nuclei) counted in the kidney. This number is divided by two, because the counting is performed both ways in the physical disector.
Tubular lengths were estimated in both cortex and medulla by
counting the number of tubule profiles sampled by a 2-dimensional
counting frame and estimating the length density, which is converted
to absolute length by multiplying with the shrinkage-adjusted renal volume

where 
 is the number of tubule profiles, 
(frame) is the area of the
test frame, 
 is the number of test points, 
(kidney) is the
number of test points which hits kidney tissue, and the volume of the
kidney is denoted 
(kidney). The factor 2 is only valid when isotropic
area probes are used, which is not the case in the medulla of the
kidney. Isotropy means that all directions have equal probability of
being chosen. This factor is therefore not correct in this study, and
the total length results are biased with an unknown factor. This, however,
applies to all four groups.
Throughout this study, we cut the tissue samples in three adjacent
sections of 2.5 μm of thickness. The first two sections were used for
counting apoptotic cells (the physical disector probe), the last section
for distinguishing between the thin limb of Henle and the capillaries,
and the distal tubules and the collecting ducts.
With regard to tissue deformation (shrinkage), small kidney samples
were weighed and the weight was converted to volume by
multiplying using 1.04 cm³/g (volume before). The volume of kidney
samples after paraffin embedding (volume after) was estimated by
point counting and the principle of Cavalieri (42). Tissue deform-
ation was equal to 1 − (volume after/volume before), which
was accounted for.
For all tissue preparation and stereological quantitation the ob-
server was naive to the origin of the histological sections.

Immunohistochemistry and collagen assessment. Apoptotic cells
were identified with the active caspase 3 technique using a rabbit
polyclonal antibody specific for the activated subunit of caspase 3
(1:200, Cell Signalling Technologies, Beverly, MA) and affinity-
purified biotinylated goat anti-rabbit IgG (Vector Laboratories, Bur-
ingame, CA). Tubular segments were identified and separated by
staining with aquaporin-2 (AQP2) antibodies for collecting duct
principal cells (N-20, 1:50, Chemicon, AH Diagnostics, Aarhus,
Denmark). The capillaries were identified using antibodies against
von Willebrand factor after demasking with proteinase K (both from
DakoCytomation, Glostrup, Denmark). The negative control included
replacement of the primary antibodies with nonimmune sera, which
showed no staining.
The extent of interstitial collagen accumulation was visualized in
sections by picro Sirius red, which stains connective tissue fibers
selectively (46).
Hydroxyproline content was measured in tissue samples from
a midsection of the kidneys in the four experimental groups. The
samples were collected and defatted in chloroform:methanol (2:1)
including protease inhibitors for 21 h at 4°C. Then, 200 μl 6 N HCl
were added to 10 mg of dried and pulverized tissue and hydrolyzed at
118°C for 18 h. This was neutralized using 120 μl 10 M sodium
hydroxide. Hydroxyproline was measured in the supernatant after
centrifugation as described elsewhere (53). The collagen content was
calculated from total hydroxyproline using 7.46 as the correction
factor and expressed as a proportion of the tissue dry weight (μg
collagen /10 mg dry weight tissue).

Statistics. Values are presented as mean (SD, [95% confidence
interval]) and compared using ANOVA and the post hoc Bonferroni
procedure. Data for tubular apoptosis, cell number, and lengths were
compared using an unpaired Student’s t-test. Values were considered
statistically significant at
.
RESULTS

All pigs had macroscopically normal-appearing left kidneys at study entry and right kidneys at study’s end. All partially obstructed kidneys were found to have significant hydronephrosis at age 30 days. Seven animals were lost due to anesthesia-related death (n = 3) or failure to thrive (n = 4). In four animals, perfusion fixation was not possible for practical reasons, leaving a total of nine sham, nine sham + can, seven PUUO, and nine PUUO + can to be included in the study. Urine culture at 30 days did not demonstrate urinary tract infection.

Kidney weight and volume. Body weight did not differ among the experimental groups, demonstrating that somatic growth was unaffected by neonatal induced PUUO and by candesartan treatment (Table 1). PUUO, on the other hand, significantly increased partially obstructed kidney weight but not kidney volume compared with sham-operated kidneys. Candesartan did not change kidney wet weight or volume in either partially obstructed or sham-operated kidneys compared with the saline-treated groups (Table 1).

PUUO increased interstitial cell apoptosis and fibrosis formation. The apoptotic interstitial cells were identified by histochemical staining for active caspase 3 as shown in Fig. 1 and quantitated using stereological techniques. Twenty-eight days of PUUO induced at age 2 days significantly increased the total number of apoptotic interstitial cells in the partially obstructed kidneys compared with sham-operated kidneys (4,568 \( \times 10^4 \) (SD 1,749 \( \times 10^4 \) [7,903–10^4, 1,234–10^4]) vs. 1,363 \( \times 10^4 \) (SD 1,230–10^4 [2,309–10^4, 418–10^4]); P = 0.004) (Figs. 1 and 2).

By use of stereological techniques and picro Sirius red staining for collagen fibers, the volume of fibrosis in the entire kidney could be estimated. The volume of fibrosis was significantly increased in the partially obstructed kidneys compared with sham-operated kidneys (4.62 (SD 2.43 [6.65, 2.59]) vs. 2.49 (SD 1.34 [3.51, 1.46]) cm^3, P = 0.03) (Figs. 3 and 4). The increased fibrosis formation in the partially obstructed kidneys was confirmed by quantitation of hydroxyproline and calculation of the collagen content (per 10 mg of dry tissue) in a section from the middle part of the kidney. The collagen density in the partially obstructed kidneys was significantly increased compared with sham-operated kidneys (443.7 (SD 176.7 [663.1, 224.4]) vs. 208.0 (SD 28.0 [276.6, 139.4]) \( \mu g/10 \) mg dry tissue, P = 0.009), confirming the results from the whole-kidney stereological analysis (Fig. 5).

Tubular apoptosis was not increased after 28 days of PUUO. To quantify the number of apoptotic cells in the different tubular segments, a new technique was developed using an additional immunostained section for segmental identification (Fig. 6). The results revealed that the number of apoptotic cells was not increased in the proximal tubules (P = 0.76), in the distal tubules (P = 0.29), or in the collecting ducts (P = 0.15) compared with sham-operated kidneys after 28 days of partial obstruction (Table 2).

Since the apoptotic response of upper tract obstruction is dynamic (15), tubular apoptosis may have peaked at an earlier time point, subsequently leading to decreased tubular length and cell number. We therefore used stereological techniques to quantify the total tubular length and the total number of tubular cells in the left kidneys of saline-treated pigs. The results revealed that partial obstruction for 28 days did not lead to a decreased number of cells in the proximal tubules (P = 0.13), the thin limb of Henle (P = 0.23), the distal tubules (P = 0.52), or the collecting ducts (P = 0.23) compared with sham-operated rats (Table 3). Furthermore, it did not lead to a decreased number of cells in the proximal tubules (P = 0.49), in the thin limb of Henle (P = 0.41), in the distal tubules (P = 0.20), or in the collecting ducts (P = 0.41) compared with sham-operated kidneys (Table 4).

Candesartan prevented the PUUO-induced interstitial cell apoptosis but not fibrosis. To elucidate the role of AT1 receptor stimulation in PUUO-induced interstitial fibrosis and apoptosis, one-half of the pigs were treated with candesartan from age 23 to 30 days. Candesartan treatment attenuated the PUUO-induced increase in interstitial apoptosis as the total number of apoptotic interstitial cells in these kidneys were not increased compared with sham-operated kidneys (1,749 \( \times 10^4 \) (SD 818 \( \times 10^4 \) [2,378–10^4, 1,121–10^4]) vs. 1,169 \( \times 10^4 \) (SD 871 \( \times 10^4 \) [2,378–10^4, 1,121–10^4]); P = 0.50). In accordance the number of apoptotic interstitial cells in the partially obstructed kidneys of candesartan-treated piglets was significantly decreased compared with partially obstructed kidneys from saline treated piglets (1,749 \( \times 10^4 \) (SD 818 \( \times 10^4 \) [2,378–10^4, 1,121–10^4]) vs.

![Fig. 2. The total number of apoptotic interstitial cells quantitated by active caspase 3 technique and stereology was significantly increased in the partially obstructed kidneys [unilateral partial ureteral obstruction (PUUO)] compared with sham-operated kidneys (*P = 0.004) and to candesartan-treated partially obstructed kidneys (PUUO + can) (*P = 0.004).](attachment:image.png)
Candesartan treatment did not reduce the PUUO-induced fibrosis formation as the total volume of fibrosis (estimated by stereological techniques) in the partial obstructed kidneys did not differ between candesartan-treated and saline-treated pigs (4.96 (SD 2.64 [7.17, 2.76]) vs. 4.62 (SD 2.43 [6.65, 2.59]) cm³, \( P = 0.73 \)) (Figs. 3 and 4). The increased fibrosis formation in both candesartan-treated and saline-treated obstructed groups was confirmed by the collagen density, which did not

Fig. 3. The total volume of fibrosis visualized by picro Sirius red staining for collagen fibers was significantly increased in PUUO kidneys (C) compared with sham-operated kidneys (A). Candesartan treatment did not reduce the PUUO-induced fibrosis (D) and did not induce fibrosis in sham-operated kidneys (B).

Fig. 4. The total volume of fibrosis quantitated by stereological methods in kidney sections stained with picro Sirius red for collagen fibers was significantly increased in PUUO kidneys compared with sham-operated kidneys (*\( P = 0.03 \)).
differ between the two groups (375.9 (SD 116.7 [473.5, 278.3]) vs. 443.7 (SD 176.7 [663.1, 224.4]) /H926210 mg dry tissue, /H11005P0.62) (Fig. 5). Candesartan did not induce fibrosis in the sham-operated kidneys (2.72 (SD 1.19 [3.63, 1.81]) cm3 and did not increase the collagen density (200.5 (SD 130 [320.7, 80.3]) /H9262/10 mg dry tissue compared with saline treatment (/H11005P0.70 and /H11005P0.89, respectively).

Candesartan prevented PUUO-induced glomerular apoptosis. Immunohistochemistry using caspase 3 antibodies (Fig. 7) and stereological methods revealed that the total number of apoptotic glomerular cells was significantly increased in kidneys subjected to partial obstruction compared with sham-operated kidneys (6,775*103 (SD 3,786*103 [10,277*103, 3,274*103]) vs. 2,520*103 (SD 2,079*103 [4,322*103, 949*103]), /H11005P0.03) (Fig. 8).

Candesartan prevented PUUO-induced glomerular cell apoptosis, since the number of apoptotic glomerular cells did not differ between partially obstructed or sham-operated kidneys in candesartan-treated pigs (3,679*103 (SD 3,100*103 [6,062*103, 1,296*103]) vs. 2,635*103 (SD 2,194*103 [4,322*103, 949*103]), /H11005P0.42) (Fig. 8).

**DISCUSSION**

In the present study, we used a well-established neonatal pig model with high analogy to the human kidney. For the first time, fibrosis formation and tubulointerstitial and glomerular cell apoptosis are quantitated in the multipapillary kidney subjected to chronic neonatal PUUO. The results demonstrated that chronic blockade of the AT1 receptor by candesartan after completion of nephrogenesis prevented PUUO-induced interstitial apoptosis but not fibrosis. Furthermore, AT1 receptor blockade prevented the increased glomerular apoptosis, demonstrating that ANG II plays a role in obstruction-induced apoptosis in this model.

Identification of apoptotic renal cells. To quantify tubulointerstitial cell apoptosis, we used antibodies against activated caspase 3. The apoptotic event is initiated by one of several
results are supported by findings in the neonatal rat kidney after 28 days of neonatal induced partial obstruction. Our distal tubule cells or in collecting duct cells was not increased.

Table 2. Tubular cell apoptosis after 28 days of partial obstruction or sham operation

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<th>Sham (n = 9)</th>
<th>PUUO (n = 7)</th>
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<tbody>
<tr>
<td>Proximal tubules</td>
<td>501 ± 10³, (797 ± 10³ [1,113 ± 10³, 0])</td>
<td>622 ± 10³, (950 ± 10³ [1,500 ± 10³, 0])</td>
</tr>
<tr>
<td>Distal tubules</td>
<td>591 ± 10³, (898 ± 10³ [1,281 ± 10³, 0])</td>
<td>2,109 ± 10³, (3,363 ± 10³ [5,219 ± 10³, 0])</td>
</tr>
<tr>
<td>Collecting ducts</td>
<td>0</td>
<td>651 ± 10³, (1,032 ± 10³ [1,605 ± 10³, 0])</td>
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</table>

Values are means (SD, [95% confidence interval]).

pathways converging into a common arm characterized by the orderly activation of a family of caspases acting as initiators, executioners, or cytokine processors, leading to the structural changes of apoptosis (43). Activated caspase 3 plays a central role in the execution and has proven a valid predictor of apoptosis (29, 55). The dynamic expression of caspases and the central role of caspase 3 have also been documented during urinary tract obstruction in mice and in human tubular cells exposed to mechanical stretch and stress (44, 49). We therefore chose to use identification of activated caspase 3 in the present study as this method specifically identifies cells undergoing apoptosis. This is opposed to the frequently used transerase-mediated dUTP-biotin nick end-labeling technique relying on DNA fragmentation, which is not specific for apoptotic cells. However, neither of these methods allows for characterization of the specific cell type undergoing apoptosis in the interstitial compartment.

The use of several antibodies and three parallel sections allowed for localization of the apoptotic cells to specific parts of the nephrons. For this purpose, we developed an improved staining technique using AQP2 antibodies to identify the collecting ducts and antibodies against von Willebrand factor to identify the capillaries. This ensured that each cell type was correctly identified and quantified.

To quantify the number of cells, a design-based stereological method (physical fractionator with varying sampling fraction) for the estimation of total numbers of “normal” and apoptotic cells was introduced. This physical fractionator with varying sampling fractions has been calibrated for tissue shrinkage, and the estimates are therefore independent of tissue deformation. To overcome the problem with estimating many different cell types in the same sampling procedure, counting frames with different sizes and varying step lengths were used in the CAST system. The aim was to make the error variance of the method (CE) so that the total variance (CV) would be less affected. In general, the aim is to design the study so that: 0.2 < CE²/ CV² < 0.5.

Neonatal PUUO did not induce tubular apoptosis after 28 days of obstruction. We found that apoptosis in proximal or distal tubule cells or in collecting duct cells was not increased after 28 days of neonatal induced partial obstruction. Our results are supported by findings in the neonatal rat kidney where partial obstruction of 2-wk duration did not induce tubular cell apoptosis (48). This is opposed to models of complete ureteric occlusion, which have demonstrated increased tubular apoptosis that in the adult mouse model peaked after 2 wk of unilateral ureteral occlusion (15, 48). The rodent studies suggest that tubular apoptosis may appear in a dynamic expression related to the severity of obstruction. It is therefore possible that tubular apoptosis had occurred at an earlier time-point, i.e., before the investigation after 28 days of PUUO, or that the obstruction was not severe enough to induce tubular apoptosis in the present study. This is consistent with the absence of apoptosis in biopsy specimens from human hydronephrotic kidneys taken at the age of 9–16 mo (37).

If tubular apoptosis had peaked at an earlier time point in the present study, it could have led to increased cell destruction and thereby decreased tubular length. Because the kidneys in our study were perfusion fixed, which preserves structural integrity, tubular lengths could be quantified accurately. We found that neonatal induced PUUO did not compromise renal growth as indicated by kidney weight and volume and did not lead to decreased tubular lengths after 28 days of partial obstruction. These observations may reflect either insignificant tubular apoptosis or compensatory tubular proliferation as described in obstructed rat kidneys subjected to treatment with epidermal growth factor (11).

Interstitial cell apoptosis and fibrosis are increased by neonatal PUUO. Twenty-eight days of PUUO increased the total number of apoptotic interstitial cells in partially obstructed kidneys in this neonatal pig model. This finding is consistent with the increased apoptotic cell density and visual impression of increased apoptotic interstitial cell numbers in completely obstructed neonatal and adult rat kidneys (10, 12, 14). Using similar apoptosis-specific methods, the levels of apoptosis signaling molecules such as Fas and Fas ligand were elevated and associated with increased caspase 3 levels and interstitial apoptotic cells in adult mice with ureteral ligation, supporting the findings of the present study (15, 49).

Using picro Sirius red staining of collagen fibers and stereological techniques for whole-kidney quantification, we found that neonatal induced PUUO significantly increased interstitial fibrosis. The increased collagen density in the partially obstructed kidneys confirmed the increased amount of fibrotic tissue. Our findings are in accordance with the increased fibrotic tissue density found in neonatal rat kidneys with severe PUUO or temporary occlusion (13, 48) and in occluded adult rat kidneys (31). Moreover, the increased collagen density is also in agreement with observations in the occluded adult mouse kidney (17).

In the present study and in previous rat studies of fibrosis and apoptosis, the harvested renal tissues are paraffin embedded before the staining procedures. The paraffin-embedding procedure is likely to induce varying degrees of tissue shrink-
Interestingly, in the present study we found that AT1 receptor blockade prevented PUUO-induced interstitial cell apoptosis for 28 days prevented interstitial fibrosis. Also, candesartan delivered by subcutaneous osmotic pumps in adult rat kidneys with complete obstruction prevented interstitial fibrosis (14). In previous studies on apoptosis interstitial fibrosis was not changed by candesartan treatment instituted after completion of nephro-obstruction, it was demonstrated that ANG II inhibition reduced fibrosis by downregulation of TGF-β (30). How-ever, ongoing studies in the present pig model suggest that candesartan may downregulate TGF-β without affecting fibrosis, suggesting other fibrogenic pathways in the neonatal pig model.

Neonatal PUUO increased glomerular apoptosis. We found a significantly increased number of glomerular apoptotic cells counterbalanced by the decreased removal of fibrogenic interstitial cells by apoptosis in the present model.

The discrepancy with findings in the rat may be related to different obstruction models as complete occlusion of the ureter creates a situation that may not be comparable to the partial obstruction induced in the present study. It may also be related to the initiation of ANG II inhibition sooner after establishment of the obstruction in the rat models than in the present pig study. This is supported by a recent study of partial obstruction in neonatal rats, where angiotensin-converting enzyme inhibition by enalapril further aggravated interstitial collagen accumulation when treatment was instituted 10 days after obstruction was induced compared with kidneys subjected to early ANG II inhibition (7). Based on these considerations, the clinical potential of blocking the AT1 receptors as suggested by some may therefore not be as beneficial for the obstructed human kidney in preventing fibrosis as previously thought.

Our findings suggest that other fibrogenic pathways acting independently of the AT1 receptors are of key importance in obstruction-induced fibrosis. This is also supported by findings in the neonatal mouse kidney, where complete absence of ANG II established by zero-copy of the angiotensinogen gene did not prevent interstitial fibrosis formation secondary to ureteral occlusion (26). TGF-β is an important regulator of many of the processes leading to fibrosis formation, including apoptosis and epithelial-mesenchymal transition (1, 32, 36). The TGF-β levels are increased in the obstructed kidney stimulated by the AT1 receptor (1), and previous studies in adult obstructed rat kidneys indicate that AT1 receptor blockade reduces fibrosis by downregulation of TGF-β (30). However, ongoing studies in the present pig model suggest that candesartan may downregulate TGF-β without affecting fibrosis, suggesting other fibrogenic pathways in the neonatal pig model.

Candesartan prevented the PUUO-induced interstitial cell apoptosis but not fibrosis. Based on knowledge obtained from gene knockout animal models, tubular cell apoptosis is likely to play an important role in the complex process leading to interstitial fibrosis (1). However, the role of interstitial cell apoptosis in the fibrogenic response remains unclear. An important finding in the present study was therefore that the PUUO-induced interstitial cell apoptosis was prevented by candesartan treatment instituted after completion of nephrogenesis (which requires an intact ANG II response) (28) but the amount of fibrotic tissue was not reduced. The methods used did not allow for identification of the interstitial cell type undergoing apoptosis. A characteristic of the renal response to obstruction is the accumulation of inflammatory cells in the interstitium, releasing fibrogenic mediators (8). It may therefore be speculated that apoptosis clears inflammatory or other fibrogenic cells from the interstitium, thereby playing a salu-atory role in the fibrogenic response. However, despite the effect on apoptosis interstitial fibrosis was not changed by candesartan in the neonatal obstructed pig kidney. In previous studies using neonatal and grown rat models of complete unilateral obstruction, it was demonstrated that ANG II inhibition reduced fibrosis (30, 31, 35, 38, 39) but did not attenuate the increased interstitial apoptosis (14). Also, candesartan delivered by subcutaneous osmotic pumps in adult rat kidneys with complete obstruction for 28 days prevented interstitial fibrosis (52). Interestingly, in the present study we found that AT1 receptor blockade reduced interstitial apoptosis but not fibrosis. It may be speculated that, in contrast to findings in the rat, the beneficial effect of ANG II inhibition on fibrosis formation was age and thereby different and unknown degrees of bias. We therefore used stereological methods calibrated for tissue shrinkage, providing estimates independently of tissue deformation. To our knowledge, such a correction has not previously been implemented in studies involving estimation of apoptotic cell or fibrosis densities in paraffin-embedded tissue from obstructed kidneys.

<table>
<thead>
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<th>Table 4. Tubular cell number after 28 days of partial obstruction or sham operation</th>
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<td><strong>Sham (n = 9)</strong></td>
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<tr>
<td>Proximal tubules</td>
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<tr>
<td>Thin limb of Henle</td>
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<tr>
<td>Distal tubules</td>
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<td>Collecting ducts</td>
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Values are means (SD, [95% confidence interval]).

Fig. 7. Immunohistochemical staining for active caspase 3 demonstrating apoptotic glomerular cells (red staining cells in glomeruli, arrows) in a representative section from PUUO kidneys of saline-treated pigs (B) as opposed to glomeruli without apoptosis in representative sections from sham-operated kidneys of saline-treated pigs (A) and PUUO kidneys of candesartan-treated pigs (C).
after 28 days of partial obstruction established during ongoing nephrogenesis in this multipapillary pig model. This suggests that increased apoptosis may play a role in the PUUO-induced loss of glomeruli as previously documented after 24 wk of partial obstruction in this model (24). The causative relationship is supported by the decrease in glomerular number occurring after end of nephrogenesis in the neonatal obstructed rat kidney, demonstrating loss of previously formed glomeruli (48).

Although only few studies exist for comparison, our findings are contradictory to rodent models where glomerular cells appear to be resistant to obstruction-induced apoptotic cell death. In adult rodent models with complete obstruction, glomerular apoptotic densities detected by the transferase-mediated dUTP-biotin nick end labeling technique were not increased (49, 50). In contrast, glomerular apoptotic densities were increased in neonatal mice with complete obstruction although the numbers did not reach statistical significance (5). The discrepancy may be explained by differences in animal species and the methods used. The renal maturational stage is also of importance as tubular apoptosis induced by ureteral occlusion is found to be twofold greater in the neonatal than in the adult rat kidney (9).

The finding of increased glomerular apoptosis was not reflected in changes in obstructed kidney glomerular filtration rate in these kidneys (25). We previously showed that a significant reduction in the number of glomeruli was coexistent with intact glomerular filtration rate and differential function values in this model (24). This supports the view that measurements of renal function may not reflect pathological mechanisms involved in congenital hydronephrosis, and a better understanding of these findings may lead to improved monitoring and treatment of human congenital hydronephrosis.

Chronic AT1 receptor blockade prevented PUUO-induced glomerular apoptosis. Blockade of the AT1 receptor by candesartan after the end of nephrogenesis prevented PUUO-induced apoptosis of glomerular cells, demonstrating proapoptotic actions of the AT1 receptor in this model. It was not possible to identify the glomerular cell type undergoing apoptosis. Our findings are in line with observations in weanling rat PUUO kidneys, where glomerular damage in the form of proteinuria was suppressed by ANG II inhibition (2). The role of ANG II in obstruction-induced apoptosis is controversial, and the pathophysiological mechanisms are not yet fully elucidated. In neonatal rat kidneys subjected to 3 days of complete occlusion, ANG II increased tubular cell apoptosis through the AT2 but not the AT1 receptor (14). However, in vitro studies suggest that both the AT1 and the AT2 receptors are involved in ANG II-induced glomerular epithelial cell and tubular cell apoptosis whereas only the AT1 receptor mediates apoptosis of glomerular podocytes subjected to mechanical strain (4, 18, 22). These results demonstrate that ANG II mediates renal cell apoptosis by complex mechanisms and that species- and perhaps cell-specific differences exist. Experimental models with high analogy to the human kidney are therefore necessary to fully elucidate the pathophysiology of congenital hydronephrosis in children and thereby identify potential targets for preventing glomerular apoptosis and destruction.

In conclusion, neonatal induced PUUO leads to increased interstitial and glomerular cell apoptosis and fibrosis in this pig model with high resemblance to the human kidney. Candesartan treatment prevented the increased interstitial and glomerular cell apoptosis but not fibrosis formation. This suggests that pathways not involving AT1 receptor stimulation contribute to neonatal obstruction-induced fibrosis or that prevention of interstitial cell apoptosis counteracts a potential antibiotic effect of AT1 receptor blockade in this pig model of congenital obstructive nephropathy. These observations may have implications for decision making and therapeutic considerations in children.

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