Trps1 Haploinsufficiency Promotes Renal Fibrosis by Increasing Arkadai Expression

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ABSTRACT

Mutations in TRPS1 cause tricho-rhino-pharyngeal syndrome (TRPS). Trps1 is essential for nephron development, acting downstream of Bmp7. Because Bmp7 counteracts epithelial-to-mesenchymal transition (EMT) and reverses chronic renal injury, we examined the function of Trps1 in renal fibrosis. Immunohistochemistry revealed Trps1 expression in proximal tubular epithelial cells of mice. Unilateral ureteral obstruction reduced mRNA and protein expression of Trps1 in wild-type and heterozygous Trps1-knockout (Trps1+/−/−) mice. Trps1 haploinsufficiency promoted tubulointerstitial fibrosis via increased phosphorylation of Smad3 and decreased Smad7 protein. In primary culture, Trps1 deficiency promoted TGF-β1-mediated EMT in proximal tubule cells. Trps1+/−/−-derived cells had higher levels of phosphorylated Smad3, and TGF-β1 induced a time-dependent decrease in Smad7 protein in wild-type and Trps1+/−/− kidneys. In addition, compared with wild-type cells, Trps1+/−/− cells had double the amount of the E3 ubiquitin ligase Arkadai, and TGF-β1 induced further Arkadai expression. Furthermore, knockdown of Arkadai inhibited TGF-β1-induced EMT in Trps1+/−/− cells. Collectively, these data suggest that Trps1 haploinsufficiency enhances TGF-β1-induced EMT and tubulointerstitial fibrosis by modulating the amount of Smad7 through Arkadai/ubiquitin-mediated degradation.


Chronic kidney disease is irreversible, but progressive renal fibrosis is commonly observed regardless of the cause of the diseases. One of the main effector cells that contribute to the development of progressive renal fibrosis is the tubulointerstitial fibroblast. Importantly, in tubulointerstitial fibrosis, a large proportion of the interstitial fibroblasts originate from tubular epithelial cells via an epithelial-to-mesenchymal transition (EMT).1,2 During the process of EMT, tubular epithelial cells lose their epithelial phenotype and acquire a mesenchymal phenotype, which includes disruption of polarized tubular epithelial cell morphology, de novo α-smooth muscle actin (α-SMA) expression and actin reorganization, loss of cell-cell adhesions through downregulation of E-cadherin, destruction of the basement membrane, and increased cell migration and invasion.3,4 Consequently, this process induces renal tubular destruction and accumulation of myofibroblasts. TGF-β1 is proposed to be the major regulator of EMT induction.5 In recent years, the receptors and signal transduction pathways mediating the effects of TGF-β in cells have been identified, and Smad proteins are critical intracellular mediators in the TGF-β signaling pathway.6–9 The transduction of TGF-β/Smad signaling occurs as follows: upon TGF-β binding to TβRII,

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TβRI is phosphorylated by TβRII, and the activated TβRI associates transiently with R-Smads (Smad2 and Smad3) and phosphorylates them. The phosphorylated R-Smads dissociate from the receptor, bind to Smad4, and translocate into the nucleus, where this Smad complex controls the transcription of TGF-β-responsive genes together with additional DNA-binding cofactors. In contrast to R-Smads, Smad7 attenuates the degradation of the complex.15,16 In contrast with Smurfs, Arkadia regulates the transcriptional activity of TGF-β via degradation of Smad7 and not through that of TGF-β receptors.14 Smad7 has been shown to function as an adaptor protein that recruits Smurf ubiquitin ligases to the TGF-β receptor complex to promote the degradation of the complex.15,16 In contrast with Smurfs, Arkadia regulates the transcriptional activity of TGF-β only through degradation of Smad7 and not through that of TGF-β receptors.14

In our previous studies, we demonstrated that Trps1 functions downstream of Bmp7 and is required for the mesenchymal-to-epithelial transition and for regulation of ureteric bud branching in the embryonic kidney.17 However, the role of Trps1 in the adult kidney is still unknown. In this study, we examined Trps1 expression in adult mouse kidneys and found that it was restricted to proximal tubular epithelial cells in the cortex region. Moreover, we used a unilateral ureteral obstruction (UOU) model of renal fibrosis and examined primary proximal tubular epithelial cells from wild-type and heterozygous Trps1-null mice. We demonstrate that heterozygous loss of Trps1 promotes renal fibrosis by activating the TGF-β/Smad3 signaling pathway.

RESULTS

Trps1 Is Localized to the Nuclei of Proximal Tubular Epithelial Cells in Adult Mice

We previously demonstrated that Trps1 was expressed in ureteric buds, renal vesicles, and cap mesenchyme cells in embryonic kidneys. To examine the precise localization of the Trps1 protein in the kidneys of adult mice, we performed immunohistochemistry with antibodies against Trps1. Positive staining was seen in the nuclei of tubular epithelial cells in the cortex region (Figure 1A, b and d). To further characterize the precise localization, antibodies directed against villin, a specific marker for the brush border of proximal tubules, was used for immunohistochemistry. The villin-positive area was restricted to the cortex regions where Trps1 was stained (Figure 1A, a and c). Thus, double immunostaining confirmed that Trps1 was localized to the proximal tubular epithelial cells (Figure 1B).

Trps1 Haploinsufficiency Promotes Renal Fibrosis after UOU

Ligation of a ureter causes renal interstitial fibrosis, characterized by tubular atrophy and deposition of interstitial matrices in the obstructed kidney. Hematoxylin and eosin staining showed representative histologic findings on day 7 and 14 after UOU or sham-operation (sham) in wild-type (WT) and heterozygous Trps1-null (HT) mice (Figure 2A). The obstructed kidneys showed tubular dilation, atrophy, a widened interstitial space, and a greater number of interstitial cells in WT and HT mice (Figure 2A, b, c, e, and f). We found that the degree of these changes was greater in HT mice than in WT mice. Interstitial collagen deposition, as determined by immunohistochemistry with an anti-type I collagen antibody, was approximately 2-fold greater in HT obstructed kidneys than in WT obstructed kidneys (Figure 2B, b, c, e, and f and Figure 2C). These data suggest that Trps1 haploinsufficiency enhances renal fibrosis after UOU.

Trps1 Haploinsufficiency Promotes TGF-β/Smad Signaling after UOU

Because TGF-β signaling is proposed to be the major contributor to renal fibrosis,3 we tested whether the promotion of renal fibrosis in HT mice was associated with TGF-β signaling. Consistent with a previ-
ous finding, real-time (RT)-PCR and ELISA assays showed that the amount of TGF-β markedly increased at the protein and mRNA levels after UUO in WT and HT mice compared with sham-operated mice. However, these levels were not different between WT and HT kidneys that received UUO (data not shown). Next, we examined the phosphorylation of Smad3, a key mediator of TGF-β in fibrosis, in UUO kidneys. Immunohistochemistry showed that nuclear phosphorylated Smad3 (pSmad3) staining was markedly increased in the UUO kidneys compared with sham-operated kidneys in WT and HT mice at day 7 and 14, and the extent of the positive staining was greater in HT kidneys than in WT kidneys (Figure 2D). A quantitative analysis showed that the amount of pSmad3 in HT kidneys was approximately 2-fold higher than in WT kidneys after UUO (Figure 2E).

The Extent of Renal Fibrosis Is Associated with Trps1 and Smad7 Protein Levels

Next, we checked the transcriptional and protein levels of Trps1 in the normal and obstructed kidneys from WT and HT mice to examine the relationship between Trps1 and renal fibrosis. As shown in Figure 3, A through C, the amount of Trps1 was lower for mRNA and protein levels on day 7 and 14 after UUO in a time-dependent manner in WT and HT kidneys and was reduced by approximately half in HT kidneys compared with WT kidneys.

Emerging evidence indicates that Smad7 is a major inhibitor of TGF-β signaling and negatively regulates its intensity and duration. Given the fact that overactivation of Smad2/3 in fibrotic kidneys is associated with a reduction in Smad7, we examined whether the expression of Smad7 was altered in UUO kidneys. Quantitative RT-PCR and western blotting showed that Smad7 protein levels declined in a time-dependent manner in the UUO kidneys from both genotypes, whereas the Smad7 mRNA levels increased (Figure 3, A through C). Interestingly, levels of Smad7 protein were 2-fold lower in the UUO kidneys from HT mice than in the UUO kidneys from WT mice (Figure 3, B and C), whereas the mRNA was 2-fold higher in the HT UUO kidneys (Figure 3A). Western blots also showed that heterozygous loss of Trps1 significantly increased the amount of α-SMA and pSmad3 in the obstructed kidneys at day 7 and 14 after UUO; levels were 2-fold higher than in UUO kidneys from WT mice (Figure 3, B and C). In parallel with α-SMA protein levels, the mRNA expression pattern was increased by RT-PCR (Figure 3A). In contrast, E-cadherin expression gradually decreased at the mRNA and protein levels according to the time course of UUO (Figure 3, A through C). From these results, it is conceivable that the amount of Trps1 protein is negatively correlated with the extent of fibrosis in obstructed kidneys and that a reduction in Trps1 promotes renal fibrosis through a reduction in Smad7 protein levels.

Trps1 Haploinsufficiency Increases TGF-β-Mediated EMT

To confirm whether decreased levels of Trps1 can influence the TGF-β/Smad3 signaling pathway, we compared the expression levels of molecules that are involved in EMT using cultured
proximal tubular epithelial cells (PTECs) from WT and HT mice. Cells from both genotypes appeared morphologically similar (Figure 4A, a and b). At 12 and 24 h after treatment with 5 ng/ml TGF-β1 to induce EMT, HT cells showed a greater degree of staining for α-SMA than WT cells (Figure 4A, c through f), indicating that more HT cells had transdifferentiated into myofibroblasts. The intensity of the signal from positive Trps1 staining decreased by 24 hours after TGF-β1 treatment in both genotypes, which is in agreement with the results of RT-PCR and western blot analysis (Figure 4A, e and f and Figure 4, B and C). Consistent with the immunostaining, RT-PCR and immunoblots revealed that the α-SMA expression was increased 2- and 4-fold, respectively, in HT cells compared with WT cells at 24 hours after TGF-β1 treatment (Figure 4, B through D). In contrast, E-cadherin expression was decreased by half in HT cells compared with WT cells (Figure 4, B through D). These results suggest that a deficiency in Trps1 expression could enhance TGF-β-induced EMT of cultured renal epithelial cells.

TGF-β1/Smad3 Signaling Is Upregulated by Reductions in Smad7 in HT Cells Compared with WT Cells During EMT

To address the molecular mechanism underlying the promotion of EMT by a deficiency of Trps1, we examined whether a reduction in Trps1 affected TGF-β1/Smad3 signaling in PTECs. Smad3 phosphorylation peaked at 6 hours and then decreased gradually in WT cells, whereas it continued to increase until it peaked 12 hours after TGF-β1 treatment in HT cells (Figure 4, C and D). On the other hand, the amount of Smad7 protein decreased in a time-dependent manner after TGF-β1 treatment in WT and HT cells. We found that the amount of Smad7 protein in HT cells was approximately half of the amount in WT cells (Figure 4, C and D), whereas the mRNA levels increased 3- and 5-fold in WT and HT cells, respectively (Figure 4B).

Trps1 Haploinsufficiency Decreases Smad7 Protein Levels by Increasing Arkadia Expression

Although the mRNA levels increased, the Smad7 protein levels gradually decreased in UUO kidney and culture cells treated with TGF-β. Therefore, we examined the expression of Smurf1, Smurf2, and Arkadia, three E3 ubiquitin ligases that degrade Smad7 and regulate TGF-β signaling in EMT of renal tubular epithelial cells.9,14 The amount of Arkadia increased at the mRNA and protein levels in a time-dependent manner in UUO kidneys and WT cells (Figure 3, A through C and Figure 4, B through D), whereas virtually no difference was seen in the levels of Smurf1 and Smurf2 (data not shown). More interestingly, heterozygous loss of Trps1 increased Arkadia protein levels 2-fold compared with those seen in UUO kidneys from WT mice and WT cells, and this difference was further augmented in a time-

Figure 3. A reduction in Trps1 promotes renal fibrosis through a reduction in Smad7 protein levels. (A) Trps1, α-SMA, E-cadherin, Smad7, and Arkadia mRNA levels were measured by quantitative RT-PCR. The results are expressed as mean ± SD (n = 6). *P < 0.05. (B) Western blot analysis of Trps1, α-SMA, E-cadherin, p-Smad3, Smad7, and Arkadia in protein extracts from kidneys of WT and HT mice. (C) Time-course densitometric data (mean ± SD) from western blots of WT and HT kidneys (n = 3).
dependent manner after UUO and TGF-β1 treatment, respectively (Figure 3, A through C and Figure 4, B through D). Taken together, these results indicate TGF-β/Smad3 signaling is promoted by reduced expression of Trps1, which results in lower Smad7 protein levels through upregulation of Arkadia expression.

Arkadia Knockdown Rescues TGF-β-Induced EMT in HT Cells

Because upregulation of Arkadia degrades Smad7 protein and accelerates EMT in HT cells after TGF-β treatment, we examined whether Arkadia inhibition by small interfering RNA (siRNA) could reverse the TGF-β-induced EMT. It was shown by western blotting that Arkadia protein levels in cells from both genotypes treated with TGF-β were decreased by Arkadia siRNA treatment (Figure 5B). Real-time PCR and immunoblots showed that the induction of α-SMA expression by TGF-β was suppressed at mRNA and protein levels in WT and HT cells by preincubation with Arkadia siRNA compared with control siRNA (Figure 5A). In addition, the reduction of Smad7 protein and the induction of pSmad3 in HT cells by TGF-β were inhibited with treatment of Arkadia siRNA (Figure 5B). These results indicate that Arkadia siRNA treatment prevents WT and HT cells from TGF-β/pSmad3-induced EMT by inhibition of Smad7 degradation.

DISCUSSION

In this study, we demonstrated that heterozygous loss of Trps1 promotes renal tubular interstitial fibrosis induced by UUO shown as a schematic representation in Figure 6. Although several factors are involved in fibrosis in renal tissues after UUO caused by the transition of renal tubular epithelial cells to extracellular-matrix-producing myofibroblasts, TGF-β has been shown to be a key mediator of this process.1,3,22 Smad3, an intracellular effector of TGF-β signaling, plays a key role in fibrosis,23 whereas Smad7 acts as an antagonist of TGF-β signaling.11,24 Therefore, when we observed an increase in fibrosis in the obstructed kidneys from HT mice compared with those from WT mice, we hypothesized that Trps1 might affect the
expression of these intracellular signaling molecules. Indeed, the amount of pSmad3 increased more in HT obstructed kidneys than in WT obstructed kidneys, whereas no differences were detected in the amounts of TGF-β/H9252 at the protein or mRNA level. In contrast, the amount of Smad7 protein decreased more in HT kidneys than in WT kidneys, with HT Smad7 protein levels being approximately half of that of WT levels, whereas the mRNA level was more elevated in HT kidneys than in WT kidneys. Previous studies of obstructive and remnant kidney diseases have found that activation of Smad2/3 within the obstructed kidney is correlated with a decrease in Smad7 protein.20,21 On the other hand, pSmad3 directly regulates the Smad7 promoter,25,26 which is considered to be a feedback suppression to TGF-β signaling. Disruption of the Smad7 gene results in a loss of the negative-feedback suppression of TGF-β signaling and promotes TGF-β/Smad activation. The results presented here are consistent with these findings.

Interestingly, the pattern of reduction in Smad7 seemed similar to that of Trps1 in our heterozygous Trps1-knockout mice, suggesting that Trps1 regulates, directly or indirectly, Smad7 expression. The levels of Smad7 and phosphorylation of Smad3 after TGF-β-induced EMT were significantly different in HT versus WT primary proximal tubular epithelial cells. This is the first demonstration that a deficiency of Trps1 modulates EMT. Because Smad7 protein levels were decreased in contrast to the elevated mRNA levels after TGF-β treatment, we assumed that the decrease in Smad7 protein in HT kidneys was caused by another unknown molecule that is upregulated by Trps1 haploinsufficiency. It has been shown that Smad7 is targeted for degradation by the ubiquitin ligases Arkadia and Smurf1/2.9,27,28 We found that the amount of Arkadia in HT tubular epithelial cells was 2-fold greater than in WT cells, whereas virtually no difference was seen in Smurf1/2 protein levels. In addition, the amount of Arkadia increased in a time-dependent manner after TGF-β treatment. It is therefore possible that Trps1 suppresses expression of Arkadia and the reduced Trps1 alleviated this suppression in HT kidneys. These molecular data are consistent with our observation that the extent of fibrosis in HT UUO kidneys is greater than in WT UUO kidneys.

Our previous report showed that Trps1 is located in ureteric buds, cap mesenchyme, and renal vesicles and it acts downstream of Bmp7 to transform mesenchymal cells into epithelial cells during renal development.17 In addition, previous studies have reported that Bmp7 expression is decreased in UUO kidneys.29 In addition, Bmp7 treatment, which counteracts the downstream mediators of TGF-β signaling (Smad proteins), rescues the phenotypic changes induced by TGF-β.22,30 From these data, it is conceivable that Bmp7 promotes the mesenchymal-to-epithelial transition and ameliorates fibrosis by inhibiting TGF-β signaling through Trps1.

This study demonstrates that Trps1 is preferentially expressed along the entirety of the proximal tubules in the adult mouse kidney. In western blot analyses of primary tubular epithelial cells, we demonstrated that the levels of Arkadia, α-SMA, E-cadherin, and Smad7 in cell lysates from WT and HT primary proximal tubular epithelial cells treated for 24 hours as indicated. For detecting pSmad3, cells were treated with reagents for 12 hours. Samples from three independent experiments were used.

Figure 5. Arkadia knockdown rescues TGF-β1-induced EMT in HT cells. (A) Total RNA samples extracted from WT and HT cells were subjected to real-time PCR for measurement of α-SMA and E-cadherin mRNA. Experiments were repeated three times, and the data are expressed as the mean ± SD. *P < 0.05. (B) Western blot analysis of Arkadia, α-SMA, E-cadherin, and Smad7 in cell lysates from WT and HT primary proximal tubular epithelial cells treated for 24 hours as indicated. For detecting pSmad3, cells were treated with reagents for 12 hours. Samples from three independent experiments were used.

In conclusion, our results suggest that Trps1 has a critical role in the modulation of renal fibrosis and the suppression of EMT. Modulation of EMT, a major contributor to tubulointerstitial fibrosis, by Trps1 through inhibition of TGF-β signal-
Figure 6. A schematic representation showing that Trps1 haploinsufficiency promotes EMT and renal tubular interstitial fibrosis induced by UUO. In Trps1 haploinsufficient (heterozygote) cells, the amount of Trps1 is reduced by half compared with wild-type cells, which induces double the amount of Arkadia that promotes proteosomal degradation (PD) of Smad7. Decreased amounts of Smad7 leads increased levels of pSmad3, which in turn amplifies the TGF-β signaling induced by UUO and causes more severe fibrosis than wild-type cells.

CONCISE METHODS

Experimental Animals, Surgery, and Tissue Preparation
Ten heterozygous Trps1-knockout (HT) and ten age-matched wild-type (WT) mice (6 to 8 weeks of age and 20 to 30 g in body weight) were used for UUO (n = 7 per genotype) or sham (n = 3 per genotype) operations. UUO surgery was performed as described previously. Male mice (body weight 20 to 25 g) received an intraperitoneal injection of sodium pentobarbital (50 mg/kg). UUO was made by ligation of the left ureter with 4-0 silk through a left-flank incision. A control group of mice was subjected to sham operations that were identical to those done for the mice with UUO except that the ureters were not ligated. At the time of sacrifice, both kidneys were removed and cut transversely and tissues were fixed with 4% paraformaldehyde for histologic and immunohistochemical analysis. 

Histologic and Immunohistochemical Analysis
The paraformaldehyde-fixed kidney tissues were embedded in paraffin, sliced into 4-μm thick sections, and stained with hematoxylin and eosin. Immunostaining was performed on paraffin sections using a microwave-based antigen retrieval technique. The antibodies used in this study included villin (Santa Cruz, SC-6762), collagen I (Southern Biotechnology, Birmingham, AL), pSmad3 (Cell Signaling, Danvers, MA), α-SMA (clone: 1A4, NeoMarkers), and E-cadherin (clone: DECMA-1 Sigma Aldrich, St. Louis, MO). The anti-Trps1 antibody was generated as described previously. For immunohistochemistry, sections were treated with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. For immunofluorescent staining, FITC-anti-rabbit IgG, Cy3-anti-goat IgG, and Cy3-anti-rabbit IgG (Sigma Aldrich) were used as secondary antibodies.

Quantitative RT-PCR Analysis
Total RNA was extracted using Trizol (Invitrogen). Reverse transcription of the RNA was performed by the first-strand cDNA synthesis kit (Invitrogen). The PCR with SYBR Green PCR Master Mix (Takara Bio, Inc., Japan) was used to analyze mRNA for α-SMA, E-cadherin, Trps1, Smad7, and Arkadia. Amplification and detection were performed using an ABI 7500 system (Applied Biosystems, Carlsbad, CA). Each sample was measured in triplicate, and the expression level of the tested gene was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. The sequences of the primers used for PCR were as follows: α-SMA, forward 5’-GTGCTATGTCGCTCTCGTTGTTGA-3’ and reverse 5’-ATGAAAGATGCTGGAGAGGGTC-3’; Smad7, forward 5’-TCAGGTGGCAGGCTCTCA-3’ and reverse 5’-GTTGATCTTCCCCGTAATTCA-3’; E-cadherin, forward 5’-CGCGTGAAAGCTGTGTGGCTG-3’ and reverse 5’-GGTGCTGTGACACAGCAGA-3’; Arkadia, forward 5’-TCTATTTGAGTTGCCTCAAACCA-3’ and reverse 5’-CCCAGTTCCCGACGCGATTTC-3’; Trps1, forward 5’-GGGACAGCCCCAATGTTG-3’ and reverse 5’-GGTATCTCTGGCAGAACAAAAA-3’; GAPDH, forward 5’-GATGTGGTTCCTGAGAGC-3’ and reverse 5’-CATCATACTTTGGCAGGTTT-3’.

Western Blot Analysis
Lysates (20 μg protein) from cultured cells and tissues were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with TBS containing 0.1% Tween 20 and 3% BSA for 1 hour at room temperature and incubated overnight at 4°C with antibodies directed against Trps1, α-SMA, E-cadherin, pSmad3 (Acris Antibodies, Herford, Germany), Smad2/3, Smad7, or Arkadia (Santa Cruz, CA). Subsequently, the blots were washed with TBS containing 0.1% Tween 20, treated with horseradish-peroxidase-conjugated secondary antibodies at room temperature for 1 hour, and developed with the ECL Plus detection system (Amersham Bioscience, Buckinghamshire, United Kingdom).

Generation of Primary PTECs
Primary PTECs were generated from the kidneys of WT and HT mice as described previously. Briefly, kidney cortices from mice (6 to 8 weeks of age) were dissected, sliced, minced, and digested in 0.25% trypsin solution (Life Technologies BRL, Grand Island, NY) in a shaking incubator at 37°C for 1 hour. Trypsin was neutralized with growth medium (DMEM and 10% FBS containing 100 U/ml penicillin and 0.1 mg/ml streptomycin). The suspension was triturated by pipetting and was passed through a 100-μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). The samples were centrifuged (600 rpm for 5 minutes) to pellet the tubules, washed with 10 ml of medium, centrifuged, and washed twice more. The final pellet, consisting mostly of renal tubules, was resuspended in culture medium (REBM...
bullets, plated onto culture dishes (Nalge Nunc International, Naperville, IL) or two-well chamber slides (Nunc Lab-Tek II-CC2, Nalge Nunc International), and incubated at 37°C in a carbon dioxide incubator with medium changes every 2 days until confluent. Experiments were carried out in serum-free DMEM. EMT was induced by the addition of 5 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) for 6, 12, and 24 hours. For the RNA interference experiment, cells were pretreated with Arkadia siRNA (100 nM) for 24 hours before exposure to TGF-β.

**Double Immunostaining of PTECs**

Proximal tubule epithelial cells treated with or without TGF-β1 were fixed with cold acetone for 20 minutes. After washing with PBS, cells were permeated with 0.5% Triton/PBS for 10 minutes and were blocked in 5% BSA/PBS for 30 minutes. Cells were then incubated with primary antibodies against α-SMA or E-cadherin overnight at 4°C. After washing, they were incubated with secondary antibodies (TRITC: anti-mouse IgG; FITC: anti-rat IgG) for 1 hour at room temperature. Sections were then incubated with the antibody against Trp1 for 1 hour and finally incubated with FITC-anti-rabbit IgG or Cy3-anti-rabbit IgG for 1 hour.

**Quantitative Analysis**

Smad3 activation was determined by the extent of its nuclear localization in stained tissue as described previously. The number of cells positive for activated Smad3 was counted in 20 consecutive tubulointerstitial areas under high-power fields (400×), excluding the glomeruli and big vessels, and was divided by the total number of cells. The accumulation of collagen type I in the entire cortical tubulointerstitium of obstructed kidneys was measured as described.

Quantification of the immunoblots was performed by measurement of the intensity of the bands with the use of NIH Image analysis software.

**Statistical Analysis**

All of the results are expressed as mean ± SD. Unpaired t tests and analysis of multiple variance by the Scheffe method were used for statistical comparisons. P < 0.05 was considered statistically significant.

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**DISCLOSURES**

None.

**REFERENCES**


