AT₁A-mediated activation of kidney JNK1 and SMAD2 in obstructive uropathy: preservation of kidney tissue mass using candesartan

Ann Wamsley-Davis,¹ Ranjit Padda,¹ Luan D. Truong,² Chun Chui Tsao,³ Ping Zhang,² and David Sheikh-Hamad¹
¹Renal Section, Department of Medicine, and ²Renal Pathology Laboratory, Department of Pathology, Baylor College of Medicine, Houston, Texas 77030

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OBSTRUCTION OF URINE FLOW complicates many human diseases, such as prostate hypertrophy, abdominopelvic malignancies, and retroperitoneal fibrosis. It is characterized by infiltration of mononuclear inflammatory cells, predominantly macrophages, and is accompanied by activation of cytokines, growth factors, and mediators of apoptosis, the net result of which is the deletion of tubular cells by apoptosis and replacement of renal parenchyma with fibrous tissue. If left untreated, chronic kidney obstruction leads to loss of functional renal parenchyma, and ultimately, the development of kidney failure. Numerous reports implicate the renin-angiotensin system in the pathogenesis of ureteric obstruction, and inhibition of the rennin-angiotensin system, using either angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers, has been shown to attenuate the renal injury that follows ureteric obstruction (19, 23, 37). Activation of the kidney renin-angiotensin system occurs immediately after ureteric obstruction, with resultant vasoconstriction and salt and water retention (14). These hemodynamic changes are followed by the activation of a number of cytokines (6, 9, 20, 21, 36, 50), some of which have been directly linked to angiotensin II, such as monocyte chemotactic protein-1 (MCP-1) (20) and transforming growth factor-β (TGF-β) (22), which appears to play a key role in the genesis of the ensuing fibrosis (32). TGF-β signaling is initiated after ligand binding to the TGF-β receptor (15, 33), leading to phosphorylation of SMAD2 or SMAD3 (1, 47, 53). SMAD proteins represent a group of transcription factors involved in gene regulation downstream of the TGF-β receptor. A heteromeric SMAD proteins complex is formed (7), resulting in translocation of the complex to the nucleus (1, 31, 47), where it can interact with transcription factors directly (7), or indirectly (27), to regulate gene expression. It was recently reported that JNK pathway (25, 51), which targets the activation of c-Jun and activated transcription factor-2 (ATF-2) (8, 12, 16, 26), is stimulated rapidly by TGF-β in human fibrosarcoma cells and that JNK activity is essential for TGF-β-induced fibronectin production (17). Furthermore, it was also suggested that SMAD proteins and the JNK1 pathway interact cooperatively in TGF-β signaling (17). These observations assume particular importance, in light of recent data that implicate JNK1 in the development of cell apoptosis after ischemia and UV irradiation in a variety of tissues (2, 3). Apoptosis is a crucial component in the pathogenesis of ureteric obstruction (5, 49). Based on these observations, we hypothesized that JNK1 may be a key contributor to the development of renal injury and fibrosis in obstructive uropathy, and thus renal protection after AT₁A-receptor blockade in obstructive uropathy may be related to the downregulation of JNK1 and SMAD proteins. Consistent with this hypothesis, our data suggest activation of SMAD2 and JNK1 signaling in obstructed kidneys in a manner that is AT₁A dependent. AT₁A-receptor blockade using candesartan downregulates JNK1 and SMAD2, decreases tissue fibrosis, and leads to tissue preservation in obstructed kidneys.

METHODS

Animals. Male Sprague-Dawley rats (200 g initial weight, Harlan Bioproducts for Science) were subjected to unilateral ureteral ligation and concomitantly treated with one of the following protocols: 1) candesartan, an AT₁A-receptor antagonist (AstraZeneca), delivered through subcutaneously implanted Alzet minipumps at a rate of 1...
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mg kg\(^{-1}\) day\(^{-1}\), or vehicle; losartan (another AT1A-receptor antagonist; Merck, Whitehouse Station, NJ) also delivered through Alzet pumps at a rate of 10 mg kg\(^{-1}\) day\(^{-1}\) or vehicle; 2) enalapril [an angiotensin-converting enzyme inhibitor (ACE); Sigma, St. Louis, MO] given in drinking water at 200 mg/l or omission of the drug; and 3) sham-operated without ureteric ligation to control for the effects of anesthesia and surgery on the renal manifestations. Rats were fed rat chow and allowed free access to tap water. The animals were killed by a lethal injection of pentobarbital sodium at predetermined intervals (0, 3, 7, 11, 17, 25, 28, 37, and 52 days), and the kidneys were harvested for analysis. Because the medullary portions of the kidney undergo effacement soon after ureteric obstruction, dissection of medullary structures from cortices becomes difficult, or nearly impossible. Thus for consistency’s sake, assays were performed on whole kidney homogenates.

Animal experiments were carried out in accordance with institutional guidelines.

Morphometric analysis. Tissue sections were evaluated by a kidney pathologist who was unfamiliar with the experimental protocol. Tubular diameter and interstitial volume of the kidneys were assessed as previously described (48). Briefly, a graded 1-cm micrometer, viewed under the \(\times 40\) objective of a Nikon microscope (Melville, NY), was used to measure tubular diameter in periodic acid Schiff-stained sections. Interstitial volume in \(\mu\)m\(^3\) was measured by \(\pi\) \(\times\) (random tubular cross sections in both cortex and medulla in each kidney section. Interstitial volume was determined using a point-counting technique on trichrome-stained sections. The interstitial volume was expressed as the percentage of grid points of a 1-cm\(^2\) grided ocular grid viewed at \(\times 20\) magnification, which lay within the interstitial area. Five to ten random fields were used for morphometry. For parenchymal depth measurements, a transverse section through the midhilary region was carried out and the distance from the cortical edge to the medullary tip was measured.

Immunohistochemistry. Kidney tissue was fixed in 10% formaldehyde followed by dehydration in graded alcohols and embedded in paraffin blocks using standard techniques. Five-micrometer sections were cut, dried, and rehydrated for labeling with antibodies against SMAD2 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), p-SMAD2 (recognizes “active” phospho-Ser465/467; 1:100 dilution, Cell Signaling Technologies, Beverly, MA), or p-JNK1 (Cell Signaling Technologies; recognizes “active” phospho-Thr183/phospho-Tyr185 JNK; 1:100 dilution), using a standard avidin-biotin peroxidase technique (Vector Laboratories) together with heat-mediated antigen retrieval. The negative control included replacement of the primary antibodies with nonimmune sera, which showed no staining. Photomicrographs were taken using a Labophot-2 Nikon microscope with a MagnaFire Olympus digital camera.

Western blotting. This method is based on Laemmli (28) with slight modifications. Briefly, equal amounts of protein were run on 12% nonreducing SDS-PAGE. Proteins were transferred overnight (4°C, 40 V) onto Hybond-ECL membranes (Amersham, Arlington Heights, IL) in Laemmli buffer (25 mM Tris, 52 mM glycine, pH 8.3) containing 10% methanol. Blots were then blocked for 1 h at room temperature (RT) in PBST [50 mM NaPO\(_4\) (pH 7.5), 100 mM NaCl, 0.05% Tween 20] containing 5% nonfat dry milk. This was followed by 1-h incubation at RT with monoclonal anti-SMAD2 antibody (BD Transduction Laboratories, San Diego, CA) diluted (1:500) in PBST containing 5% nonfat dry milk. After a 15-min wash in PBST, blots were incubated for 1 h (RT) with peroxidase-conjugated secondary antibody, diluted (1:1,000) in PBST containing 5% nonfat dry milk. Blots were then washed for 15 min in PBST, and protein bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer’s instructions.

Kinase assays. MAPK activity was determined as previously described (35, 43) with slight modifications. Briefly, tissue was lysed using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) in Triton lysis buffer [TLB; consisting of 20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM Na pyrophosphate, 10% glycerol, 1 mM PMSF, and 1 μg/ml leupeptin]. The resultant lysate was centrifuged at 15,000 g for 10 min, and the supernatant was collected. Anti-ERK antibody (recognizes ERK1 and ERK2; Upstate Biotechnology, Waltham, MA) was bound to protein A&G agarose (Santa Cruz Biotechnology). Cell lysate (100 μg protein) was then added to the agarose beads, and ERK 1 and 2 were precipitated by the antibody-agarose complex. The beads were washed three times in TLB, followed by three additional washes in kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl\(_2\), 2 mM DTT, 0.1 mM Na vanadate). Myelin basic protein (Sigma) in kinase buffer containing 25 μM [α\(^{32}\)P]dATP was added to the beads and incubated for 20 min at 30°C. The kinase reaction was stopped by centrifugation at 12,000 g for 2 min. The supernatant was then resolved on a 15% SDS-PAGE, and the gel was dried and autoradiographed. The activity of ERK1 and 2 was determined by the extent of \(^{32}\)P incorporation in the myelin basic protein. For JNK1 and p38 kinase assays, the above procedure was used except for the following: anti-p38 or anti-JNK1 antibodies (Santa Cruz Biotechnology) were used for immunoprecipitation, and Pansobrin (Calbiochem-Novabiochem, San Diego, CA) was used to immobilize the antibodies; ATF-2 antibody (1:1000 dilution) was used as the substrate for both p38 kinase and JNK1. The reaction supernatant was resolved on 10% SDS-PAGE. Gels were dried and exposed to X-ray film. Films were scanned using ScanMaker 8700 (Microtect, Carson, CA), and band intensities were quantitated using ImageTool software (University of Texas Health Science Center, San Antonio, TX).

Statistical analysis. The means and SE were calculated using paired and unpaired t-tests and ANOVA as appropriate. Results were considered statistically significant if \(P\) was <0.05.

RESULTS

Preservation of tissue mass in obstructed kidney with candesartan treatment. In the following experiment, we determined the effect of AT1A blockade on renal tissue mass and morphology after unilateral ureteric obstruction for 28 days. This time point was selected, as it represents intermediate stage in the spectrum of obstructive uropathy in the rat, in which obstruction for 50 days results in end-stage kidney damage. Ureteric obstruction for 28 days caused tubular atrophy in both the cortex and medulla, interstitial fibrosis, and decreased corticomedullary thickness. Candesartan treatment, however, resulted in significant preservation of tissue mass and morphology in the obstructed kidneys (Fig. 1 and Table 1). Of interest, AT1A blockade provided better preservation of tubular diameter and interstitial volume of the kidneys (48). Of note, JNK1 activity in nonobstructed contralateral controls, the following experiment, we examined JNK1 activity in obstructed kidneys and nonobstructed contralateral controls, starting from day 3 after surgery and ending with day 52. JNK1 activity is markedly increased throughout the time course in obstructed kidneys. The highest activity was observed at day 11 (7.5-fold induction; Fig. 2) and appeared to correlate with the peak apoptotic index, as shown previously by our laboratories (5, 49). Of note, JNK1 activity in nonobstructed contralateral kidneys showed a modest increase as well; however,
the downregulation of JNK1-mediated apoptotic signals. The results with ACE inhibition were not surprising, however, as non-ACE-mediated renal production of angiotensin II was likely sustained despite ACE inhibition (18, 38). Consistent with this hypothesis, previous data by Otsuka et al. (41, 42) and Noda et al. (39, 40) suggested greater renoprotective effects when direct AT1A inhibition was utilized compared with ACE inhibition in various models of renal injury.

_Candesartan inhibits the rise in SMAD2 protein in obstructed kidneys._ Recent data suggest that kidney tissue injury that follows ureteric obstruction may be related, in part, to angiotensin II-mediated activation of TGF-β signaling. In addition, it has also been reported that TGF-β signaling is mediated through SMAD transcription factors (17, 30). In the following experiment, we examined the effect of candesartan treatment on SMAD2 protein abundance after ureteric obstruction for 28 days. Ureteric obstruction increased the expression of SMAD2 protein, consistent with induction and activation of SMAD signaling. Candesartan treatment, however, significantly attenuated the rise in SMAD2 protein level in obstructed kidneys (Fig. 4, A and B). Consistent with the Western blot analysis data, immunohistochemical analysis revealed increased SMAD2 protein level in obstructed kidneys, which was observed in all cells (Fig. 5). In addition, a great proportion of SMAD2 protein existed in the activated form, as evidenced by its phosphorylation and nuclear translocation (p-SMAD2). Similarly, JNK1 activity (p-JNK) was enhanced in obstructed kidneys; however, unlike SMAD2, JNK1 activation did not involve all cells and appeared restricted to some tubular structures. The significance of this variance in SMAD2 and JNK activation remains to be determined. Candesartan treatment attenuated SMAD2 protein levels and activity and decreased JNK1 activation in obstructed kidneys. Collectively, our data suggest that ureteric obstruction leads to activation of JNK1- and SMAD-mediated signaling pathways; these, in turn, are attenuated by AT1A blockade, an effect that may be responsible for the renoprotective effects observed with candesartan treatment.

**DISCUSSION**

While there is consensus among investigators on the final outcome of chronic ureteric obstruction, that is, fibrosis and loss of normal tissue architecture and function (34, 52), there does not appear to be a clear understanding of the mechanisms involved. Of particular note is the role of AT1A receptor blockade in mediating renoprotective effects observed in this model of kidney injury. It is important to consider that not all AT1A receptor antagonists exhibit similar renoprotective effects, and the timing and duration of treatment differ significantly. For example, the renoprotective effects observed with candesartan were sustained throughout the entire experimental period (28 days), whereas losartan treatment led to a transient improvement in renal function that was not sustained beyond the initial period of treatment. These observations suggest that the renoprotective effects of AT1A receptor antagonists are dependent on the duration of treatment and the specific pharmacological properties of the drug.
that dominate the pathophysiological processes leading to renal failure. Over the past two decades, several crucial links in the pathogenesis of chronic obstructive uropathy have been elucidated (13, 23, 24). It is now well documented that obstruction of urine outflow activates the intrarenal renin-angiotensin system, resulting in elevated levels of in situ angiotensin II and an imbalance between renal vasodilators and vasoconstrictors in favor of the latter. Vasoconstriction-induced ischemia, stimulation of angiotensin II receptors on tubular cells, mechanical stretch due to the increase in intratubular pressure, or the generation of cytotoxic oxygen species in tubular cells may constitute initial triggers for the pathogenetic processes that lead to tissue injury (44). The injured tubular cells are the source of proinflammatory mediators, such as osteopontin and MCP-1, which are responsible for the recruitment of inflammatory cells to the kidney, especially macrophages (9, 10). Distribution and retention of these inflammatory cells are promoted by the ubiquitous adhesion molecules ICAM-1 and VCAM-1 (45). The recruited inflammatory cells, in turn, are the source of additional proinflammatory and profibrogenic cytokines, which establish a vicious cycle that leads to disordered regulation of apoptosis and proliferation and, ultimately, loss of functional parenchyma and fibrosis (9).

Several lines of evidence implicate angiotensin II in the proliferative and apoptotic responses observed in various cells, which occur through either direct stimulation of the angiotensin II receptor or the activation of intermediate molecules such as TGF-β or PDGF (14). Studies by Morrissey and Klahr (37) suggested that AT2-receptor antagonism during experimental chronic ureteric obstruction does not alter the fibrotic changes after obstruction (37). In fact, studies by Ma et al. (32) suggested that AT2-receptor stimulation during ureteric obstruction may provide a protective effect, as obstructed kidneys of AT2-receptor null mice develop accelerated fibrosis and...
Fig. 4. Candesartan attenuates the rise in SMAD2 protein in obstructed kidneys. Unilateral ureteric ligation in Sprague-Dawley rats was maintained for 28 days. Kidneys from sham-operated, obstructed and vehicle-treated, or obstructed and candesartan-treated rats were analyzed for SMAD2 protein level using Western blotting; \( n = 3 \) animals/group. A: representative blot. B: graph depicts SMAD2 protein level in the kidneys of sham-operated, obstructed without candesartan treatment, or obstructed plus candesartan-treated rats. ** \( P < 0.0011 \), paired \( t \)-test.

Fig. 5. Candesartan attenuates SMAD2 protein levels and diminishes the activities of SMAD2 and JNK1 in obstructed kidney. Twenty-eight days after sham operation, left ureteric obstruction plus vehicle treatment, or left ureteric obstruction plus candesartan treatment, kidney tissue was obtained for immunohistochemistry. Staining was carried out with anti-SMAD2, anti-p-SMAD2, or anti-pJNK1. Brown represents positive staining for the respective antibody. G, glomerulus; T, tubule. Magnification: \( \times 300 \).
collagen deposition compared with obstructed kidneys of wild-type mice. On the other hand, it was reported by Chevalier et al. (4) that AT\(_\text{1}\)-receptor stimulation was responsible for the proliferative changes as well as apoptosis in a model of ureteric obstruction. Thus the utilization of AT\(_\text{1}\)- and AT\(_\text{2}\)-receptor knockout mice points to AT\(_\text{1}\)-receptor activation as a key mediator of kidney tissue injury in models of ureteric obstruction.

TGF-\(\beta\) exerts its effects on cell proliferation, differentiation, and migration, in part, through modulation of extracellular matrix components, such as fibronectin and plasminogen activator inhibitor-1 (PAI-1) (17). Although SMAD proteins represent a key component in TGF-\(\beta\)-signaling, other pathways, such as JNK1, appear to play a crucial role in mediating TGF-\(\beta\) effects in a manner that is SMAD independent (11, 17). These findings suggest activation of JNK1 and SMAD proteins by TGF-\(\beta\) in a parallel yet seemingly independent fashion. However, while activators of JNK1 only partially stimulated transcriptional responses characteristic of TGF-\(\beta\) without concomitant SMAD pathway activation, JNK1 has been shown to phosphorylate SMAD3, and hence, facilitate its activation by TGF-\(\beta\) (11). Indeed, SMAD proteins have been shown to cooperate with Jun/Fos (downstream targets of JNK1) in TGF-\(\beta\)-induced transcription (53), suggesting an interdependent relationship between JNK and SMAD pathways in TGF-\(\beta\)-mediated gene expression. Thus SMAD and JNK cascades are being recognized as essential components of TGF-\(\beta\) signaling machinery and are implicated in common transcriptional responses downstream of TGF-\(\beta\) (11, 17, 53). Angiotensin II receptor stimulation, and specifically that of the AT\(_{1A}\) subtype, is essential for TGF-\(\beta\)-activation and the development of tissue fibrosis in obstructive uropathy (46). Therefore, inhibition of AT\(_{1A}\) receptors in the setting of obstructive uropathy has the potential of attenuating TGF-\(\beta\)-mediated tissue fibrosis and renal injury. Candesartan has high binding affinity to the angiotensin II receptor (29) and thus may be ideal for AT\(_{1A}\)-receptor inhibition, without interference with the potentially beneficial AT\(_{2}\) stimulation, and consequently, may effectively inhibit angiotensin II-mediated tissue injury in obstructed kidneys. Our data are consistent with this notion and suggest that AT\(_{1A}\)-receptor stimulation is essential for JNK1 and SMAD activation in obstructive uropathy and that AT\(_{1A}\) blockade provides an effective tool to inhibit both JNK1 and SMAD pathways. This inhibition, in turn, may be responsible for the renoprotective effects afforded by candesartan treatment in the setting of obstructive uropathy.

**REFERENCES**


**GRANTS**

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