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Hyperosmolarity Enhanced Susceptibility to Renal Tubular Fibrosis by Modulating Catabolism of Type I Transforming Growth Factor-β Receptors

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ABSTRACT

Hyperosmolarity plays an essential role in the pathogenesis of diabetic tubular fibrosis. However, the mechanism of the involvement of hyperosmolarity remains unclear. In this study, mannitol was used to evaluate the effects of hyperosmolarity on a renal distal tubule cell line (MDCK). We investigated transforming growth factor-β receptors and their downstream fibrogenic signal proteins. We show that hyperosmolarity significantly enhances the susceptibility to exogenous transforming growth factor (TGF)-β1, as mannitol (27.5 mM) significantly enhanced the TGF-β1-induced increase in fibronectin levels compared with control experiments (5.5 mM). Specifically, hyperosmolarity induced tyrosine phosphorylation on TGF-β RII at 336 residues in a time (0–24 h) and dose (5.5–38.5 mM) dependent manner. In addition, hyperosmolarity increased the level of TGF-β RI in a dose- and time-course dependent manner. These observations may be closely related to decreased catabolism of TGF-β RI. Hyperosmolarity significantly downregulated the expression of an inhibitory Smad (Smad7), decreased the level of Smurf 1, and reduced ubiquitination of TGF-β RI. In addition, through the use of cycloheximide and the proteasome inhibitor MG132, we showed that hyperosmolarity significantly increased the half-life and inhibited the protein level of TGF-β RI by polyubiquitination and proteasomal degradation. Taken together, our data suggest that hyperosmolarity enhances cellular susceptibility to renal tubular fibrosis by activating the Smad7 pathway and increasing the stability of type I TGF-β receptors by retarding proteasomal degradation of TGF-β RI. This study clarifies the mechanism underlying hyperosmotic-induced renal fibrosis in renal distal tubule cells. J. Cell. Biochem. 109: 663–671, 2010.

KEY WORDS: HYPEROSMOLARITY; TGF-β; SMAD; RECEPTORS; SMURF; HALF-LIFE

Diabetic nephropathy is a major complication of diabetes and accounts for up to 40% of cases with end-stage-renal disease (ESRD). Many studies suggest a key role for hyperglycemia in the pathogenesis of this class of complications [Hodgkinson et al., 2003; Dave and Kalia, 2007; Schrijvers and De Vriese, 2007]. Indeed, elevated osmosis has been shown to stimulate synthesis of extracellular matrix (ECM), a process that is mediated by stimulation of transforming growth factor-β (TGF-β) production [Küper et al., 2007]. Furthermore, the pathogenic role of TGF-β in diabetic renal complications is supported by experiments that have blocked TGF-β using neutralizing antibodies or antisense strategies in experimental models of diabetes [Hoffman et al., 1998; Goldfarb and Ziyadeh, 2001; Benigni et al., 2003]. However, details about the underlying signaling pathways involved in the hyperosmolarity-mediated induction of renal fibrosis in diabetes remain unclear.

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The TGF-β signal is transduced by a pair of transmembrane serine/threonine kinases, known as type I and type II receptors, which are present on almost cell types that have been analyzed [Zhang, 2004]. The type I receptor does not bind TGF-β in the absence of the type II receptor. The binding of TGF-β to the type II receptor results in the recruitment, phosphorylation, and concomitant activation of the type I receptor [Ng et al., 2003]. The activated type I receptor phosphorylates a family of downstream mediators, termed Smads, transmitting the signal and resulting in the regulation of target gene expression [Moustakas et al., 2001; Mehra and Wrana, 2002; Itoh and ten Dijke, 2007]. Based on this pathway, the TGF-β RII is essential for the transduction of the extracellular TGF-β signal into cells. In fact, previous studies from our lab have shown that one of the mechanisms of captopril, a clinically effective drug for the treatment of diabetic nephropathy, is suppression of the increased expression of TGF-β receptors. Moreover, we previously determined that the high-glucose-induced increase in TGF-β RII expression is correlated with the secretion of fibronectin [Guh et al., 1996; Yang et al., 1997a,b, 1998; Chuang et al., 2003]. Thus, understanding the mechanism underlying hyperosmolarity-induced signaling of TGF-β receptors may aid in the development of therapeutic approaches toward prevention of diabetic renal fibrosis. In addition to regulation of TGF-β RII, the regulation of TGF-β RI kinase activity might also ameliorate renal fibrosis [Petersen et al., 2007; Fu et al., 2008]. In this study, we investigated the effects of osmotic pressure (i.e., mannitol) on the expression of both types of TGF-β receptors and their downstream signal regulators (Smads).

Ubiquitination-mediated proteolysis regulates the activity of diverse receptor systems and is involved in some signal transduction pathways. Recently, the homologous to E6-associated protein C-terminus (HECT) family ubiquitin ligases known as Smurfs have been shown to bind R-Smads (i.e., Smad2/3) and have been implicated in the specific ubiquitination of these targets [Zhu et al., 1999]. Interestingly, Smurfs also bind to I-Smads (i.e., Smad7). Following prolonged treatment of cells with TGF-β, the Smad7–Smurf complex forms, exits the nucleus, and binds to the activated TGF-β receptor complex. Smurf then ubiquitinates the receptor, resulting in rapid proteasomal degradation of the receptor protein [Kavsak et al., 2000]. Based on these findings, we chose to investigate the role of Smurf1 and the ubiquitination level of the TGF-β receptors under hyperosmotic stress.

In this study, we elucidate the interactions between hyperosmolarity and renal tubule fibrosis. We investigate the mechanism underlying TGF-β-induced post-receptor signal transduction under high osmotic stress. We found that hyperosmolarity may be a risk factor that enhances susceptibility to cellular fibrosis by upregulating the expression of TGF-β receptors in renal distal tubule cells.

MATERIALS AND METHODS

Glucose and mannitol were purchased from Sigma (St. Louis, MO). TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Protein A/G agarose gels were purchased from Calbiochem (Germany). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove). The first-strand cDNA synthesis kit and the GeneClean™ DNA elution kit were purchased from Clontech (Clontech Laboratories, Inc., USA). The fibronectin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Fibronectin Antigen ELISA (Cat # 11030, Kordia Life Sciences). MDCK (CCL 34) cells were cultured as described in our previous study [Yang et al., 1998]. All antibodies were purchased from Santa Cruz Biotechnology (CA).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

We used the ELISA assay to evaluate the expression of secreted fibronectin and TGF-β1. For quantification of fibronectin or TGF-β1 in the supernatant of cultured MDCK cells, conditioned culture media were collected and centrifuged at 1,200 rpm for 5 min to remove particulates; the cleared supernatant was collected, concentrated, and stored at −80°C until use. Immediately prior to performing the ELISA, samples were acidified by addition of 1 N HCl followed by the addition of NaOH to the original pH condition. For detection of TGF-β1, we used a commercial sandwich ELISA kit from R&D Systems. For detection of fibronectin, we used a commercial sandwich ELISA kit from Takara Bio, Inc. (Shiga, Japan). The protocol was performed according to the manufacturer's instruction. The absorbance (450 nm) for each sample was analyzed by an ELISA reader. The absorbances for TGF-β1 and fibronectin were assayed and the concentrations of each were determined by interpolation against a standard curve.

IMMUNOBLOTTING

We used Western blot assays to evaluate the expression of the protein levels of TGF-β RII, TGF-β RI, and Smad signal molecules (Smad2/3, pSmad2/3, Smad7). In brief, cells were lysed in lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 20 μg/ml Aprotinin, 20 μg/ml Leupeptin, 1 mM DTT, and 50 μg PMSF) and the crude protein lysate was resolved by 7.5%, 10%, or 12.5% SDS–PAGE. After protein transfer to a polyvinylidene difluoride (PVDF) membrane on an electrotransfer unit, the PVDF membrane was blocked with 10% (w/v) non-fat milk in Tris-buffered saline (TBS-T) for 2 h at 37°C. The blots were probed with a 1:1,000 (v/v) dilution of primary antibody. The primary antibodies used were as follows: anti-pSmad2/3 (sc-11769), anti-Smad7 (sc-11392), anti-TGF RI (sc-9048), anti-TGF-β RII (sc-1700). All antibodies were purchased from Santa Cruz Biotechnology. After hybridization at 37°C, the blots were washed and hybridized with 1:2,000 (v/v) dilutions of goat anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (Calbiochem), or donkey anti-goat IgG-HRP (Santa Cruz Biotechnology). The blocking procedure was performed in 5% non-fat milk in TBS-T buffer. The signal was generated by adding enhanced chemiluminescent reagent. β-actin was used as an internal control.

IMMUNOPRECIPITATION

Briefly, cell lysates were centrifuged at 12,000*g* for 10 min at 4°C. The resulting supernatants were collected for immunoprecipitation. After preclearing with normal host IgG, the lysates were immunoprecipitated overnight at 4°C with 2 μg of various primary antibodies, followed by precipitation with 30 μl of protein A/G

Plus-Agarose (Santa Cruz Biotechnology) for 3 h at 4°C. The primary antibodies used were as follows: anti-TGF-β RI (sc-9048), anti-Smurf1 (sc-25510), and anti-ubiquitin (sc-9133). All antibodies were purchased from Santa Cruz Biotechnology. The precipitated complexes were boiled for 5 min in SDS sample buffer and immunoblotted with various antibodies, as indicated.

ASSAY FOR PROTEIN HALF-LIFE

Cells were incubated with cycloheximide (CHX: 100 μg/ml) in the presence or absence of mannitol (27.5 mM) for various periods of time (2, 4, and 8 h), as indicated. In brief, cells were lysed in lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 20 μg/ml Aprotinin, 20 μg/ml Leupeptin, 1 mM DTT, 50 μg PMSF) and the crude protein lysate was resolved by 10% SDS–PAGE. The blots were probed with a 1:1,000 (v/v) dilution of primary antibody. The primary antibodies used were as follows: anti-TGF RI (sc-9048) from Santa Cruz Biotechnology. The signal was generated by addition of enhanced chemiluminescent reagent. β-actin was used as an internal control.

FLOW CYTOMETRY

Cells were fixed in 2% formaldehyde for 10 min at 37°C, followed by permeabilization with ice-cold 100% methanol for 30 min at 4°C. After washing twice with PBS (in 4% FCS), cells were treated with anti-phosphotyrosine-specific TGF-β RII goat polyclonal antibodies (1 μg/100 μl in PBS plus 4% FCS) for 15 min at room temperature. Cells were then washed twice with PBS, incubated with fluorescein-conjugated anti-goat antibodies (1:200 dilution, 5 μg/ml) in PBS plus 4% FCS for 15 min at room temperature. Cells were then washed twice and a FACScan™ system was used to collect the data. Each measurement consisted of gating 10^5 cells that were then analyzed with the CellQuest™ software.

STATISTICS

All results are expressed as the mean standard errors of the mean (SEM). Unpaired Student's *t*-tests were used for comparisons between two groups. One-way analysis of variance followed by unpaired *t*-tests was used for comparisons among more than three groups. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

As shown in Figure 1A, osmotic pressure (i.e., mannitol treatment) per se did not affect the expression level of fibronectin in the culture media. However, in the presence of 10 ng/ml TGF-β1, mannitol accentuated the secretion of fibronectin by about fivefold ($P < 0.01$) in a dose-dependent manner (5.5–38.5 mM). Moreover, the increase in secretion of fibronectin induced by TGF-β1 was significantly enhanced under high osmotic conditions (i.e., 27.5 mM of mannitol), as compared with control conditions (i.e., 5.5 mM of mannitol), as shown in Figure 1B. This observation implies that high osmotic pressure may enhance fibrogenic susceptibility to TGF-β1 in distal tubule cells. To elucidate the underlying mechanism, we performed TGF-β1 ELISA assays and Western blots. Our results showed that

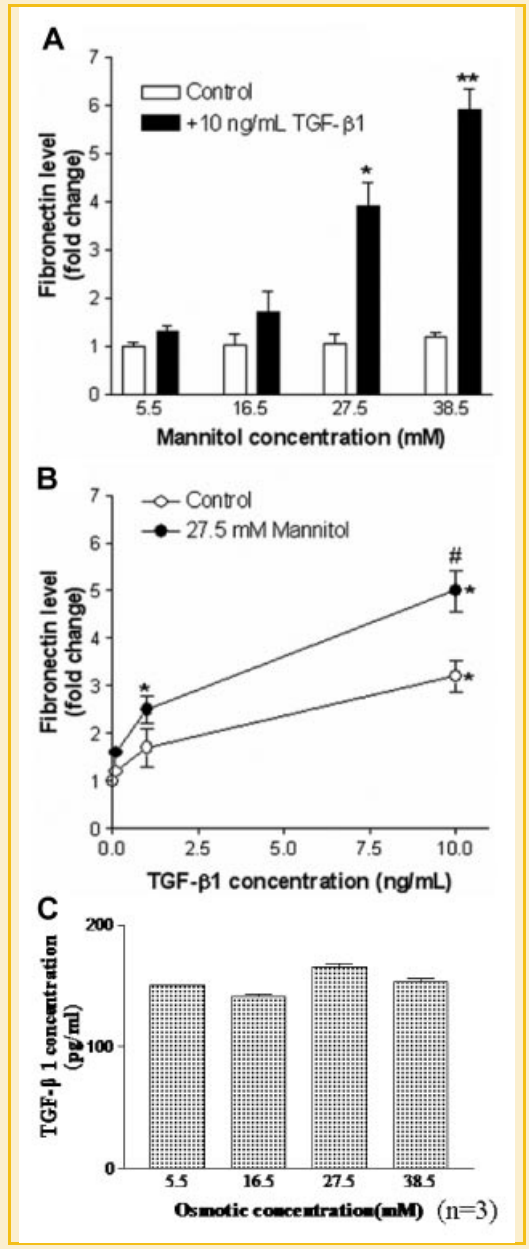

Fig. 1. Effects of osmotic concentration and exogenous TGF-β1 on the secretion of fibronectin; high osmolarity does not induce TGF-β1 secretion. A: We used mannitol to mimic high osmotic pressure. MDCK cells were treated with different osmotic concentrations (5.5, 16.5, 27.5, or 38.5 mM) in the presence or absence of TGF-β1 (10 ng/ml) in 0.5% fetal bovine serum for 24 h. B: As in (A), cells were subjected to treatment with 5.5 mM (designated as "control") or high osmotic pressure (27.5 mM mannitol) in the presence of TGF-β1 (0, 0.1, 1, or 10 ng/ml) in serum free medium for 24 h. C: MDCK cells were cultured in serum-free medium (MEM with 0.5% FBS) with the addition of different concentrations of mannitol (5.5, 16.5, 27.5, and 38.5 mM) for 24 h. Cell culture media were collected and fibronectin and TGF-β1 levels were evaluated by ELISA, as described in the Materials and Methods Section. All experiments were repeated four times (n = 3) and similar results were obtained. *P*-values of less than 0.05 were considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$). It is evident that in the presence of TGF-β1, high osmotic concentrations accentuate the secretion of fibronectin but do not induce the secretion of TGF-β1 in MDCK cells. $^{\#}P < 0.05$ (27.5 mM mannitol curve vs. control curve) by two-way ANOVA.

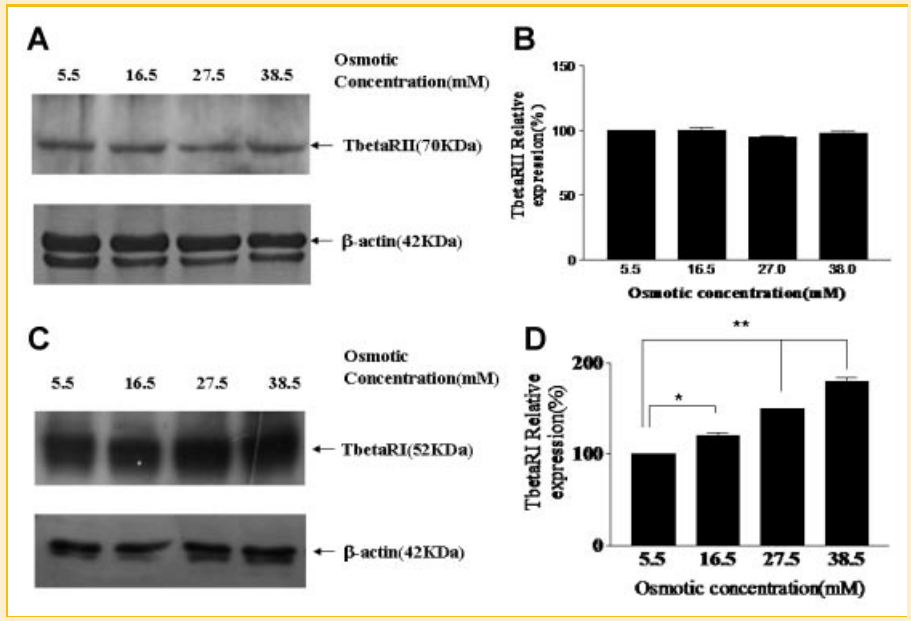

Fig. 2. High osmotic pressure does not induce an increase in the protein level of TGF-β RII but it does induce an increase in the level of TGF-β RI in MDCK cells. MDCK cells were cultured in 75 cm^2 flasks. Cells were serum starved (0.5% FBS) and incubated in different osmotic concentrations (5.5, 16.5, 27.5, and 38.5 mM) for 24 h. Cells were lysed in lysis buffer, as described in the Materials and Methods Section. A: Two hundred fifty micrograms of protein lysate was resolved by 10% SDS–PAGE. Western blot was performed with polyclonal antibodies against the type II TGF-β receptor, followed by the addition of secondary antibodies (1:4,000). The expression of β-actin was used as an internal control. B: Data from TGF-β RII was scanned and normalized to the respective β-actin samples. It is evident that high osmotic pressure does not induce an increase in the protein level of TGF-β RII. C: Sixty micrograms of protein lysate was resolved by 10% SDS–PAGE. Western blot was performed using polyclonal antibodies against the type I TGF-β receptor, followed by the addition of secondary antibodies (1:4,000). The expression of β-actin was used as an internal control. D: Data from TGF-β RI was scanned and normalized to the respective β-actin samples. It is evident that high osmotic pressure does induce an increase in the protein level of TGF-β RI. Experiments were repeated three times and similar results were observed. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (5.5 mM osmotic concentration).

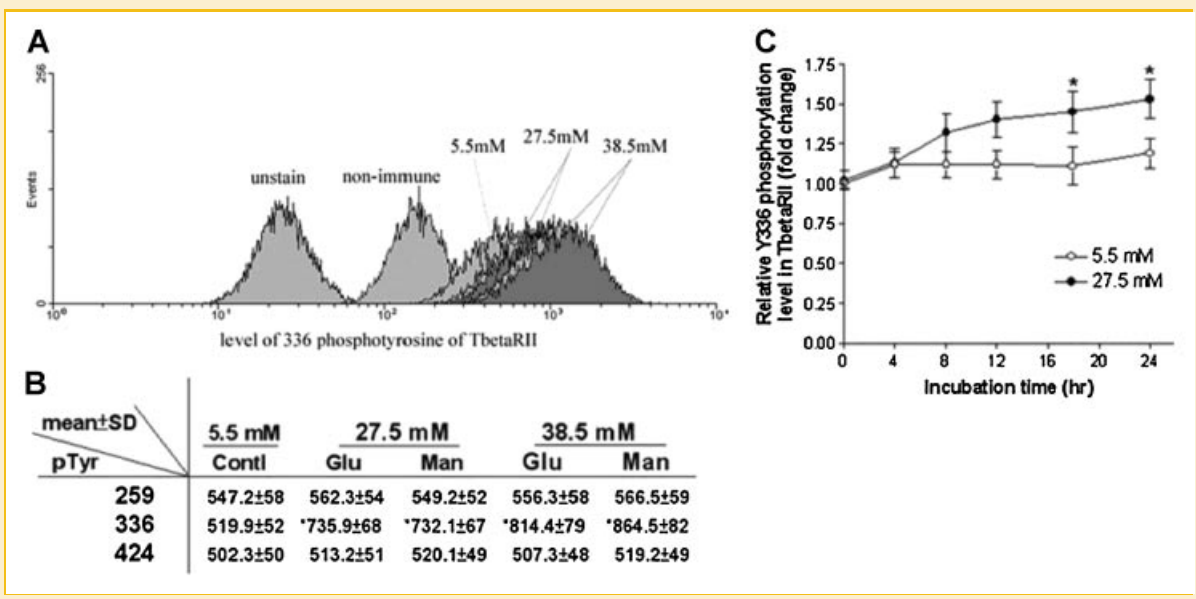

Fig. 3. High osmotic concentration induces phosphorylation of TGF-β RII at tyrosine 336 in MDCK cells in a dose- and time-dependent manner. MDCK cells were cultured in 75 cm^2 flasks. Cells were serum starved (0.5% FBS) and incubated in low osmosis (5.5 mM) or high osmosis (27.5 or 38.5 mM) conditions for 24 h. Cells were harvested and stained with TGF-β RII phosphotyrosine-specific antibodies, as described in the Materials and Methods Section. A: Cells were subjected to flow cytometry analysis (note: only the pTyr336 data is shown in Fig. 4A). B: Data were formatted as a table. C: A time-course response. MDCK cells were cultured in 75 cm^2 flasks. Cells were serum starved (0.5% FBS) and incubated in low osmosis (5.5 mM) or high osmosis (27.5 mM) conditions for the indicated time period (0, 4, 8, 12, 18, or 24 h). Cells were analyzed as described in Figure 4A. Experiments were repeated three times and similar results were observed. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (5.5 mM osmotic concentration).

high osmotic pressure did not induce the secretion of TGF-β1 in MDCK cells. Thus, we investigated whether high osmotic pressure might affect the expression of either type of TGF-β receptor. Since TGF-β RII is responsible for the direct binding of TGF-β at the cell surface and initiates the phosphorylation of type I TGF-β receptors, we examined the expression of TGF-β RII. As shown in Figure 2A,B, high osmotic pressure does not induce an increase in the protein level of TGF-β RII. However, as shown in Figure 2C,D, we found that high osmotic pressure does induce an increase in the protein level of TGF-β RI in a dose-dependent manner.

TGF-β RII is a serine/threonine/tyrosine kinase receptor and its activity is regulated by protein phosphorylation. Three tyrosine (Y) residues on TGF-β RII have been reported as phosphorylation-regulated and related to the activity of TGF-β RII. To evaluate phosphorylation of these sites, we used antibodies specific for pTyr 259, pTyr 336, and pTyr 424 of TGF-β RII in a flow cytometry assay. As shown in Figure 3, osmotic concentration induced the phosphorylation of TGF-β RII at pTyr 336 in MDCK cells in a time-course and dose-dependent manner. Thus, we conclude that phosphorylation of TGF-β RII might be related to hyperosmolarity-induced susceptibility to cellular fibrosis in renal distal tubule cells.

Moreover, high osmotic pressure induced an increase in the protein level of Smad2/3 and pSmad2/3 in MDCK cells, as shown in Figure 4. In addition, the inhibitory Smad7 is significantly suppressed under hyperosmotic conditions. The change in expression level of TGF-β RI might be associated with sensitivity to cellular fibrosis. As shown in Figure 5, high osmotic pressure increased the protein level of TGF-β RI in a time-course (0, 4, 12, and 24 h) and dose-dependent (5.5 or 38.5 mM) manner.

We investigated whether hyperosmolarity affects the protein half-life of TGF-β RI. To this end, we used the protein synthesis inhibitor, CHX, to determine the half-life of TGF-β RI. As can be seen in Figure 6, high osmotic conditions significantly increased the half-life of TGF-β RI. Thus, we suggest that hyperosmolarity significantly increases the stability of the TGF RI protein.

Since the Smurf proteins have been implicated in ubiquitination of surface receptors, resulting in rapid proteasomal degradation of those targeted receptors, we investigated whether Smurf1 might mediate the catabolism of TGF-β RI. As shown in Figure 7, we

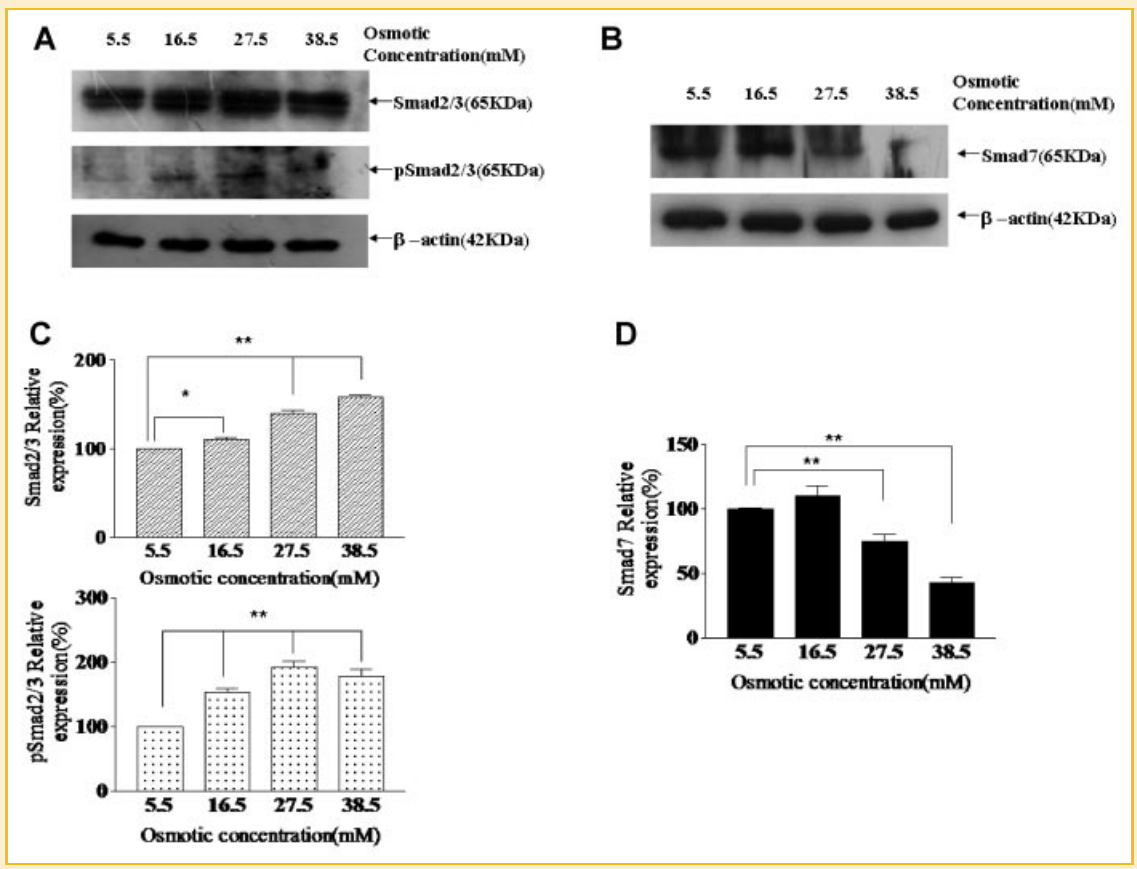

Fig. 4. Effects of osmolarity on the protein levels of Smad2/3, pSmad2/3, and Smad7 in MDCK cells. MDCK cells were cultured in 25 cm^2 flasks. Cells were serum starved (0.5% FBS) and incubated at different osmotic concentrations (5.5, 16.5, 27.5, and 38.5 mM) for 24 h. Cells were lysed in lysis buffer as described in the Materials and Methods Section. Eighty micrograms of the cell lysate was resolved by 10% SDS–PAGE. A,B: Western blot analysis was performed using antibodies specific for Smad2/3, pSmad2/3, Smad7, or β-actin, followed by the addition of secondary antibodies (1:4,000). C,D: The expression of β-actin was used as an internal control. Data were scanned and normalized to that of β-actin. It is evident that osmotic concentration induced the expression of Smad2/3 and pSmad2/3 and suppressed the expression of Smad7 in a dose-dependent fashion. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (5.5 mM osmotic concentration).

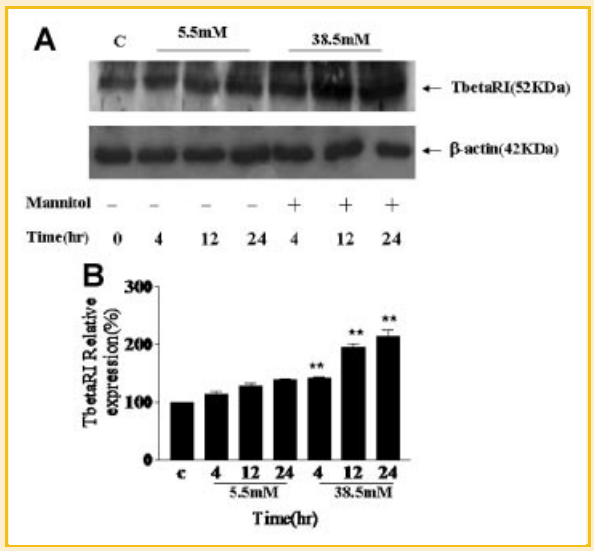

Fig. 5. High osmotic concentration induced the protein expression of TGF-β RI in MDCK cells. MDCK cells were cultured in 25 cm^2 flasks. Cells were serum starved (0.5% FBS) and incubated in low osmosis (5.5 mM) or high osmosis (38.5 mM) conditions for the indicated time periods. Cells were lysed in lysis buffer, as described in the Materials and Methods Section. A: Sixty micrograms of cell lysate was resolved by 10% SDS–PAGE. Western blots were performed using polyclonal anti-TGF-β RI antibodies, followed by the addition of secondary antibodies (1:4,000). The expression of β-actin was used as an internal control. B: The expression level of TGF-β RI was scanned and normalized to that of β-actin. It is evident that high osmotic pressure induces an increase in the protein level of TGF-β RI in a time-dependent manner. Experiments were repeated three times and similar results were observed. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (5.5 mM osmotic concentration).

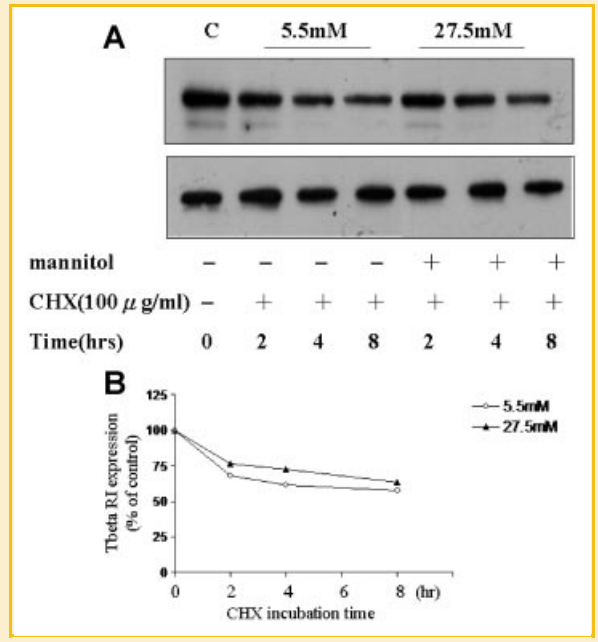

Fig. 6. High osmolarity promotes TGF-β1 type I receptor protein degradation in MDCK cells. A: MDCK cells were incubated with cycloheximide (CHX: 100 mg/ml) in the absence or presence of mannitol (27.5 mM) for various periods of time (2, 4, and 8 h), as indicated. Sixty micrograms of cell lysate was resolved by 10% SDS–PAGE. Western blots were performed using polyclonal anti-TGF-β RI antibodies, followed by the addition of secondary antibodies (1:4,000). The expression of β-actin was used as an internal control. B: Graphic representation of the relative abundance of the TGF-β 1 type I receptor protein shown in (A). TGF-β 1 type I receptor abundance is displayed as the percentage of the value in the control samples (100). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (5.5 mM osmotic concentration).

examined the interaction between Smurf1 and TGF-β RI by immunoprecipitation assay. Our data indicate that hyperosmolarity significantly reduced the interaction between TGF-β RI and Smurf1, as well as reducing interaction between TGF-β RI and ubiquitin. The results showed that hyperosmolarity induces significant down-regulation of protein catabolism of TGF-β RI by reducing the TGF-β RI binding of both Smurf1 and ubiquitin. To investigate the underlying mechanism, the level of TGF-β RI ubiquitination was examined by immunoprecipitation in the presence of a proteasomal inhibitor (MG132). As shown in Figure 7B, hyperosmolarity reduced both the ubiquitination level and the catabolism of the TGF-β RI protein in a time-dependent manner.

Taken together, our data indicate that high osmolarity accentuates susceptibility to fibrosis, possibly by activating TGF-β receptors (i.e., phosphorylating type II TGF-β receptors and increasing the level of type I TGF-β receptors) and their downstream Smad-related pathways. Potential effects of hyperosmolarity on distal tubule cells are shown in Figure 8.

DISCUSSION

The pathogenesis of diabetic nephropathy is not well understood. Nonetheless, renal fibrosis may be an important determinant in this process [Tamaki and Okuda, 2003]. In this regard, TGF-β has been suggested as the primary candidate for mediating diabetic nephropathy [Pantsulaia, 2006]. Our study provides the first demonstration that hyperosmolarity may enhance sensitivity to cellular fibrosis in renal distal tubule cells.

In this study, we showed that mannitol per se can enhance susceptibility to cellular fibrogenesis in MDCK cells. Moreover, the underlying mechanism of this process may be associated with the upregulation of type I TGF-β receptors, the induction of phosphorylation of type II TGF-β receptors, and the activation of Smad pathways. This observation is the first demonstration that high osmotic conditions may play a direct role in the induction of cellular susceptibility to renal cellular fibrosis.

High glucose levels exert their effects partly through the glucose molecule directly and partly through the increase in osmolarity resulting from the high glucose levels. Previous studies have shown that high ambient glucose levels induce the secretion of TGF-β in MDCK cells [Yang et al., 1998]. We have shown that high osmolarity did not affect the level of TGF-β secretion (Fig. 1). Thus, it is likely glucose directly, rather than its effect on osmolarity, that induces the secretion of TGF-β. Similarly, we found that high osmolarity did not enhance the expression of type II TGF-β receptors. However, our previous studies have shown that high ambient glucose levels (27.5 mM) can induce a significant increase in the expression levels

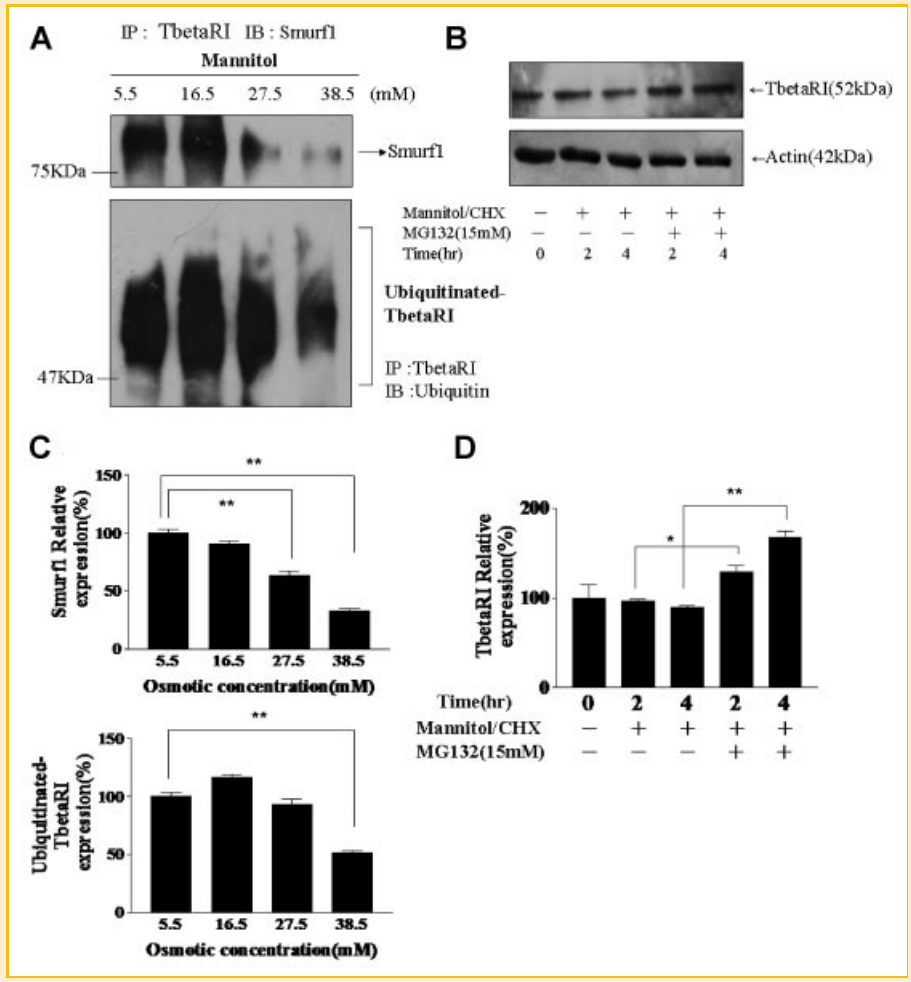

Fig. 7. High osmotic pressure reduced TGF-β RI protein degradation through an ubiquitination- and proteasome-dependent pathway. A: MDCK cells were cultured in 25 cm^2 flasks. Cells were serum starved (0.5% FBS) and incubated at different osmotic concentrations (5.5, 16.5, 27.5, and 38.5 mM) for 24 h. Whole-cell lysates (1 ml) were immunoprecipitated with polyclonal anti-type I TGF-β receptor antibodies, followed by immunoblotting with anti-ubiquitin (1:4,000) or anti-Smurf1 (1:4,000) antibodies. It is evident that high osmotic pressure significantly reduced the expression of Smurf1. In addition, hyperosmolarity inhibited Smurf1-mediated ubiquitination of the type I TGF-β receptor. B: Western blot analysis shows that treatment with the proteasome inhibitor (MG132) abolished the mannitol-mediated degradation of the type I TGF-β1 receptor. MDCK cells were pre-incubated with or without 15 μM MG132 for 5 h, followed by treatment with CHX (100 μg/ml) and mannitol (27.5 mM) for various periods of time (2, and 4 h), as indicated. C: Data from smurf1 and ublquitinated-TGF-β RI was scanned and normalized to the respective β-actin samples. D: The expression level of TGF-β RI was scanned and normalized to that of β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (5.5 mM osmotic concentration).

of type II TGF-β receptors [Yang et al., 1998]. Thus, it is glucose itself, rather than its osmotic effect, that induces the expression of type II TGF-β receptors.

Moreover, we have shown that osmotic concentration induced the phosphorylation of TGF-β RII at tyrosine 336 in MDCK cells in a time-course and dose-dependent manner (Fig. 3). This observation is consistent with Lawler et al. [1997], who showed that phosphorylation at tyrosine residues might contribute to the activation of TGF-β RII, a serine/threonine/tyrosine receptor kinase. We suggest a novel concept that hyperosmolarity might exert its biological effects by modulating the phosphorylation status of TGF-β receptors. High osmolarity resulted in a significant increase in expression of type I TGF-β receptors (Figs. 2 and 5). This observation is consistent with our previous study showing that high glucose levels (27.5 mM) induced an increase in expression of type I TGF-β receptors, as compared with control levels (5.5 mM) [Yang et al., 1998]. Thus, in addition to high glucose, high osmolarity in general exerts its fibrogenic effects on distal tubule cells by upregulating the level of TGF-β receptors, thereby enhancing susceptibility to tubular fibrosis.

Hyperosmotic conditions significantly increased the expression of TGF-β RI, but not TGF-β RII, in MDCK cells. Our work provides the first demonstration showing that hyperosmolarity may increase renal cellular fibrosis by inducing the expression of TGF-β receptors. Moreover, these results prompted us to investigate whether Smad signaling might be affected by hyperosmolarity. As shown in Figure 4, high osmotic pressure induced the expression of Smad2/3 and pSmad2/3 in MDCK cells, in addition to significantly suppressing the expression of the inhibitory Smad7. These expression changes may be the result of a feedback mechanism that involves specific protein ubiquitination and proteasomal degradation of Smads and TGF-β receptors. Ubiquitination plays a key role in a number of biological processes, including signal

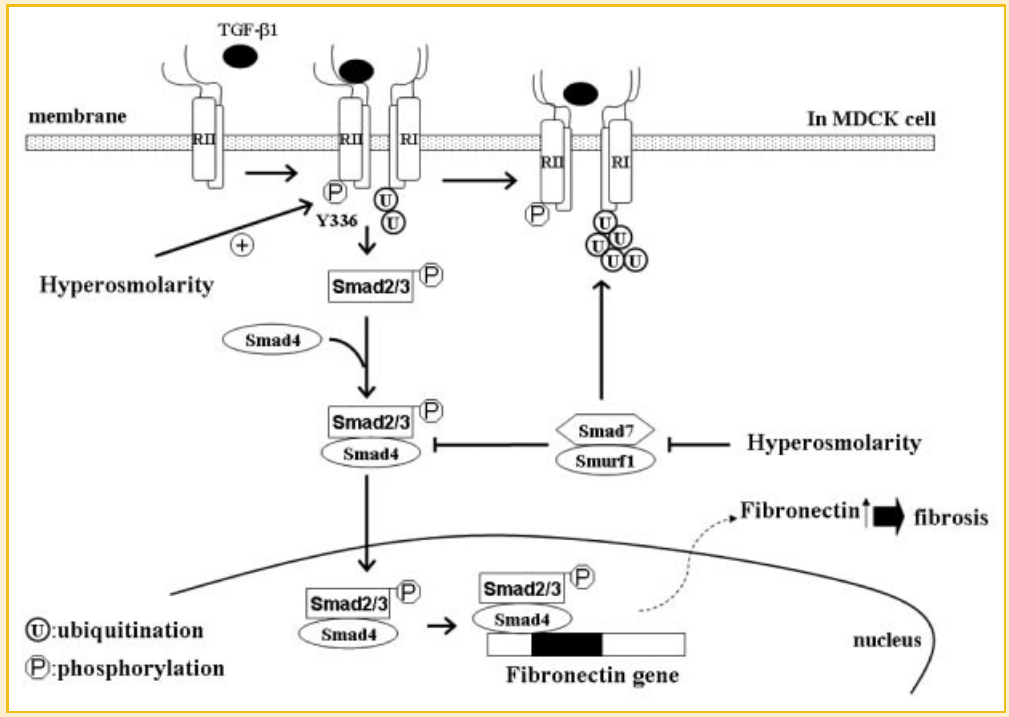

Fig. 8. Proposed mechanism for the interaction between hyperosmolarity and fibrosis status in renal distal tubule cells. Active TGF-β1 interacts sequentially with two membrane receptors. TGF-β1 binds first to TGF-β RI, which is phosphorylation-regulated, and then the ligand–receptor complex associates with TGF-β RI. Activated TGF-β RI propagates the signal downstream by directly phosphorylating Smad2 and Smad3. These molecules form complexes with Smad4 and translocate into the nucleus where they regulate the expression of extracellular matrix-related genes (e.g., fibronectin). In this study, we found that high osmolarity enhances the phosphorylation of TGF-β RI at pTyr336. In addition, high osmolarity increases the level of TGF-β RI expression. Moreover, high osmolarity induces the activation of the Smad2/3 pathway and suppresses the expression of the inhibitory Smad7. Taken together, these data indicate that high osmolarity accentuates sensitivity to cellular fibrosis by activating TGF-β-related pathways in MDCK cells.

transduction and gene expression [Guterman and Glickman, 2004]. Recently, ubiquitin ligases known as Smurfs have been shown to bind to Smad7 and have been implicated in the ubiquitination and catabolism of TGF-β RI [Yamaguchi et al., 2006]. In this study, we demonstrated that hyperosmolarity, generated by 27.5 mM mannitol, significantly increased the protein half-life of TGF-β RI (Fig. 6). Treatment with MG132 significantly attenuated the CHX-induced decrease in the level of expression of TGF-β RI, indicating that proteasomal activity seems to be responsible for the instability of TGF-β RI (Fig. 7B).

In conclusion, high osmolarity may increase distal tubule fibrosis by increasing the level of type I TGF-β receptors, phosphorylating type II TGF-β receptors, and activating the downstream Smad pathway (upregulating Smad2/3 and inhibiting Smad7). In addition, our experiments involving CHX and a proteasome inhibitor indicate that high osmolarity significantly increases the half-life and reduces the protein levels of the TGF-β receptor I via polyubiquitination and proteasomal degradation. Thus, we suggest that high osmolarity may accentuate cellular fibrosis in distal tubules by reducing catabolism of TGF-β receptor I in MDCK cells.

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