Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-β1-induced renal injury

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Noh H, Oh EY, Seo JY, Yu MR, Kim YO, Ha H, Lee HB. Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-β1-induced renal injury. Am J Physiol Renal Physiol 297: F729–F739, 2009. First published June 24, 2009; doi:10.1152/ajprenal.00086.2009.—Excessive accumulation of extracellular matrix (ECM) in the kidneys and epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial cells contributes to the renal fibrosis that is associated with diabetic nephropathy. Histone deacetylase (HDAC) determines the acetylation status of histones and thereby controls the regulation of gene expression. This study examined the effect of HDAC inhibition on renal fibrosis induced by diabetes or transforming growth factor (TGF)-β1 and determined the role of reactive oxygen species (ROS) as mediators of HDAC activation. In streptozotocin (STZ)-induced diabetic kidneys and TGF-β1-treated normal rat kidney tubular epithelial cells (NRK52-E), we found that trichostatin A, a nonselective HDAC inhibitor, decreased mRNA and protein expressions of ECM components and prevented EMT. Valproic acid and class I-selective HDAC inhibitor SK-7041 also showed similar effects in NRK52-E cells. Among the six HDACs tested (HDAC-1 through -5 and HDAC-8), HDAC-2 activity significantly increased in the kidneys of STZ-induced diabetic rats and db/db mice and TGF-β1-treated NRK52-E cells. Levels of mRNA expression of fibronectin and α-smooth muscle actin were decreased, whereas E-cadherin mRNA was increased when HDAC-2 was knocked down using RNA interference in NRK52-E cells. Interestingly, hydrogen peroxide increased HDAC-2 activity, and the treatment with an antioxidant, N-acetylcysteine, almost completely reduced TGF-β1-induced activation of HDAC-2. These findings suggest that HDAC-2 plays an important role in the development of ECM accumulation and EMT in diabetic kidney and that ROS mediate TGF-β1-induced activation of HDAC-2.

reactive oxygen species; extracellular matrix; epithelial-to-mesenchymal transition

PROGRESSIVE ACCUMULATION of extracellular matrix (ECM) in glomerular mesangium and tubulointerstitium is the hallmark of diabetic nephropathy (21). Epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial cells, characterized by loss of epithelial phenotype and gain of profibrotic features that are characteristic of mesenchymal cells, has been implicated in the accelerated fibrogenesis that is associated with diabetic nephropathy (41). Transforming growth factor (TGF)-β1 seems to be the key stimulus for ECM accumulation (25) and EMT (4, 28) and has been recognized as the final common pathway to renal fibrosis. TGF-β1 mRNA and protein levels are increased in the kidneys of diabetic animals (22, 32–34, 39) and humans (11, 40). Type II receptor for TGF-β1 is also upregulated in diabetic kidneys (5, 33). Short-term treatment with neutralizing anti-TGF-β antibody prevents glomerular hypertrophy and attenuates the increase in the mRNAs encoding collagen-α1 (IV) and fibronectin in streptozotocin (STZ)-induced diabetic mice (33, 44). Long-term effect of neutralizing anti-TGF-β antibody on glomerulosclerosis and renal insufficiency was also shown in db/db mice (44). Reactive oxygen species (ROS) are well recognized as important signaling molecules mediating renal injury in diabetes. We and others have shown that TGF-β1-induced Smad2 phosphorylation (20) and the ROS-extracellular-regulated protein kinase (ERK)1/2 axis (29) mediated EMT in a normal rat kidney tubular epithelial cell line, NRK52-E.

Gene transcription in eukaryotic cells is profoundly influenced by the manner in which DNA is packaged (38). Post-translational modifications of histones, including acetylation, phosphorylation, methylation, and ubiquitination, play an important role in transcriptional regulation (1, 30). In particular, acetylation of histone tails, mediated by histone acetyltransferase, is associated with activation of gene transcriptional activity, whereas decreased acetylation levels, mediated by histone deacetylase (HDAC), are associated with transcriptional repression (38). Earlier studies indicated a potential antifibrotic effect of HDAC inhibitors in liver (23), skin (31), and heart (13, 16). Recently, a report has suggested that global suppression of HDAC activities by inhibitors targeting both class I and class II HDACs inhibits TGF-β1-induced EMT in human renal proximal tubular epithelial cells (42). However, it is extremely important to identify which isoform of HDAC is involved, since each HDAC appears to have a distinct function. For example, class I HDACs appear to induce cardiac hypertrophy in response to angiotensin II infusion and aortic banding (13), whereas class II HDACs function to antagonize cardiac hypertrophy, as shown in mutant mice lacking the class II HDACs (43). Therefore, the present study was aimed at 1) determining the effect of HDAC inhibition on glomerular and tubular ECM accumulation and EMT in diabetic kidneys, 2) identifying the isoforms of HDACs participating in this process, and 3) examining the role of ROS as a downstream signaling molecule mediating diabetes or TGF-β1-induced dysregulation of HDAC.

MATERIALS AND METHODS

Animals. All animal work was approved by Ewha Womans University’s institutional animal care and use committee and handled in strict accordance with good animal practice as defined by the relevant national animal welfare bodies. Male Sprague-Dawley rats (Jai II Experimental Animal, Ansung, Korea) weighing 250 g were random-
ized to four groups: control (n = 9), control + trichostatin A (TSA; n = 9), diabetes (n = 10), and diabetes + TSA (n = 12). Diabetes was induced by intraperitoneal injection of STZ (70 mg/kg; Sigma, St. Louis, MO) in sodium citrate. Nondiabetic control rats were injected with an equivalent volume of sodium citrate buffer alone. Control or diabetic rats were randomized to receive 500 μg/kg TSA (Sigma) in 50 μl of DMSO or 50 μl of DMSO alone by subcutaneous injection once daily for 4 wk. At week 4, all rats were euthanized, and both kidneys were removed and weighed. Glomeruli and tubules were separated using a standard sieving method. To measure HDAC activity, we also used diabetic 20-wk-old db/db mice and age-matched control db/m mice. All animals were fed standard chow and water ad libitum.

Metabolic data. Blood glucose was measured by the glucose oxidase method (Sigma). Plasma and urinary creatinine was measured by a modified Jaffe method and adjusted for the interference by glucose. Urinary protein concentration was determined by the Bradford method, using the Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA).

Cell culture and treatments. NRK52-E cells were purchased from American Type Culture Collection (Rockville, MD). Near-confluent cells were incubated with serum-free media for 24 h to arrest and synchronize cell cycle. Before stimulation with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) or H2O2 (Duksan, Ansan, Korea), cells were pretreated with various concentrations of TSA, valproic acid (Sigma), SK-7041 (kindly supplied by In2gen, Seoul, Korea), or N-acetylcystein (NAC; Sigma) for the specified duration. In some experiments, the media containing TSA, SK-7041, or TGF-β1 were changed every 48 h.

Western blot analysis. Tissue and NRK52-E cell lysates were centrifuged to remove cell debris, and supernatant was mixed with SDS loading buffer. Samples were then heated at 100°C for 5–10 min before loading, separated through SDS-polyacrylamide gels, and subjected to Western blot as previously described (29). Antibodies to

Table 1. Characteristics of STZ-induced diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose, mg/dl</th>
<th>Kidney Weight/Body Weight, g/kg</th>
<th>Plasma Creatinine, mg/dl</th>
<th>Urine Volume, ml/day</th>
<th>Urine Protein/Creatinine, mg/mg</th>
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<tbody>
<tr>
<td>Control, n = 9</td>
<td>134.1 ± 7.1</td>
<td>3.36 ± 0.14</td>
<td>0.54 ± 0.10</td>
<td>13.3 ± 1.6</td>
<td>0.59 ± 0.10</td>
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<tr>
<td>Control + TSA, n = 9</td>
<td>132.8 ± 9.7*</td>
<td>3.00 ± 0.06</td>
<td>0.52 ± 0.11</td>
<td>11.0 ± 1.5</td>
<td>0.60 ± 0.08</td>
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<tr>
<td>DM, n = 10</td>
<td>582.8 ± 41.5*</td>
<td>5.00 ± 0.29*</td>
<td>0.42 ± 0.09</td>
<td>81.9 ± 17.8*</td>
<td>2.69 ± 0.45*</td>
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<tr>
<td>DM + TSA, n = 12</td>
<td>540.2 ± 60.2*</td>
<td>4.72 ± 0.25*</td>
<td>0.53 ± 0.08</td>
<td>90.3 ± 18.6*</td>
<td>1.70 ± 0.39*</td>
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Data are means ± SE. DM, streptozotocin (STZ)-induced diabetic rats. Trichostatin A (TSA; 500 μg/kg) was administered by sc injection once daily for 4 wk. *P < 0.05 vs. control, †P < 0.05 vs. DM.
collagen I (1:1,000; Southern Biotech, Birmingham, AL), α-smooth muscle actin (SMA; 1:5,000; Sigma), E-cadherin (1:5,000; BD Transduction Laboratories, San Jose, CA), acetylated histone H3 (1:2,000; Cell Signaling Technology), total histone H3 (1:2,000; Upstate, Lake Placid, NY), acetylated (1:2,000; Upstate) and total histone H4 (1:2,000; Upstate) were used. For fibronectin, peroxidase-conjugated rabbit anti-human fibronectin (1:10,000; DAKO, Glostrup, Denmark) was used.

**Analysis of mRNA levels.** Total RNA was extracted from tissues and NRK52-E cells with TRIzol (Sigma) according to standard procedures. Total RNA was reverse-transcribed to cDNA and amplified with specific primers by real-time PCR.

**Fig. 2.** Effect of TSA (A and B) and valproic acid (C) on ECM expression and EMT in NRK52-E cells. Cells were treated with transforming growth factor (TGF)-β1 in the presence or absence of TSA or valproic acid. A: after 6-h treatment with TGF-β1 with or without 6-h pretreatment of TSA, fibronectin, collagen I, α-SMA, and E-cadherin mRNAs were analyzed by real-time PCR. B: after 96-h treatment with TGF-β1 with or without 6-h pretreatment of TSA, fibronectin, collagen I, α-SMA, and E-cadherin protein expressions were analyzed by Western blot analysis. Media containing TGF-β1 or TSA were changed every 48 h. Data are means ± SE of 3–6 experiments. C: cells were treated with TGF-β1 for 6 h with or without 48-h pretreatment of valproic acid (2 mM) and analyzed for α-SMA and E-cadherin mRNAs by RT-PCR. Data are means ± SE of 6 experiments. *P < 0.05 vs. control [TGF-β1 (−) and TSA (−)], †P < 0.05 vs. TGF-β1 only.
protocol. For semiquantitative RT-PCR, the cDNA product was synthesized and amplified by PCR using the following primers: HDAC-2 sense 5'-ATT CGA GCA TCA GAC AAG CG-3', antisense 5'-TTT GGC TCC TTT GGT GTC TG-3'; α-SMA sense 5'-GAT CAC CAT CGG GAA TGA ACG-3', antisense 5'-GTT AGA AGC ATT TGC GGT GGA-3'; E-cadherin sense 5'-GAA GGG ACC GTC AAC AAC TG-3', antisense 5'-GCT GCC TTC AGG TTT TC TCG-3'; fibronectin sense 5'-GCC ACA CCT ACA ACC AGT AT-3', antisense 5'-ATG ACC ACT CAG AAA TGG AG-3'; and GAPDH sense 5'-ATG GTC TAC ATG TTC CAG TA-3', antisense 5'-TCA GAT CCA CAA CGG ATA CA-3'. The PCR was initiated by 15 min of incubation at 95°C, followed by 26 (fibronectin), 30 (HDAC-2 and GAPDH), 35 (α-SMA), or 40 (E-cadherin) cycles of 30 s at 94°C, 30 s at 55–56°C, and 1 min at 72°C. The reaction products were analyzed by agarose gel electrophoresis.

Real-time PCR was performed using the SYBR Green system (iCycler Real-Time PCR Detection System; Bio-Rad Laboratories) as previously described (19). For linear amplification of fibronectin,

Fig. 3. (A) Effect of TSA on histones H3 and H4 acetylation. NRK52-cells were treated with TGF-β1 in the presence or absence of TSA, and then histone was isolated as described in MATERIALS AND METHODS. Histone acetylation was measured by Western blot analysis. B and C: chromatin immunoprecipitation (ChIP) assay of E-cadherin and fibronectin promoter regions in NRK52-E cells. After 6-h TGF-β1 treatment with or without TSA, immunoprecipitation was performed using specific antibodies against acetyl H3 and H4, and semiquantitative (B) or real-time PCR (C) was performed using specific primers for E-cadherin and fibronectin promoter regions. Data are means ± SE of 5 experiments. *P < 0.05 vs. control, †P < 0.05 vs. TGF-β1 only.
collagen I, α-SMA, E-cadherin, and GAPDH, SYBR Green Master Mix (Invitrogen, Carlsbad, CA) and the following primers were used: fibronectin sense 5'-CGA GAG TAA ACC TGA AGC TG-3', antisense 5'-CCT GGT GAC CTC AAG ATG TG-3'; collagen I sense 5'-TGC CGT GAC CTC AAG ATG TG-3', antisense 5'-CAC AAG CGT GCT GTA GGT GA-3'; α-SMA sense 5'-GAT CAC CAT CGG GAA TGA ACG C-3', antisense 5'-CTT AGA AGC ATT TGC GGT GAA TGA ACG C-3'; and GAPDH sense 5'-GAC AGU UAG G-3', antisense 5'-GGT TGG ATT CAG AGC TTT GC-3'.

Isolation of histone. Histone was isolated by acid extraction from NRK52-E cell lysates as previously described (12). Briefly, NRK52-E cells were lysed in 10 mM HEPES, 1.5 mM MgCl2 (pH 7.9), 10 mM KCl, 0.5 mM DTT, and protease inhibitors. Sulfuric acid (50 µl, 2 M) was added dropwise into 200 µl of lysate while being gently vortexed. Samples were placed on ice for 1 h with vigorous vortexing every 10 min. Samples were centrifuged at 4° for 10 min at 12,000 rpm. Histone proteins were precipitated with three volumes of 20% trichloroacetic acid with vigorous vortexing every 10 min for 1 h. Samples were then washed twice with acidified acetone (0.1% (1 HCl) and twice with acetone. After desiccation, pelleted histones were resuspended in water, separated on a 15% SDS-PAGE gel, and immunoblotted.

Chromatin immunoprecipitation assay. A chromatin immunoprecipitation (ChIP) assay was performed as previously described (15), using an EZ ChIP kit (Upstate) according to the manufacturer’s description. Cells (107) were treated with 1% formaldehyde for 60 min at 25°C and were then sonicated to fragment the genomic DNA. The cross-linked DNA-protein complexes were immunoprecipitated (ChIP) assay was performed as previously described (15), with acetone. After desiccation, pelleted histones were resuspended in water. Histone was isolated by acid extraction from NRK52-E cell lysates as previously described (12). Briefly, NRK52-E cells were lysed in 10 mM HEPES, 1.5 mM MgCl2 (pH 7.9), 10 mM KCl, 0.5 mM DTT, and protease inhibitors. Sulfuric acid (50 µl, 2 M) was added dropwise into 200 µl of lysate while being gently vortexed. Samples were placed on ice for 1 h with vigorous vortexing every 10 min. Samples were centrifuged at 4° for 10 min at 12,000 rpm. Histone proteins were precipitated with three volumes of 20% trichloroacetic acid with vigorous vortexing every 10 min for 1 h. Samples were then washed twice with acidified acetone (0.1% HCl) and twice with acetone. After desiccation, pelleted histones were resuspended in water, separated on a 15% SDS-PAGE gel, and immunoblotted.

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serum- and antibiotic-free conditions for 24 h and treated with TGF-β1 (10 ng/ml). Stealth RNAi Negative Control Duplex (Invitrogen) was used as a negative control. The transfection efficiency was determined using Cy3-labeled GAPDH siRNA by flow cytometry.

Statistical analyses. The mean values obtained from each group were compared by ANOVA with subsequent Fisher’s least significant difference method. Unpaired two-tailed Student’s t-test and nonparametric analyses were also used where appropriate. Data are presented as mean ± SE. A P value < 0.05 was used as the criterion for a statistically significant difference.

RESULTS

Prevention of enhanced ECM expression and EMT by a nonselective HDAC inhibitor in STZ-induced diabetic kidneys. We first examined whether nonselective HDAC inhibition can prevent ECM accumulation and EMT in diabetic kidneys. In STZ-induced diabetic rats at 4 wk of disease, blood glucose, kidney-to-body weight ratio, and urinary protein excretion significantly increased compared with control rats (Table 1). A nonselective HDAC inhibitor targeting both classes I and II TSA had no effect on blood glucose or kidney/body weight ratio in diabetic or control rats. Urinary protein/creatinine excretion significantly decreased by TSA in diabetic rats but remained elevated compared with control rats. Glomerular (Fig. 1, A and C) and tubular (Fig. 1, B and D) fibronectin and collagen I and tubular α-SMA expression (Fig. 1, B and D) significantly increased, whereas tubular E-cadherin expression (Fig. 1, A and B) was significantly decreased in diabetic rats compared with control rats. TSA effectively prevented the increases in fibronectin, collagen I, and α-SMA and the decrease in E-cadherin expression at both mRNA (Fig. 1, A and B) and protein (Fig. 1, C and D) levels in diabetic rats, whereas it had no effect in control rats.

Prevention of enhanced ECM expression and EMT by nonselective HDAC inhibitors in NRK52-E cells. To elucidate the mechanism of an antifibrotic effect of TSA in diabetes, we performed an in vitro study using NRK52-E cells stimulated with TGF-β1. The dose and time for TGF-β1 were determined through a preliminary study. TGF-β1 at 10 ng/ml significantly increased fibronectin, collagen I, α-SMA mRNA, and protein expression and significantly decreased E-cadherin mRNA and protein expression. TSA effectively prevented all of these changes (Fig. 2, A and B). TSA up to 300 nM had no effect on cell survival determined by lactic dehydrogenase assay or on basal expression of fibronectin, collagen I, α-SMA, and E-cadherin in NRK52-E cells (data not shown). Another struc...
urally different nonselective HDAC inhibitor, valproic acid (8), also showed reversal of TGF-β1-induced EMT assessed by the mRNA expression of α-SMA and E-cadherin (Fig. 2C).

To determine whether the effects of TSA correlated with increases in histone acetylation, the effect of TSA treatment on histones H3 and H4 acetylation was determined by immunoblot analysis. As shown in Fig. 3A, TSA increased acetylation of histones H3 and H4 in a dose-dependent manner in basal as well as TGF-β1-stimulated NRK52-E cells. We next performed a ChIP assay to test whether TSA-induced hyperacetylation of histone was associated with alteration of transcriptional activity. Histones H3 and H4 were chosen for the ChIP assay because they are targets of class I HDAC. The ChIP assay confirmed that TSA-induced acetylation of H3 was

![Image of immunoblot analysis](https://example.com/immunoblot_analysis.png)

**Fig. 6.** A: NRK52-E cells were transfected with Cy-3-labeled GAPDH siRNA, and transfection efficiency was determined by flow cytometry. B: effect of HDAC-2 silencing on ECM expression and EMT in NRK52-E cells. NRK52-E cells were transfected with siRNA using Lipofectamine reagent for 24 h and treated with TGF-β1 (10 ng/ml) for 6 h. mRNA levels for HDAC-2, α-SMA, E-cadherin, and fibronectin were analyzed by RT-PCR. NT, nontreated; MOCK, Lipofectamine only; NC, Stealth RNAi negative control duplex; S1 and S2, siRNA of HDAC-1/2. Data are means ± S.E. of 5 experiments. *P < 0.05 vs negative control.
associated with upregulation of E-cadherin and downregulation of fibronectin gene transcription. Acetylation of H4 had a similar effect (Fig. 3, B and C).

Prevention of enhanced ECM expression and EMT by a class I HDAC-selective inhibitor in NRK52-E cells. To test whether TGF-β1-induced ECM expression and EMT could be prevented by class I-selective HDAC inhibition, we treated NRK52-E cells with SK-7041. SK-7041 [3-(4-substituted phenyl)-N-hydroxyl-2-propenamide] is one of the novel hybrid HDAC inhibitors synthesized by combining the hydroxamic acid of TSA and the pyridyl ring of MS-275, a synthetic benzamide derivative (14). Previously, it had been found to have high selectivity to class I HDACs (26). SK-7041 induced a qualitatively similar effect to TSA on TGF-β1-induced expression of fibronectin, collagen I, α-SMA, and E-cadherin in these cells (Fig. 4), suggesting that class I HDAC is involved in ECM accumulation and EMT.

Increased HDAC-2 activity in types 1 and 2 diabetic kidneys and TGF-β1-treated NRK52-E cells. To examine whether HDAC activity and/or expression is increased in diabetic kidneys or tubular epithelial cells cultured under TGF-β1 and which isoform of HDAC is activated, we measured activity of several HDACs belong to class I or II. Induction of diabetes by STZ for 4 wk was associated with elevated HDAC-1, -2, and -4 activities in the renal cortex (Fig. 5A). In the renal cortex of diabetic db/db mice at the age of 20 wk, diabetes increased activity of HDAC-2 and -4 (Fig. 5B). However, in TGF-β1-treated NRK52-E cells, only HDAC-2 activity was found to be increased (Fig. 5C). Expressions of HDAC-2 mRNA (Fig. 5D) and protein (Fig. 5E) were not different by TGF-β1 treatment.

Silencing of HDAC-2 reduces TGF-β1-induced fibronectin expression and EMT. Because elevated HDAC-2 activity is a common finding in both types 1 and 2 diabetic kidneys and TGF-β1-treated NRK52-E cells, we next tested whether siRNA targeting HDAC-2 can block TGF-β1-induced ECM expression and EMT. With 50% of transfection efficiency of siRNA (Fig. 6A), two different HDAC-2-specific siRNAs could efficiently knock down HDAC-2 mRNA expression (Fig. 6B). Interestingly, in parallel, fibronectin and α-SMA mRNA expressions were also attenuated and E-cadherin mRNA expression was increased by HDAC-2 siRNA compared with scrambled oligonucleotides (Fig. 6B). These results provide evidence for the involvement of HDAC-2 in ECM accumulation and EMT.

ROS mediate activation of HDAC-2 induced by TGF-β1. Several lines of evidence show that ROS play a major role in hyperglycemia-induced ECM upregulation in both cultured mesangial cells and diabetic glomeruli and in TGF-β1-induced EMT. We therefore hypothesized that ROS mediate TGF-β1-induced activation of HDAC-2. In NRK52-E cells, H₂O₂...
significantly elevated HDAC-2 activity (Fig. 7A) and an antioxidant, NAC, almost completely blocked TGF-β1-induced activation of HDAC-2 (Fig. 7B). Expressions of HDAC-2 mRNA (Fig. 7C) and protein (Fig. 7D) were not affected by H₂O₂.

DISCUSSION

This study demonstrates that diabetes and TGF-β1 activate HDAC-2 in the kidneys, which may be involved in the subsequent accumulation of ECM and EMT. In addition, we have shown that ROS can directly increase HDAC-2 activity and mediate TGF-β1-induced HDAC-2 activation. These new observations strengthen the concept that HDAC may play a central role in the pathogenesis of diabetic nephropathy and may improve our understanding of the beneficial effect of HDAC inhibitors on tissue fibrosis.

The development of diabetic nephropathy is a multigenic, integrative response involving signal integration of multiple pathways. Through the gene-specific transcriptional regulation, posttranslational modification of nucleosomal histone proteins may have a role in the genesis of diabetic nephropathy. In mammalian cells, HDACs constitute a family of 18 enzymes and are separated into three classes on the basis of their similarity to various yeast HDACs. HDAC-1-3, -8, and -11 constitute class I HDACs and share homology with the yeast RPD3 protein. Class II HDACs, including HDAC-4-7, -9, and -10, have similarities to yeast HDA1. Class III HDAC, or silent information regulator-2, is an NAD⁺-dependent HDAC for the removal of acetyl groups from acetylated lysine residues (7).

Recent reports have indicated that changes in the histone acetylation are correlated with several pathological conditions such as cancer (3), cardiac hypertrophy (13, 43), and chronic obstructive pulmonary disease (10). HDAC inhibitors are now considered among the promising anti-cancer agents, leading to growth arrest, differentiation, and apoptosis of transformed cells.

Interestingly, several earlier studies also suggested a potential antifibrotic effect of HDAC inhibitors. TSA was found to suppress myofibroblastic differentiation in rat hepatic stellate cells (23) and TGF-β1-induced fibrogenesis in rat skin fibroblasts (31). It was shown that mammalian Snail requires HDAC activity to repress the E-cadherin promoter and that treatment with TSA is sufficient to block the repressor effect of Snail (27). In this study, we clearly demonstrate that HDAC-2 in renal tubular epithelial cells and diabetic kidneys in both types 1 and 2 is profibrotic, because HDAC-2 activity was increased and SK-7041, a class I-selective HDAC inhibitor, and silencing of HDAC-2 blocked the development of ECM expression and EMT. Although valproic acid inhibits multiple HDACs from class I and II (8), a study demonstrated that valproic acid, but not TSA, induced proteosomal degradation of HDAC-2 (18). The results obtained in the present study showing that TSA prevented ECM upregulation and EMT in NRK52-E cells are consistent with a previous report by Yoshikawa et al. (42). Those authors showed that TSA prevented TGF-β1-induced downregulation of E-cadherin and upregulation of collagen I in human renal proximal tubular epithelial cells, in association with induction of inhibitors of DNA binding/differentiation 2 (Id2) and bone-morphogenic protein-7 mRNAs, inhibitors of TGF-β1 signals (42). However, information on each HDAC isoform should be clarified individually, given that class I and II HDACs can exert antagonistic actions even on cardiac hypertrophy (13, 43). In this regard, this study reveals that HDAC-2 functions selectively as a key molecule in response to diabetes or TGF-β1.

Since increased levels of histone acetylation are generally associated with transcriptional activation, it is not clear how HDAC inhibition can lead to transcriptional repression of profibrotic genes in our study. We confirmed by ChIP assay that histone acetylation was involved in the regulation of fibronectin transcription. Although the mechanisms remain unclear, transcriptional activation of repressors and their binding to the promoters of profibrotic genes and acetylation of nonhistone proteins may, in part, explain their antifibrotic effects.

Cellular ROS are increased in diabetes and contribute to the pathogenesis of diabetic vascular complications, including nephropathy (6, 9, 24). We have shown that high glucose and TGF-β1 increase dichlorofluorescein-sensitive ROS in renal cells (25) and that antioxidants inhibit glomerular TGF-β1 and fibronectin mRNA in diabetic rats (9). Furthermore, we also found that TGF-β1-induced phosphorylation of mitogen-activated protein kinase and EMT in NRK52-E cells is mediated by ROS (29). Our current data further support the concept that ROS are the key regulators of renal injury induced by diabetes.

Fig. 8. Proposed role of HDAC-2 in diabetes and TGF-β1-induced renal injury. Diabetic stress induces reactive oxygen species (ROS) generation through TGF-β1-dependent or -independent mechanisms. ROS may induce activation of HDAC-2 activation through yet-identified kinase. Increased HDAC-2 activity is then expected to induce renal fibrosis either by inhibiting antifibrotic gene expression such as inhibitors of DNA binding/differentiation 2 (Id2), bone-morphogenic protein (BMP)-7, and friend leukemia virus integration 1 (FLI1) or by suppressing a gene such as E-cadherin. Dotted lines, unknown and putative mechanisms.

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or TGF-β1. In NRK52-E cells, HDAC activity was elevated after exposure to H₂O₂, and antioxidant reduced TGF-β1-induced HDAC-2 activity. There are conflicting reports whether ROS induce HDAC activity and consequent hypoacetylation of histones. Miura et al. (21a) have reported that hepatitis C virus (HCV)-induced ROS caused hypoacetylation of histones by increased HDAC activity in HCV replicon cells. However, Berthiaume et al. (2) have suggested that strong oxidative stress induces hypoacetylation, whereas weak oxidative stress induces hyperacetylation even in the same cell line. Thus, the relationship between ROS and HDAC activity may depend on the intensity of oxidative stress, endogenous HDAC activity, and cell types. In the present study, neither TGF-β1 nor H₂O₂ altered HDAC-2 mRNA or protein expression, suggesting that posttranslational modification of HDAC-2 may play an important role in TGF-β1- and H₂O₂-induced HDAC-2 activity. Phosphorylation is considered the leading posttranslational mechanism controlling enzymatic activities of HDAC (37). It has been reported that phosphorylation of HDAC-2 is necessary for both enzymatic activity and the association with the corepressors mSin3 and Mi2 (36). Protein kinase CK2 has been shown to control HDAC activity (37). Therefore, it will be interesting to determine whether ROS increase HDAC-2 phosphorylation. A model for HDAC-2-mediated diabetic renal fibrosis signaling pathway is shown in Fig. 8.

In conclusion, the present study demonstrates that HDAC-2 plays an important role in the development of renal fibrosis induced by diabetes or TGF-β1 and that ROS mediate increased HDAC-2 activity. These data suggest that new therapeutic strategies based on HDAC-2 inhibition could prove beneficial in diabetic nephropathy.

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