Pathobiology of renal-specific oxidoreductase/myo-inositol oxygenase in diabetic nephropathy: its implications in tubulointerstitial fibrosis

Ping Xie,1 Lin Sun,2 Peter J. Oates,3 Satish K. Srivastava,4 and Yashpal S. Kanwar1

1Departments of Pathology and Medicine, FSM, Northwestern University, Chicago, Illinois; 2Department of Nephrology, Central South University, Hunan, PR China; 3Department of Cardiovascular Metabolic and Endocrine Diseases, Pfizer Global Research and Development, Groton, Connecticut; and 4Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas

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Xie P, Sun L, Oates PJ, Srivastava SK, Kanwar YS. Pathobiology of renal-specific oxidoreductase/myo-inositol oxygenase in diabetic nephropathy: its implications in tubulointerstitial fibrosis. Am J Physiol Renal Physiol 298: F1393–F1404, 2010. First published March 24, 2010; doi:10.1152/ajprenal.00137.2010.—Renal-specific oxidoreductase/myo-inositol oxygenase (RSOR/MIOX) is expressed in renal tubules. It catabolizes myo-inositol and its expression is increased in diabetic mice and in LLC-PK1 cells under high-glucose ambience. Aldose reductase (AR) is another aldo-keto reductase that is expressed in renal tubules. It regulates the polyol pathway and plays an important role in glucose metabolism, osmolyte regulation, and ECM pathobiology via the generation of advanced glycation end products, reactive oxygen species, and activation of transforming growth factor (TGF)-β. In view of the similarities between AR and RSOR/MIOX, the pathobiology of RSOR/MIOX and some of the cellular pathways affected by its overexpression were investigated. An increased expression of fibronectin was noted by transfection of LLC-PK1 cells with pcDNA3.1-RSOR/MIOX. Similar changes were observed in LLC-PK1 cells under high-glucose ambience, and they were notably lessened by RSOR/MIOX-small interfering (si) RNA treatment. The changes in tubulointerstitial fibronectin expression were also observed in the kidneys of db/db mice having high levels of RSOR. The pcDNA3.1-RSOR/MIOX transfectedants had an increased NADH/NAD⁺ ratio, PKC and TGF-β activity, Raf1:Ras association, and p-ERK phosphorylation. These changes were significantly reduced by the inhibitors of PKC, aldose reductase, Ras farnesylation, and MEK1. Similar increases in various the above-noted parameters were observed under high-glucose ambience. Such changes were partially reversed with RSOR-siRNA treatment. Expression of E-cadherin and vimentin paralleled in cells overexpressing RSOR/MIOX or subjected to high-glucose ambience. These studies suggest that RSOR/MIOX modulates various downstream pathways affected by high-glucose ambience, and conceivably it plays a role in the pathobiology of tubulointerstitium in diabetic nephropathy.

Fibronectin; db/db mice; sorbinil

Diabetes mellitus is a common metabolic disorder in which hyperglycemia afflicts injury on multiple organ systems in humans, and frequent lesions that have been well described include diabetic microangiopathy, neuropathy, retinopathy, and nephropathy (6, 33, 47, 54). The changes in the kidney occur in more than one-third of the patients with diabetes mellitus, and they have been exhaustively described in the literature (27, 46, 51, 53, 57, 62). The hyperglycemia-induced renal changes may be acute and reversible or chronic and irreversible in nature, and these kidney lesions may serve as a prototypical example that would be applicable to other mammalian organ systems. The acute metabolic changes include increased activity of the polyol pathway, altered redox state of pyridine nucleotides (NADPH/NADP⁺ and NADH:NAD⁺ ratios), perturbations in the myo-inositol and diacylglycerol levels, activation of protein kinase C, glycation of proteins, and generation of reactive oxygen species (ROS) (6, 27, 53, 57, 61, 62, 67). The chronic irreversible changes are insidious and cumulative in nature, and they affect the proteins with a relatively slow metabolic turnover, e.g., collagen, eye lens proteins, and DNA (27, 35, 67). A prototype of chronic change includes the formation of glycation products that undergo Amadori rearrangements to generate advanced glycation end products (AGEs), which adversely affect the pathobiology of the extracellular matrix (ECM) (58). In addition, AGE-RAGE interaction can further lead to the generation of ROS, activation of PKC, various other cellular kinases, e.g., MAPKs, and several transcription factors, e.g., NF-κB and activator protein (AP)-1, and cytokine transforming growth factor (TGF)-β (27, 35, 50, 67), the latter being a fibrogenic cytokine that exerts its effects on ECM proteins like collagen and fibronectin via various Smad signaling pathways (35, 52, 67). Collectively, these metabolic events lead to an aberrant expression of ECM, especially that of the renal glomerulus, which is regarded as the hallmark characteristic of diabetic nephropathy (14, 35, 46), although injury to the tubulointerstitial compartment is also an important correlative of the derangements in renal function (16, 38, 39, 40, 66).

In addition to the glomerulus, the tubular or tubulointerstitial compartment is adversely affected especially by the enhanced activity of the polyol pathway that is regulated by a rate-limiting enzyme, aldose reductase (AR) (10). AR belongs to the family of NAD(P)H-dependent oxido-reductases, and it is abundantly expressed in the tubules where it regulates the osmotic homeostasis of the kidney (13, 24, 43). Another related ~32-kDa cytosolic enzyme, known as renal-specific oxido-reductase, RSOR, that is relevant to the pathogenesis of diabetic nephropathy, has been recently described (64). Like AR, RSOR is also heavily expressed in the renal tubular compartment and is also known as myo-inositol oxygenase (MIOX) (2, 41). Previous in vivo and in vitro studies indicate that the activity of RSOR/MIOX is modulated by diverse mechanisms, and it is endowed with dual properties to channel glucose intermediaries, characteristic of hepatic aldehyde reductases, and to maintain osmoregulation, a function of renal tubules where it regulates the polyol pathway and plays a crucial role in glucose metabolism, osmolyte regulation, and ECM pathobiology via the generation of advanced glycation end products, reactive oxygen species, and activation of transforming growth factor (TGF)-β. In view of the similarities between AR and RSOR/MIOX, the pathobiology of RSOR/MIOX and some of the cellular pathways affected by its overexpression were investigated. An increased expression of fibronectin was noted by transfection of LLC-PK1 cells with pcDNA3.1-RSOR/MIOX. Similar changes were observed in LLC-PK1 cells under high-glucose ambience, and they were notably lessened by RSOR/MIOX-small interfering (si) RNA treatment. The changes in tubulointerstitial fibronectin expression were also observed in the kidneys of db/db mice having high levels of RSOR. The pcDNA3.1-RSOR/MIOX transfectedants had an increased NADH/NAD⁺ ratio, PKC and TGF-β activity, Raf1:Ras association, and p-ERK phosphorylation. These changes were significantly reduced by the inhibitors of PKC, aldose reductase, Ras farnesylation, and MEK1. Similar increases in various the above-noted parameters were observed under high-glucose ambience. Such changes were partially reversed with RSOR-siRNA treatment. Expression of E-cadherin and vimentin paralleled in cells overexpressing RSOR/MIOX or subjected to high-glucose ambience. These studies suggest that RSOR/MIOX modulates various downstream pathways affected by high-glucose ambience, and conceivably it plays a role in the pathobiology of tubulointerstitium in diabetic nephropathy.

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Address for reprint requests and other correspondence: Y. S. Kanwar, Dept. of Pathology, FSM, Northwestern Univ., 303 E. Chicago Ave., Ward 3–140, Northwestern Univ. Medical School, Chicago, IL 60611 (e-mail: y-kanwar@northwestern.edu).

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In view of the fact that AR plays a crucial role in channeling of glucose intermediaries and it may share certain similarities with RSOR/MIOX, studies were initiated to assess the relevance of RSOR/MIOX in the pathogenesis of hyperglycemia-induced renal tubulointerstitial injury, utilizing both in vivo and in vitro systems. In addition, since the AR inhibitors are known to suppress the activity of the polyol pathway and thereby a multitude of downstream events with eventual amelioration of the ECM complications in diabetic nephropathy (27, 35), the investigation was extended to assess the efficacy of these agents in inhibiting the signaling events initiated by RSOR/MIOX.

MATERIALS AND METHODS

Reagents. The LLC-PK₁ cell line (renal tubular cell line) was purchased from the American Type Culture Collection. The pcDNA3.1 vector Lipofectamine 2000, G418, zeocin, and psirNA-h7SKGFPpeo G1 vector were obtained from Invitrogen (siRNAs denote small interfering RNA). Anti-RSOR polyclonal antibody was prepared in our laboratory previously (41, 64). Other reagents were purchased from the following vendors: Sigma-Aldrich: polyclonal anti-fibronectin and monoclonal β-actin antibodies, lactate, pyruvate, NAD⁺, NADH, lactate dehydrogenase, and M199 media; Santa Cruz Biotechnology: mouse monoclonal anti-fibronectin, rabbit polyclonal anti-PKC-α, and mouse monoclonal anti-vimentin antibody; Amersham Biosciences, enhanced chemiluminescence (ECL) kit, protein A-Sepharose and monoclonal antibody (Invitrogen) for immunoprecipitation and Western blot analyses. For immunoprecipitation, aliquots of 200 μg of cellular lysates from each of the experiments in 500-μl volume RIPA buffer were precleared with 50 μl of protein A-Sepharose CL-4B (Amersham Biosciences) slurry as described previously (56). After centrifuging at 600 g for 5 min, the supernatants were incubated with 2 μg of rabbit anti-Raf-1 polyclonal antibody at 4°C for 4 h, followed by addition of 50 μl of protein A-Sepharose CL-4B. The mixture was incubated at 4°C for another 4 h with gentle orbital shaking. The complexes (protein-Sepharose + antibody + Raf-1) were pelleted by centrifuging at 600 g for 5 min. The pellet was washed with RIPA buffer, and the process was repeated twice. The final pellet was dissolved in the lysis buffer. After addition of equivalent amounts of SDS sample buffer, the samples were boiled for 5 min and subjected to SDS-PAGE.

Similarly, the cell/tissue lysates containing equal amounts of protein from each of the experiments were dissolved in SDS sample buffer, boiled, and subjected to SDS-PAGE. The fractionated proteins were then transferred to nitrocellulose membranes. Western blot analyses were carried out using the ECL kit (Amersham Biosciences) by following the manufacturer’s protocol.

Northern blotting studies. Total RNA was prepared from kidneys by the acid guanidinium isothiocyanate-phenol-chloroform extraction method (9). About 30 μg of total RNAs extracted from db/db and db/db kidneys was glyoxylated, subjected to 1% agarose gel electrophoresis, and then capillary-transferred to Hybond N⁺ nylon membrane (Amersham Biosciences). After cross-linking of RNA to the membrane, prehybridization and hybridization of various membrane blots were carried out with various α-[32P]dCTP-labeled (1×10⁶ cpm/ml) cDNA probes of fibronectin or RSOR/MIOX (63, 64) (where cpm denotes counts per minute). Following the preparation of autoradiograms, the membrane blots were stripped by boiling in 0.1% SDS buffer for 2 min and reprobed with radiolabeled β-actin cDNA probe.

Immunofluorescence studies. The immunofluorescence studies were performed as described previously (63). One- to two-mm-thick slices of kidney were embedded in optimum cutting temperature compound (Sakura Finetek) and chilled in liquid nitrogen. About 4-μm-thick cryostat sections of kidneys were prepared, mounted on glass slides, and air dried. The sections were hydrated in PBS for 10 min and incubated either with rabbit polyclonal anti-RSOR or -fi-
bronectin antibody at 37°C for 1 h. Following a rinse with PBS, the slides were incubated with goat anti-rabbit antibody conjugated with FITC at 37°C for another hour, and examined with an UV light microscope equipped with Epi-illumination.

For cell immunofluorescence studies, the cells were fixed in cold methanol at −20°C for 4 min and immersed in PBS solution. Following blocking with 3% of BSA, the slides were incubated with mouse monoclonal anti-fibronectin at 37°C for 1 h, washed with PBS, and then reincubated with anti-mouse IgG conjugated with FITC (Sigma-Aldrich) at 22°C for another hour. The sections were counterstained with 4,6-diamidino-2-phenylindole to highlight the nuclei and then examined.

**RSOR/MIOX activity analysis.** The RSOR/MIOX activity in the cells or its His-tagged recombinant protein was analyzed as described previously (8, 41). Cells that had undergone various treatments were homogenized in a buffer containing 20 mM sodium acetate (pH 6.0), 2.0 mM L-cysteine, 1 mM ferrous ammonium sulfate, 1 mM glutathione, and 1 mM PMSF. The homogenate was centrifuged at 20,000 g for 30 min, and the supernatants were saved for an RSOR/MIOX activity assay. The RSOR/MIOX activity assay was performed in 1-ml reaction mixture including 50 mM sodium acetate buffer (pH 6.0), 2.0 mM L-cysteine, 1 mM ferrous ammonium sulfate, 60 mM myo-inositol, and either 100 μl of the cellular supernates or 1 μg His-RSOR/MIOX fusion protein. The reaction mixtures were shaken vigorously at 30°C for 15 min, and the reaction was terminated by the addition of 100 μl of 25% TCA. The precipitated protein was centrifuged at 1,000 g. The supernatant was saved for measurement of β-gluconic acid by the orcinol method.

**Lactate assay.** Lactate concentration was determined by a modification of a previously described method in which the NADH is converted to lactate by lactate dehydrogenase (LDH) (20). Diluted cell culture supernatant was retained as the “membrane” fraction. Both membrane and cytosolic fractions were passed through DEAE-52 cellulose (Pharmacia) columns, washed three times with 5 ml of buffer B, and then eluted with 0.5 ml of buffer B containing 200 mM NaCl.

PKC activity was measured by its ability to transfer γ-[32P]ATP into specific substrate QKRPSQRSKYL (single-letter amino acid abbreviations) in the presence of PKA/CalM inhibitor cocktail, per instructions in the PKC assay kit manual. The phosphorylated substrate was then separated from the unincorporated γ-[32P]ATP using P81 phosphocellulose paper and measured by using a scintillation counter. PKC activity was calculated by subtracting the nonspecific kinase activity measured in the absence of Ca2+, phosphatidylserine, and diacylglycerol (DAG). Protein determination was performed using the method of Bradford.

**ELISA of fibronectin and TGF-β.** Fibronectin in the culture supernatants of conditioned media was measured by using a quantitative competitive ELISA kit according to the vendor’s instructions (Assaypro). Fibronectin amount was expressed as per milligram of total cellular protein. The TGF-β1 in culture supernatants of conditioned media (100 μl) was first activated by the addition of 20 μl of 1 N HCl and then incubating at 25°C for 10 min, followed by neutralization with 20 μl of 1 N NaOH. Total TGF-β1 was measured by using a sandwich ELISA kit according to the manufacturer’s instructions (Assaypro). The TGF-β1 level in the samples was expressed as per milligram total cell protein.

**Bioassay of TGF-β.** Bioactivity of TGF-β1 was determined by using a mink lung epithelial cell line which had been stably transfected with PAI-1 promoter linked to the luciferase reporter gene (1). Mink cells were seeded onto 96-well tissue culture plate (1.6 × 10⁴/well) and allowed to adhere for 3 h. Serial dilution of TGF-β1 standard or conditioned media from LLC-PK1 cells (1:1 dilution) was added to the medium of mink cells and incubated for 14 h. Luciferase activity was determined as described by using a Promega luciferase assay reagent, and the values were compared with various TGF-β1 standards.

**Statistical analysis.** All results are depicted as means ± SE. Data were analyzed by one-way analysis of variance, and a P value of <0.05 was considered statistically significant.

**RESULTS**

In our initial transfection studies, we inadvertently observed that overexpression of RSOR-MIOX resulted in an increased cellular expression of fibronectin. Since RSOR-MIOX is intimately involved in glucose metabolism where increased expression of ECM is a common occurrence, this led us to explore the mechanisms that may be responsible for the induction of the fibronectin gene.

**Induction of fibronectin by overexpression of RSOR/MIOX**

In our initial studies, the findings that there is an induction of fibronectin comparable to high glucose-induced protein expression were validated. By immunofluorescence microscopy, an increased cellular expression of fibronectin was observed in stably transfected LLC-PK1 cells with RSOR/MIOX pcDNA 3.1 under low-d-glucose (5 mM) ambience (Fig. 1, B and A). To assess the specificity of the RSOS/MIOX effect, the cells were treated with RSOR-siRNA while scrambled oligo serving as a control. Under low-glucose ambience, there were no discernible differences among the cells treated with scrambled oligo or RSOR-siRNA (Fig. 1, C and D). Under high-d-glucose ambience (30 mM), the cells treated with scrambled oligo exhibited comparable immunofluorescence that is observed in cells transfected with RSOR-pcDNA (Fig. 1, E vs. B). While the siRNA treatment resulted in a notable reduction of immunofluorescence under high-glucose ambience (Fig. 1, F vs. E), suggesting that the induction of fibronectin expression is specifically mediated by...
RSOR/MIOX, and the findings are reminiscent of those observed for other ECM proteins in a diabetic milieu. Fibronectin expression was also measured in the culture media by ELISA assay. The culture media of pcDNA3.1-RSOR/MIOX transfectants had significantly higher concentration of fibronectin (39.5 ± 3.1 ng/mg protein) compared with media of cells transfected with pcDNA3.1 alone (14.3 ± 1.8 ng/mg protein) (Fig. 1G). The amount of secreted fibronectin in cells treated with siRNA under low-glucose ambience was comparable to those transfected with empty vector alone. The media from cells subjected to high glucose and treated with scrambled oligo had a high concentration of fibronectin. However, with siRNA treatment it was reduced by ~40% (42.5 ± 4.2 → 25.4 ± 2.4 ng/mg protein) (Fig. 1G), and the fibronectin reduction was similar to that observed in the cellular compartment by immunofluorescence microscopy (Fig. 1F).

Expression of RSOR/MIOX and fibronectin in diabetic db/db mice. The next question that needed to be addressed was whether there is any correlation between RSOR/MIOX and fibronectin expression in vivo in kidneys of diabetic mice. Northern blot analyses showed an increase in the intensity of bands reflective of enhanced fibronectin expression in the cortices of db/db mice compared with the control db/m mice at 16 wk of age, when the diabetic state is well established (Fig. 2A). This increase was associated with increased expression of RSOR/MIOX (Fig. 2B), while no significant differences in the expression of β-actin were observed among these strains of mice (Fig. 2C). Similar to the gene expression, Western blot analyses revealed an increase in the expression of fibronectin...
was much higher than that of pcDNA3.1 transfectents alone (26.8 ± 2.3) (Fig. 4A). Minimal change in the NADH/NAD⁺ ratio (27.3 ± 2.4) was observed in cells transfected with inactivated 124MU RSOR/MIOX mutant (Lys124 → Ala, Fig. 4A). Since RSOR is an oxido-reductase like the aldose reductase, therefore, the effect of inhibitors of the latter enzyme, such as, sorbinil or zopolrestat, was assessed. Interestingly, addition of 10 μM sorbinil or zopolrestat in the media of pcDNA3.1-RSOR/MIOX transfectents reduced the ratio to 35.6 ± 3.3 and 38.8 ± 3.9, respectively (Fig. 4A). As in glomerular cells, the high-N-glucose ambience of 30 mM was found to increase the NADH/NAD⁺ ratio (47.6 ± 3.5) compared with low glucose (21.6 ± 1.8) in LLC-PK₁ cells (Fig. 4B), and the treatment with scrambled oligo had a minimal effect. Exposure of 30 mM l-glucose did not significantly change the NADH/NAD⁺ ratio observed in cells subjected to low-glucose ambience (not shown). Suppression of RSOR/MIOX by siRNA significantly reduced the ratio (47.6 ± 3.5 → 32.7 ± 2.9) under high-glucose ambience (Fig. 4B), suggesting that there are certain similarities with respect to the metabolic activities of RSOR/MIOX and AR, an enzyme that plays a key role in glucose metabolism (41). The intimate relationship of RSOR/MIOX with glucose metabolism impelled us to investigate the signaling events related to the pathogenesis of diabetic nephropathy.

Increased membrane translocation expression of PKC-α by overexpression of RSOR/MIOX. First, the status of PKC-α was investigated. Following transfection of LLC-PK₁ cells and treatments with various inhibitors (sorbinil and zopolrestat) for 24 h, plasmalemmal and cytosolic fractions were fractionated and extracted to determine the protein content and activity of PKC-α as previously described (25). The membrane PKC

associated with an increase in RSOR/MIOX expression in db/db mice compared with control db/m mice (Fig. 3, E and F). To delineate the spatial expression of fibronectin in the kidney, immunofluorescence studies were carried out. By immunofluorescence microscopy, an increase in the intensity of fibronectin staining was observed in the renal cortices (Fig. 3, B vs. A). Immunoreactivity was mainly confined to the interstitial compartment, but a mild increase above the background of control db/m mice was also observed in the cellular compartment of db/db mice (Fig. 3B). The cellular expression of RSOR/MIOX was also markedly increased in db/db mice compared with control db/m mice (Fig. 3, D vs. C). RSOR/MIOX expression was not observed in any of the glomerular cell types. These studies suggested that there may be a causal relationship between the expression of fibronectin and RSOR/MIOX.

Modulation of NADH/NAD⁺ ratio by RSOR/MIOX in LLC-PK₁ cells. Since the perturbation in the NADH/NAD⁺ ratio reflects the state of cellular redox in high-glucose ambience and both NADH and NAD⁺ are intimately involved in RSOR/MIOX metabolism, studies were initiated to assess their status by measuring the lactate/pyruvate ratio in the media following the transfection of LLC-PK₁ cells in the absence of FBS for 24 h. The ratio of lactate/pyruvate (NADH/NAD⁺) in culture media of pcDNA3.1-RSOR/MIOX transfectents (57.2 ± 5.2)
activity of pcDNA3.1 transfectents was designated as to be 100%, and it increased significantly (232 ± 25.4%) upon transfection of LLC-PK₁ cells with pcDNA3.1-RSOR/MIOX in the membrane fraction (Fig. 5A). By Western blot analyses, an increase in intensity of the membrane PKC-α band in pcDNA3.1-RSOR/MIOX transfectents was also observed compared with that in pcDNA3.1 transfectents in the membrane fraction (Fig. 5C, red arrowhead). A mild decrease in cytosolic PKC activity and protein amount was observed in the pcDNA3.1-RSOR/MIOX transfectant group (Fig. 5, A and C). Similarly, by immunofluorescence microscopy, increased intensity of cytoplasmic and plasmalemmal staining of PKC-α was observed by overexpression of RSOR/MIOX in cells cultured under low-glucose ambience (Fig. 5, F vs. E). Transfection of the RSOR/MIOX (K124A) mutant had no notable effect on the activity and expression of PKC-α (Fig. 5, A and C). Treatment of pcDNA3.1-RSOR/MIOX transfectents with 10 μM sorbinil or zopolrestat for 24 h significantly reduced the PKC activity confined to the membrane fraction (232 ± 25 → 152 ± 18%, sorbinil group, and → 169 ± 15%, zopolrestat group) (Fig. 5A). This was associated with a reduction to the basal level of the increase in membrane protein content of PKC-α induced by the overexpression of RSOR/MIOX (Fig. 5C).

The membrane activity and protein expression of PKC-α were elevated in cells subjected to high-glucose ambience in the presence of scrambled oligo (189 ± 16.6%) (Fig. 5, B and arrowhead in D). Similarly, there was an increased intensity of plasmalemmal and cytoplasmic staining of PKC-α in LLC-PK₁ cells subjected to high-glucose ambience (Fig. 5G). The treatment of 30 mM of D-glucose did not appreciably increase PKC-α activity compared that observed with low-glucose ambience. The high glucose-induced incremental effects on PKC-α activity and its protein content were notably reduced by the treatment with RSOR/MIOX-siRNA (Fig. 5, B and D). The intensity of PKC-α staining was also reduced in both the cellular compartments, but more so in the plasmalemma (Fig. 5H).

**Role of RSOR/MIOX on MAPK signal pathway.** Second, the molecules modulating the MAPK pathway were investigated since it has been demonstrated to be intricately involved in the pathogenesis of diabetic nephropathy. Overexpression of RSOR/MIOX in LLC-PK₁ cells increased the binding between Ras with Raf-1, as reflected by accentuated intensity of the band following immunoprecipitation and Western blotting procedures (Fig. 6A, arrowhead). No significant change in expression of total Ras or Raf-1 was observed. Concomitantly, there was an increased phosphorylation of p-ERK, while no significant change was noted in total ERK (Fig. 6C, arrowhead). Incubation with PKC inhibitors calphostin C (200 nM) or Gö6967 (1 μM), or sorbinil (10 μM) for 24 h partially reduced the Ras:Raf-1 association and phosphorylation of ERK (Fig. 6, A and C). The binding between Ras with Raf-1 and phosphorylation of ERK were not affected by the transfection of LLC-PK₁ cells with the RSOR/MIOX (A124K) mutant.

Incubation of LLC-PK₁ cells with high D-glucose for 24 h induced Ras:Raf-1 associations and phosphorylation of ERK without affecting total Ras, Raf-1, or ERK expression (Fig. 6, B and D, arrowheads). Such changes were not observed with D-glucose treatment. Treatment with RSOR/MIOX-siRNA reduced the high glucose Ras:Raf-1 binding and ERK phosphorylation (Fig. 6, B and D).

**Modulation of TGF-β1 expression and activity by RSOR/MIOX in LLC-PK₁ cells.** Since the PKC and MAPK signaling pathways modulate the activity of TGF-β, a cytokine that has been intimately linked to the pathogenesis of diabetic nephropathy, the status of TGF-β was thus investigated. ELISA revealed an increased secretion of TGF-β (792 ± 71 pg/mg
There was a mild increase in the activity with the transfection of the RSOR/MIOX (A124K) mutant. Like the TGF-β secretion into the media, calphostin, Gö6967, sorbinil, and zopolrestat treatments also significantly decreased the bioactivity of TGF-β bioactivity in LLC-PK₁ cells transfected with pcDNA3.1-RSOR/MIOX (Fig. 7B). However, exposure of pcDNA3.1-RSOR/MIOX transfectents with manumycin and PD98059 marginally affected TGF-β bioactivity (Fig. 7B).

Fig. 6. Effect of RSOR/MIOX overexpression and HG on Raf1:RAS association and ERK activity. Overexpression of RSOR/MIOX enhanced the binding between Ras with Raf-1, as indicated by increased intensity of the band following immunoprecipitation and Western blotting procedures (A, arrowhead). In parallel, there was increased phosphorylation of P-ERK (C, arrowhead). Incubation with PKC inhibitors calphostin C and Gö6967 or aldose reductase inhibitor sorbinil partially reduced the Ras:Raf-1 association and phosphorylation of ERK (A and C). The Ras:Raf-1 and phosphorylation of ERK were not significantly affected by the 124MU mutant. Similar to the transfection of pcDNA3.1-RSOR/MIOX, HG enhanced the Raf1:Ras association and ERK phosphorylation, and it was remarkably reduced with the RSOR-siRNA treatment (B and D). The transfection of cells or HG treatment had no significant effect on β-actin that served as a control.

Fig. 7. Effect of RSOR/MIOX and various inhibitors of PKC, aldose-reductase, Ras, and MEK, and of HG on transforming growth factor (TGF)β expression and activity. TGF-β expression and activity were increased following the pcDNA3.1-RSOR/MIOX transfection, which was almost twofold more than observed by the transfection pcDNA3.1 alone (380 ± 25.9 pg/mg protein) (Fig. 7A). The transfection of the (A124K) mutant also increased the TGF-β content in the media (503.8 ± 39.8 pg/mg protein), but it was significantly less than those of cells transfected with pcDNA3.1-RSOR/MIOX. Calphostin (200 nM), Gö6967 (1 μM), sorbinil (10 μM), and zopolrestat (10 μM) reduced the pcDNA3.1-RSOR/MIOX-induced increased TGF-β content in the culture media to 584 ± 44, 526 ± 55, 540 ± 39.2, and 541 ± 58 pg/mg protein, respectively (Fig. 7A). Exposure of pcDNA3.1-RSOR/MIOX transfectents with manumycin (an inhibitor of Ras farnesylation, 1 μM) and PD98059 (MEK1 inhibitor, 5 μM) had minimal effect on TGF-β content in the culture media (Fig. 7A).

The bioactivity of TGF-β was expressed as 100% in LLC-PK₁ cells transfected with pcDNA (Fig. 7B). With the overexpression of RSOR/MIOX, the activity more than doubled.
Exposure to LLC-PK₁ cells with 30 mM D-glucose for 24 h increased the amount of TGF-β1 (356.6 ± 37 → 633.8 ± 58 pg/mg protein) (Fig. 7C), and its bioactivity almost doubled in the culture media compared with that seen under low-glucose ambience (Fig. 7D). The T-glucose treatment resulted in a minimal increase in TGF-β expression and its activity. The siRNA of RSOR/MIOX notably reduced the D-glucose-induced increase in TGF-β1 bioactivity (Fig. 7, C and D).

Modulation of fibronectin, E-cadherin, and vimentin expression by inhibitors of various signaling pathways in LLC-PK₁ cells overexpressing RSOR/MIOX. The next set of experiments addressed the question of whether the various inhibitors of signaling pathways involved in diabetic nephropathy modulate pcDNA 3.1 RSOR/MIOX-induced fibronectin expression and whether the molecules such as E-cadherin and vimentin, reflective of altered phenotype (epithelial → mesenchymal type) of LLC-PK₁, are affected. Figure 1 (see above) illustrates that overexpression of RSOR/MIOX induces increased synthesis of fibronectin, as assessed by ELISA of the culture media. The transfection of the RSOR/MIOX (A124K) mutant also increased the secreted fibronectin (25.4 ± 2.4 ng/mg protein); however, it was comparatively much less than observed in the pcDNA3.1 RSOR/MIOX transfecents group (42.1 ± 3.1 ng/mg protein) (Fig. 8A). Calphostin (200 nM) and Gö6967 (1 μM), sorbinil (10 μM), and zopolrestat (10 μM) downregulated the RS/OR/MIOX-induced increased fibronectin expression to 24.1 ± 2.2, 23.9 ± 2.1, 25.0 ± 2.5, and 28.8 ± 2.7 ng/mg protein, respectively (Fig. 8A). Exposure of pcDNA3.1-RSOR/MIOX transfectents to manumycin (1 μM) and PD98059 (5 μM) also reduced the induced fibronectin expression to 31.4 ± 3.5 and 28.4 ± 2.3 ng/mg protein, respectively (Fig. 8A).

Overexpression of RSOR/MIOX decreased the protein expression of E-cadherin with a concomitant increase in the expression of vimentin in LLC-PK₁ cells (Fig. 8B, arrowheads). The PKC inhibitors calphostin (200 nM) and Gö6967 (1 μM), AR inhibitors sorbinil (10 μM) and zopolrestat (10 μM), and the inhibitors of the ERK signal pathway manumycin and PD98059 normalized the altered E-cadherin and vimentin expression that was induced by overexpression of RSOR/MIOX. A marginal decrease in E-cadherin and increase in vimentin was observed in the RS/OR/MIOX (A124K) mutant group compared with that of the empty vector pcDNA (−) group. Exposure LLC-PK₁ cells to high-glucose ambience (30 mM) for 72 h decreased E-cadherin expression and inversely increased that of vimentin in the presence of scrambled oligo compared with the low-glucose ambience (5 mM) group (Fig. 8C, arrowheads). This high-glucose-induced altered expression of E-cadherin and vimentin was normalized to a large extent when LLC-PK₁ cells were treated with RSOR-siRNA under high-glucose ambience (Fig. 8C).

DISCUSSION

The observations described in the previous section highlight two major aspects of this investigation. First, there are certain biological similarities between the well-characterized renal tubular enzyme AR that is critical in the pathogenesis of diabetic nephropathy and RS/OR/MIOX. Second, the changes in fibronectin expression under high-glucose ambience parallels those observed upon overexpression of RSOR/MIOX resulting in tubulointerstitial fibrosis, as seen during the progression of diabetic nephropathy in humans and animal models.

Tubulointerstitial fibrosis invariably contributes to renal dysfunctions, leading to end-stage renal failure seen in a wide variety of kidney diseases, including in diabetic nephropathy, the latter being the net result of derangements in a multitude of metabolic and signaling pathways. Conceivably, one of the signaling events initiated in response to high-glucose ambience is increased activity of the accessory polyol pathway in tubular epithelia with upregulation of AR, subsequent conversion of NAD⁺ to NADH, leading to a redox imbalance. (59) (Fig. 9). Incidentally, inhibition of the polyol pathway by specific AR inhibitors like sorbinil or zopolrestat had limited success in the amelioration of diabetic nephropathy, perhaps since there are other molecules and pathways that are involved in its pathogenesis. RS/OR/MIOX, a tubular enzyme, metabolizes myo-inositol and, like AR, it has been reported to be upregulated in the diabetic state, which apparently leads to an altered redox balance related to NAD⁺ and NADH interconversions during various steps in the RS/OR/MIOX metabolic pathway (42).
Furthermore, since the urinary excretion of myo-inositol, a substrate of RSOR/MIOX, significantly increases in patients with diabetes mellitus and murine models of diabetes (28, 30), this led us to investigate the mechanisms that could be relevant in the pathogenesis of renal tubulointerstitial fibrosis. An additional incentive to pursue these studies came from the fact that the increased expression of the ECM protein fibronectin paralleled that of RSOR in db/db mice (Figs. 2 and 3), a model of type 2 diabetes mellitus. An increase in ECM proteins and tubulointerstitial fibrosis has been observed in db/db mice at \( \sim 16 \) wk of age when hyperglycemia is well established in this murine model. (4, 53).

The first question that needed to be addressed was to establish a causal relationship between the expression of RSOR/MIOX and ECM fibronectin. Overexpression of RSOR/MIOX in LLC-PK\(_1\) cells by transfecting with pcDNA3.1-RSOR/MIOX resulted in an upregulated cellular expression of fibronectin along with the increased secretion into the culture medium (Fig. 1). The increase in fibronectin was comparable in LLC-PK\(_1\) cells treated with 30 mM glucose. Interestingly, high glucose-induced fibronectin expression was significantly reduced by the treatment with RSOR/MIOX siRNA, suggesting that the effects are specific and RSOR/MIOX is indeed causally linked to increased expression of fibronectin. With the establishment of this link, we proceeded to delineate the relevance of RSOR/MIOX in pathways/events that are affected by high-glucose ambience while analyzing its biological similarities/dissimilarities with AR.

It is well known that the altered redox balance and perturbations in the NADH/NAD\(^+\) ratio in a diabetic microenvironment play a role in the pathogenesis of diabetic nephropathy since it favors free radical generation and increased synthesis of DAG, which is an activator of conventional PKC isoforms (48). An increased cytosolic NADH/NAD\(^+\) ratio has been reported in high-glucose ambience with enhanced activity of glycolytic and sorbitol pathways in the retina (60). In the glucuronate-xylulose pathway RSOR/MIOX initiates the processing of myo-inositol. Conceivably, during the intermediary steps of the latter metabolic pathway, L-glucuronate dehydrogenase and D-xylulose reductase catalyze NAD\(^+\) to produce NADH, thus perturbing the redox balance (8, 23). In line with this notion are our findings which indicate an increased NADH/NAD\(^+\) ratio, as reflected by the increased lactate/pyruvate ratio in the culture media under high-glucose ambience (Fig. 4). A similar increase in the lactate/pyruvate ratio was observed with the RSOR/MIOX overexpression in LLC-PK\(_1\) cells, and this effect was not discernible with the transfection of the RSOR/MIOX 124MU mutant. These redox perturbations were significantly reduced by siRNA treatment, suggesting that the heightened activity of the myo-inositol metabolic pathway may alter the NADH/NAD\(^+\) ratio in diabetic nephropathy. Interestingly, the perturbed NADH/NAD\(^+\) ratio induced by overexpression of RSOR/MIOX was partially corrected by the treatment with AR inhibitors sorbinil and zopolrestat (Fig. 4). Most likely, this may be due to the inhibition of glucuronate reductase activity in the myo-inositol metabolic pathway, when one draws a parallel between the various intermediary steps of myo-inositol with polyol pathways. These observations suggest that these two pathways do perturb the cellular redox balance under high-glucose ambience with a similar conceivable final outcome, i.e., increased synthesis of fibronectin. Nevertheless, it seems necessary to do a comparative analysis between the activities of other signaling pathways that are affected by these two metabolic pathways to arrive at some consensus that the events leading to aberrant ECM fibronectin synthesis is indeed a convergent outcome. In this regard, the status of signaling molecules like PKC, ERK, Ras, Raf1, and the profibrogenic cytokine TGF-\(\beta\) was addressed in the next set of studies discussed in the following section.

As mentioned above, an increased NADH/NAD\(^+\) ratio leads to perturbation in the cellular redox, which induces the synthesis of DAG, an activator of conventional PKC. PKC, in turn, can induce transcription of various growth factors, including that of TGF-\(\beta\). The latter contains an AP-1 binding site in its promotor region, where proto-oncogenes like c-fos and c-jun can also bind and modulate transcription of ECM genes (11, 31), which would suggest a critical role of PKCs in the pathobiology of diabetic nephropathy (7). The PKCs include a dozen isoforms that are designated as conventional isoforms, including \(\alpha, \beta, \gamma\), and \(\gamma\); novel isoforms, including \(\delta, \epsilon, \eta, \) and \(\theta\); and atypical isoforms, including \(\lambda, \upsilon, \) and \(\xi\). These multiple isoforms of PKC have variable tissue distributions, biochemical regulations, and physiological functions that at times are cell specific. Many of them, including \(\alpha, \beta, \delta, \epsilon, \) and \(\xi\), have been reported to modulate hyperglycemia-induced renal injury, consequentially leading to fibrosis (21, 61). In addition, it has been shown that the activity of PKC parallels that of TGF-\(\beta\) and fibronectin protein expression, which can be blocked by inhibition of PKC (19, 52). Among various isoforms, PKC-\(\beta\) appears to be the major isozyme that is respon-
sible for the initiation of synthesis of ECM proteins in glomerular mesangial cells (61), while in renal proximal tubular cells PKC-α appears to be the dominant isoform that modulates their biology (37, 49). With this paradigm of cell specificity, the expression of PKC-α was investigated under high-glucose ambience and upon overexpression of RSOR/MIOX in LLC-PK₁ cells, a porcine cell line derived from proximal tubular cells. Both high glucose treatment and RSOR/MIOX overexpression increased the membrane translocation and activity of PKC-α (Fig. 5). This increase was inhibited by the specific RSOR/MIOX siRNA and AR inhibitors sorbinil and zopolres-tat, thus suggesting the relevance of PKC-α in the pathobiology of RSOR/MIOX, like that of AR, in hyperglycemia-induced cellular events in proximal tubular cells.

Other signaling pathways that have been described to modulate hyperglycemia-induced events include a number of phosphorylating enzymes that are commonly referred to as phosphorylating kinases. In this regard, the MAPK signaling pathway is considered to play a vital role in the pathogenesis of diabetic nephropathy (12, 15). Three of the subfamilies of MAPK, including ERK1/2, JNK, and p38 MAPK, have been well characterized, and their activities are known to be influenced by PKC or ROS (22, 44). For instance, upregulation of phosphorylated ERK in the renal tubules of diabetic rats and increased phosphorylation of ERK in LLC-PK₁ cells treated with high glucose have been reported in the literature (15).

Another set of signaling molecules that can conceivably modulate the activities of ERK include GTP binding proteins such as Ras or Rap, which via their association with the Raf family of kinases, can phosphorylate MEK and consequently MAPK and thereby increase the expression of ECM fibronectin in mesangial cells (34). In this study, overexpression of RSOR/MIOX increased the binding between Ras and Raf-1 and also enhanced the expression of the phosphorylated form of ERK1/2. In addition, the high glucose-induced activation of Ras and ERK1/2 or the Ras:Raf1 association was found to be reversed by RSOR/MIOX siRNA treatment of LLC-PK₁ cells. In addition, inhibitors of PKC (calphostin and Gö6967) or AR (sorbinil and zopolres-tat), and Ras (manumycin), suggesting a commonality with conventional signaling pathways activated by high-glucose ambience (Fig. 8). However, it is of interest here that RSOR/MIOX overexpression also altered the biochemical phenotype of the tubular cells. Like under high-glucose ambience, overexpression led to an increase in the expression of vimentin and a reciprocal decrease in E-cadherin, a marker of epithelial phenotype, suggesting an in vitro epithelial-mesenchymal transition (EMT) (Fig. 8). EMT has been regarded by some investigators as a process that is an antecedent of tubulointerstitial fibrosis (26). It is characterized by loss of cellular polarity, reduction of E-cadherin, ZO-1, and disarray of cytoskeleton, with concomitant acquisition of mesenchymal markers like α-smooth muscle actin (α-SMA), FSP-1, N-cadherin, and vimentin (45, 65). Interestingly, expression of both E-cadherin and vimentin was reversed with the treatment of inhibitors of high glucose-induced signaling pathways and RSOR/MIOX siRNA. This phenotypical change observed was regarded as specific since it was not seen with the transfection of the RSOR/MIOX 124MU mutant, in which the region spanning the residues that regulate the activity of this enzyme are apparently localized (5). Intriguingly, one of the major markers of EMT, α-SMA was not detected in RSOR/MIOX-overexpressing cells (not shown). It is conceivable that LLC-PK₁ cells are not amenable to such a phenotypical change since treatment with very high dosages of TGF-β (ng/ml instead of pg/ml) or its direct transfection has been shown to induce α-SMA expression (36).

To sum up, this communication compares the biological properties of RSOR/MIOX to another renal tubular enzyme, AR, which is known to play a role in the pathogenesis of...
diabetic nephropathy. In addition, the study underscores the relevance of this enzyme in the pathobiology of ECM, which is usually an eventual outcome in progression of diabetic nephropathy leading to end-stage renal disease. Finally, it is anticipated that the findings of this study would yield impetus for future investigations highlighting the role of RSOR/MIOX in the pathogenesis of diabetic nephropathy affecting the tubulointerstitial compartment of the kidney.

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


