Diabetic nephropathy (DN) is characterized by mesangial cell (MC) hypertrophy and progressive accumulation of glomerular extracellular matrix (ECM). It was reported recently that 12/15-lipoxygenase (12/15-LO) expression is increased in high-glucose (HG)-stimulated MC and in experimental DN. 12-LO products could also directly induce MC hypertrophy and ECM expression and mediate growth factor effects, thus implicating the 12/15-LO pathway in DN. Because TGF-β is a major player in the pathogenesis of DN, whether there is an interplay between the TGF-β and 12/15-LO pathways in MC was evaluated.

Treatment of rat MC (RMC) with TGF-β significantly increased levels of the 12/15-LO product 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] and also 12/15-LO mRNA and protein expression. HG-induced TGF-β mRNA expression in RMC was inhibited by a specific ribozyme and siRNA targeted to knockdown rat 12/15-LO. It is interesting that direct treatment of RMC with 12(S)-HETE increased TGF-β mRNA and protein levels, as well as p-Smad2/3, which are TGF-β-specific target transcription factors. 12(S)-HETE also increased transcription from a minimal TGF-β promoter. Furthermore, TGF-β expression and p-Smad2/3 levels were lower in MC from 12/15-LO knockout mice relative to control mice. Reciprocally, mouse MC stably overexpressing 12/15-LO had greater TGF-β mRNA and also nuclear p-Smad2/3 relative to mock-transfected cells. 12/15-LO and TGF-β could functionally signal and increase ECM expression via the p38 mitogen-activated protein kinase signaling pathway. These results indicate for the first time that the 12/15-LO and TGF-β pathways can cross-talk and activate each other. These novel interactions may amplify the signal transduction cascades and molecular events that lead to DN.

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nal disease, very little is known regarding the role of lipids in mediating TGF-β actions or its expression.

Lipoxygenases (LO) are a family of nonheme iron-containing enzymes that insert molecular oxygen into polyunsaturated fatty acids such as arachidonic and linoleic acids (21,22). They are classified as 5-, 8-, 12-, and 15-LO on the basis of the carbon atom of arachidonic acid at which oxygen is inserted (21,22). 12-LO activation can lead to the formation of oxidized lipids such as 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] (21). Human and rabbit 15-LO as well as the leukocyte-type 12-LO have high homology and are classified as 12/15-LO (especially in rat and mouse) because they can form both 12(S)-HETE and 15(S)-HETE from arachidonic acid (21,22). Recently, there has been heightened interest in 12/15-LO pathway because of key data implicating it in the pathogenesis of atherosclerosis, restenosis, and hypertension (23–25).

Increased urinary 12(S)-HETE excretion was noted in patients with diabetes, suggesting a renal origin (26). 12/15-LO is present in the kidney (27,28), and recent data indicate its potential significance and function. Thus, 12/15-LO mRNA and protein are increased in glucose-stimulated MC and in experimental DN (27). Furthermore, in vivo expression of the matrix protein FN and TGF-β in diabetic rat glomeruli were associated with increased glomerular 12/15-LO expression (27), thereby implicating the 12/15-LO pathway in the pathogenesis of DN. Factors that are relevant to the pathogenesis of DN, such as HG, angiotensin II (Ang II), and PDGF, increased leukocyte-type 12/15-LO activity and expression in rat MC (RMC) and vascular smooth muscle cells (VSMC) (27,29–31). The growth-promoting and matrix-inducing effects of Ang II in MC were blocked by pharmacologic 12-LO inhibitors as well as a novel 12-LO ribozyme (31). Furthermore, 12(S)-HETE could directly induce cellular hypertrophy and expression of the ECM protein FN in RMC with similar potency as Ang II (31). 12(S)-HETE-induced effects were mediated at least in part by p38 MAPK and its target transcription factor, cAMP response element binding protein (31,32).

We also recently demonstrated the potential in vivo functional role of 12/15-LO in MC growth related to glomerulosclerosis and nephropathy by comparing the properties of mouse MC (MMC) derived from 12/15-LO knockout (LOKO) mice with those obtained from genetic control wild-type mice (WT) (33). Cell Culture

Primary cultures of RMC from Sprague-Dawley rats were obtained and cultured as described previously (27). Cells between 5 and 12 passages were used. An immortalized MMC cell line was cultured as described (33). Primary cultures of MMC from genetic control (C57BL/6, WT) and leukocyte-type 12/15-LOKO mice (Jackson Laboratory; strain B6.129S2-Alox15tm1Fun) were obtained as described (33) and according to a protocol approved by the Institutional Research Animal Care Committee.

12(S)-HETE Assay

Serum-depleted RMC were treated with recombinant human TGF-β (10 ng/ml) for 30 min or 1 h. Culture dishes were cooled, and the supernatants were aspirated and saved at −70°C. Cell pellets were lysed and deacetylated. 12(S)-HETE levels in cell extracts and supernatants were quantitated by a specific RIA as described previously (29,34).

RNA Isolation and RT-PCR

Isolation of total RNA and RT-PCR using gene-specific primers along with 18S RNA primers as internal standards were performed as described previously (31). The following gene-specific primers were used in the RT-PCR reactions: Rat 12/15-LO (290-bp product): sense 5'-ATT GCA TC3 and antisense 5'-GGA GCC AC-3'; mouse TGF-β1 (300-bp product): sense 5'-CAA CGC CAT CTA TGA GAA ACC A-3' and antisense 5'-AAC CTG AAG AGA CC-3'; rat collagen I (413-bp product): sense 5'-GGA GTC GGG ACC ACC ATC C-3' and antisense 5'-ATT CCG TCT CGT C-3'; rat collagen I (413-bp product): sense 5'-GGT AAC CGG GCA AAC AAG GT-3' and antisense 5'-GAG ACC AGC AGA GAA GCC AC-3'; rat FN (446-bp product): sense 5'-GCA AGG CTC AAG CTC AGA AGA AGA C-3' and antisense 5'-CTT GGT GGC TCT ATG ATT GTA C-3'.

Western Blotting

Cells were lysed in Laemmli’s sample buffer, fractionated on 10% SDS-PAGE gels (Bio-Rad, Hercules, CA), and immunoblotted with antibodies to 12/15-LO (1:400) (30,33), phospho-p38 MAPK (1:1000), phospho-ERK1/2 (1:1000), or phospho-Smad2/3 (1:200) as described (31,32). The blots were stripped and then reprobed with an antibody to β-actin (1:5000), total p38 MAPK (1:1000), total ERK1/2 (1:1000), or...
Smad2/3 (1:200). Immunoblots were scanned using GS-800 densitometer, and protein bands were quantified with Quantitation One software (Bio-Rad).

**Immunofluorescence**

Confluent MMC on glass slides were fixed with 2% paraformaldehyde in PBS for 15 min. Cells were permeabilized with 0.2% Triton-X100 in PBS and washed. For reducing nonspecific binding, cells were treated with 5% goat serum in PBS and then incubated with 1:40 p-Smad2/3 antibody, followed by incubation with rhodamine-conjugated secondary antibody (1:200) and viewed using a fluorescence microscope.

**Transfection of Rat 12/15-LO Ribozyme**

RMC were plated in 60-mm culture dishes and transfected the next day (80% confluent) with a chimeric DNA-RNA hammerhead ribozyme (Rz) targeted to cleave rat leukocyte-type 12/15-LO (Rz) or control catalytically inactive mutant 12/15-LO Rz (mRz) oligonucleotides using Lipofectamine 2000 as described (31). After 6 h, cells were washed and fresh medium that contained 1% FCS was added. The next day, cells were placed in serum-free RPMI 1640 medium that contained 0.2% BSA and 5.5 mM glucose (normal glucose) or 25 mM glucose (high glucose [HG]). Twenty-four hours later, total RNA was extracted and TGF-β mRNA levels were determined by RT-PCR.

**Construction of Rat Leukocyte Type 12/15-LO–Specific Short Hairpin RNA Vectors and Transfection**

We also used RNA interference initiated by small interfering RNA (siRNA) to effectively suppress 12/15-LO gene expression. For more stable suppression of gene expression, we constructed Pol III U6 promoter-driven short hairpin RNA (shRNA) targeting rat leukocyte-type 12/15-LO using a modification of a protocol that uses a PCR-based strategy for rapid screening of siRNA accessibility sites (35,36). Briefly, the siRNA target sequences on rat 12/15-LO mRNA are GCAACTGGATTTCTGTGAAGG (m233) and GAAGCGGATTTCTCCTTCTG (m826). We used a combination of shRNA to both of these sites because we obtained better gene suppression with two together than individually as also reported for other genes (35,36). The final PCR products included the U6 promoter, followed by 21nt sense siRNA sequence, 9nt hairpin, and 21nt antisense sequence. For PCR, the vector pTZU6 + 1 was used as template. The universal U6 forward primer was 5'-GGAAGATCTGGATCCAAGGTCGGGCAGG-5', and the reverse primers were 5'-CCAAGCTTCCCGGGAAAAAAGCAA-CTGGATTTCTGGAAGGCTACACAACTCCATGATTCTGCTGCTTCCTTCTCC-3' (for m233) and 5'-CCAAGCTTCCCGGGAAAAAAGCAA-CTGGATTTCTGGAAGGCTACACAACTCCATGATTCTGCTGCTTCCTTCTCC-3' (for m826). These PCR products were inserted into pCR3.1 vector to generate the shRNA (pCR3.1-m233 or -m826 siRNA) first and then an EGFP fragment was inserted into these pCR3.1-shRNA vectors to track transfection efficiency and facilitate cell sorting. This was done by first digesting the vector pEGFP-N1 at Asel and Afl II sites followed by ligation of the CMV promoter and EGFP fragments to Hinc II and SacI sites of pCR3.1-shRNA to generate the pCR3.1/EGFP shRNA vectors. We also used GGATATATTCCCGAACTAGACA sequence to make a control scrambled shRNA unrelated to any gene. The empty vector has everything except siRNA. RMC were transfected with the shRNA using Lipofectamine 2000 reagent. Forty-eight hours after transfection, the cells were selected with 400 μg/ml G418 for 1 wk.

**Transient Transfection and Reporter Gene Assays**

Immortalized MMC were plated in 12-well plates and transfected the next day with 1 μg/well TGF-β1-Luc promoter construct (pA835) (6) using Lipofectamine 2000. Cells then were allowed to recover overnight in serum that contained medium and then transferred to serum-free medium that contained 0.2% BSA. The next day, they were stimulated with 12(S)-HETE (0.1 μM). Cells then were lysed, and luciferase activities were assayed as described (32,33).

**ELISA**

Cell supernatants were frozen at −20°C until assay by a sandwich TGF-β ELISA kit according to the manufacturer’s specifications. Acid activation of supernatants of MMC was first performed to convert latent TGF-β into active TGF-β form that can be recognized by the antibody. Total (latent + active) TGF-β in a sample was compared with known standards and read as ng/ml.

**Statistical Analyses**

Data are expressed as mean ± SEM of multiple experiments. Paired t tests were used to compare two groups, or ANOVA with Dunnet’s post test for multiple groups using PRISM software (Graph Pad, San Diego, CA). Statistical significance was detected at the 0.05 level.

**Results**

**TGF-β Induces 12/15-LO mRNA and Protein Expression in RMC**

Quiescent MC were stimulated with TGF-β for 0 to 24 h, and 12/15-LO mRNA expression was determined by RT-PCR. Figure 1A shows that TGF-β (10 ng/ml) treatment can induce 12/15-LO mRNA expression in RMC by 6 h, and this remains
sustained for 24 h. 12/15-LO transcript levels were quantified as the ratio of density of 12/15-LO to 18S band and depicted by the bar graph in Figure 1B. To determine whether this increase in 12/15-LO mRNA was associated with an increase in 12/15-LO protein expression, we performed immunoblotting using 12/15-LO antibody. Figure 1C shows that TGF-β treatment for 8 h increased 12/15-LO protein levels in RMC even at 1 ng/ml.

**TGF-β Increases Levels of the 12/15-LO Product 12(S)-HETE in RMC**

We next examined whether TGF-β alters 12/15-LO activity by examining levels of cell-associated and released 12/15-LO product, 12(S)-HETE by a RIA specific to 12(S)-HETE (29). Figure 2 shows that TGF-β treatment significantly increased the levels of cell-associated 12(S)-HETE and released 12(S)-HETE in cell supernatants, respectively, in RMC at 30 min as well as 1 h. Thus, TGF-β increases 12/15-LO activity by 30 min to 1 h, whereas protein and mRNA expression are increased by 6 h.

**12(S)-HETE Induces TGF-β mRNA Expression in RMC**

We hypothesized that 12/15-LO activation can in turn increase TGF-β expression. Therefore treated RMC with 12(S)-HETE for time periods ranging from 1 to 24 h and examined rat TGF-β mRNA expression by RT-PCR. Figure 3A shows that 12(S)-HETE can increase TGF-β mRNA expression by 2 h, and this remains sustained for 24 h. Bar graph quantification is seen in Figure 3B. We also confirmed that this effect of 12(S)-HETE was specific because a stereoisomer, 12(R)-HETE, which is not a LO product, did not increase TGF-β mRNA expression (Figure 3C).

**12(S)-HETE Increases the Transcriptional Activity of TGF-β1 Promoter in MMC**

Because 12(S)-HETE increased TGF-β mRNA levels, we next examined whether 12(S)-HETE could also increase the transcriptional activity of the TGF-β1 promoter. MMC were transiently transfected with a plasmid pA835, which contains the luciferase gene under the control of a minimal TGF-β1 promoter (6) and were left untreated or stimulated with 12(S)-HETE or HG for 18 h. Figure 4 shows that 12(S)-HETE could induce the transcriptional activity of TGF-β1 promoter as demonstrated by significant increase in luciferase activity. HG also increased the transcriptional activity as expected. Furthermore, the effects of HG and 12(S)-HETE together were also synergistic indicating a potential cross-talk between the HG and 12(S)-HETE activated pathways leading to TGF-β transcription.

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**Figure 2. Effect of TGF-β on formation of the LO product 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] in RMC.** Serum-depleted cells were left alone or treated with TGF-β for 30 min or 1 h. Cell-associated 12(S)-HETE in cell lysates (A) and cell-released 12(S)-HETE in culture supernatants (B) were extracted, and levels were quantified by a specific RIA. The results were expressed as pg/10⁶ cells. Data represent mean ± SEM of three independent experiments (A: *P < 0.01 versus Ctrl; **P < 0.05 versus 1 h Ctrl, B: *P < 0.01 versus 30 min Ctrl and 1 h Ctrl).

**Figure 3. 12(S)-HETE induces TGF-β mRNA expression in RMC.** (A) Quiescent RMC were treated with 12(S)-HETE for indicated time periods, and expression of TGF-β mRNA was determined by relative RT-PCR using 18S RNA as an internal control. (B) The ratio of TGF-β mRNA to internal standard 18S RNA was determined by densitometric analysis. There was significant increment in TGF-β/18S mRNA in RMC that were exposed to 12(S)-HETE from 2 to 24 h. Data represent mean ± SEM of three independent experiments (*P < 0.01 versus Ctrl). (C) RMC were treated with 12(S)-HETE or 12(R)-HETE (0.1 μM each) for 24 h, and TGF-β mRNA expression was analyzed by RT-PCR.
Inhibition of HG Induced TGF-β mRNA Expression by a 12/15-LO Ribozyme or 12/15-LO shRNA in RMC

We have previously shown that HG culture of RMC significantly increased 12/15-LO activity and expression (27). Evidence also shows that hyperglycemia in RMC and human and experimental DN are associated with enhanced production and action of TGF-β (14,37,38). We therefore hypothesized that HG-induced TGF-β may be mediated at least in part by 12/15-LO activation. To test this, we used a novel short hammerhead Rz as a molecular tool to cleave and inactivate rat 12/15-LO (31). Control for the Rz was an mRz with a point mutation at the catalytic site. RMC were transfected with the Rz or the mRz and then treated with HG (25 mM) for 24 h. Results shown in Figure 5A indicate that the 12/15-LO Rz could clearly reduce HG-induced rat TGF-β mRNA expression by >50%. The control mRz, however, had no effect.

Furthermore, we also used novel U6 promoter-driven shRNA to evaluate this further. We designed and tested siRNA to rat 12/15-LO using a novel rapid PCR method. The siRNA were cloned into pCR3.1 to obtain shRNA expression vectors. RMC were transfected with 12/15-LO shRNA to two different sites (m223 and m826), which we found had the optimum gene knockdown effects. We confirmed that 12/15-LO shRNA but not scrambled control can effectively knock down 12/15-LO protein expression in RMC (Figure 5B). Results shown in Figure 5, C and D, indicate that the 12/15-LO shRNA significantly reduced HG-induced TGF-β mRNA in RMC. These new results suggest that HG-induced TGF-β expression is mediated, at least in part, by the 12/15-LO pathway.

12(S)-HETE Treatment Increases Smad2/3 Phosphorylation in RMC

Because 12(S)-HETE could increase TGF-β expression, we anticipated that it should also increase the activity of Smad2/3, which are TGF-β–specific transcription factors. RMC were treated with 12(S)-HETE for various time periods, and Smad2/3 phosphorylation was evaluated by immunoblotting with an antibody that recognizes p-Smad2 and 3. 12(S)-HETE (0.1 μM) could increase p-Smad2/3 levels in a time-dependent manner, peaking at 6 h and declining by 24 h (Figure 6A, top). No change in total
Smad2/3 was seen under these conditions (Figure 6A, bottom). The ratio of pSmad2/3 and total Smad2/3 in control samples and cells stimulated with 12(S)-HETE for 6 h is shown in Figure 6B.

**Comparison of TGF-β and Phospho-Smad2/3 Levels in MMC Derived from WT versus 12/15-LOKO Mice**

Because LO products could increase TGF-β expression and MMC overexpressing 12/15-LO had elevated TGF-β levels, we next hypothesized that MMC derived from LOKO mice would have lower levels of TGF-β than those from control WT mice.

**Role of MAPK Activation in Mediating 12(S)-HETE and TGF-β Effects in RMC**

We next examined the role of specific common signal transduction mechanisms by which 12(S)-HETE and TGF-β exert...
their effects and potentially mediate a cross-talk. We therefore examined the activation of key growth- and stress-related MAPK. Serum-depleted RMC were treated with 12(S)-HETE (0.1 μM) or TGF-β (10 ng/ml) for various time periods, and MAPK activation was determined by Western blotting with phosphospecific antibodies that recognize only the activated kinases. As shown in Figure 9A, a marked increase in p38 MAPK activation could be seen within 1 h after stimulation with 12(S)-HETE and remained elevated until 24 h. However, 12(S)-HETE did not significantly activate ERK1/2. These results demonstrate that 12(S)-HETE is a potent activator of p38 MAPK but not ERK1/2 in RMC. As shown in Figure 9B, TGF-β in turn could increase both p38 MAPK and ERK1/2 activation from 1 to 6 h.

Given that 12(S)-HETE increased both p38 MAPK phosphorylation and TGF-β mRNA expression, we posed the question of whether the p38 MAPK pathway was involved in mediating 12(S)-HETE-induced TGF-β mRNA expression. Serum-depleted RMC were pretreated with the p38 MAPK inhibitor SB202190 (10 μM) for 30 min and then stimulated with 12(S)-HETE for 24 h. SB202190 significantly inhibited the activation of p38 MAPK by 12(S)-HETE (data not shown). In the presence of SB202190, 12(S)-HETE–induced increase in TGF-β mRNA was clearly attenuated (Figure 10A, top). Furthermore, we examined whether MAPK activation plays a functional role in ECM accumulation by 12(S)-HETE. We observed that 12(S)-HETE increased mRNA expression of the ECM proteins collagen I and FN, and these were blocked by the p38 MAPK inhibitor SB202190 (Figure 10A, middle and bottom).

Next, we evaluated whether the p38 MAPK and ERK1/2 pathways were involved in mediating TGF-β–induced 12/15-LO expression. In the presence of SB202190 (10 μM) or the ERK pathway inhibitor PD98059 (10 μM), TGF-β–induced 12/15-LO mRNA expression was markedly attenuated (Figure 10B, top). Furthermore, TGF-β–induced collagen I and FN mRNA expression were inhibited by both SB202190 and PD98059 (Figure 10B, middle and bottom). SB202190 or PD98059 alone had no effect. These results support the operation of a p38 MAPK-dependent cross-talk between the two pathways.

Inhibition of 12(S)-HETE–Induced Collagen α1(I) and FN mRNA Expression by Neutralization with a TGF-β Antibody, and Inhibition of TGF-β–Induced Collagen α1(I) and FN mRNA Expression by 12/15-LO shRNA

We next examined whether 12(S)-HETE–induced collagen α1(I) and FN expression can be blocked by neutralization with a TGF-β antibody in RMC. RMC were serum-depleted for 48 h and then stimulated with 12(S)-HETE for 24 h after 1 h of preincubation with pan-specific TGF-β antibody (25 μg/ml) or neutralizing antibody. Given the importance of p38 MAPK in mediating 12(S)-HETE–induced collagen I and FN expression, we evaluated whether the p38 MAPK inhibitor SB202190 would block TGF-β–induced collagen I and FN mRNA expression. As shown in Figure 10C, SB202190 significantly inhibited the activation of both p38 MAPK and ERK1/2 from 1 to 6 h.

Figure 8. TGF-β expression and p-Smad2/3 activities in WT and 12/15-LOKO MMC. MMC from WT and 12/15-LOKO were serum-depleted for 48 h and then stimulated with FCS (10%) for 24 h. (A) TGF-β protein was measured by ELISA in supernatants of cells that were unstimulated (Basal) or stimulated with 10% FCS (FBS). (B) Cells were used to extract RNA for the determination of mouse TGF-β mRNA expression by RT-PCR. (C) p-Smad2/3 activity was examined by immunoblotting of cell lysates with anti–p-Smad2/3 or Smad2/3 antibodies. (D) Graphical presentation shows that the ratio of phosphorylated to total Smad2/3 was significantly decreased in LOKO cells compared with control cells. Data represent mean ± SEM of four independent experiments (*P < 0.01 versus Ctrl).

Figure 9. Effect of 12(S)-HETE or TGF-β on p38 mitogen-activated protein kinase (MAPK) and ERK1/2 phosphorylation in RMC. Quiescent RMC were treated in serum-free medium with 12(S)-HETE (A) or TGF-β (B) for various time periods, and p38 MAPK and ERK1/2 phosphorylation were determined by Western blotting with phosphospecific antibodies (p-38 and p-ERK1/2). Blots were stripped and reprobed using total p38 and ERK1/2 antibodies as internal controls. Data shown are representative of three similar experiments.
control IgG. Figure 11A shows that in 12(S)-HETE–treated cells, collagen α1(I) and FN mRNA expression were increased by 24 h, and this was clearly attenuated by pretreatment with the TGF-β antibody but not control IgG. These results suggest that 12(S)-HETE can induce collagen α1(I) and FN mRNA expression in RMC through TGF-β signaling. The former may be mediated by Smad, whereas MAPK may be involved in the latter (13,17). Furthermore, Figure 11B shows that in TGF-β–treated cells, collagen α1(I) and FN mRNA expression were increased by 24 h, and this was clearly attenuated by pretreatment with 12/15-LO shRNA but not control scrambled shRNA (Scr). These results suggest that TGF-β can induce collagen α1(I) and FN mRNA expression in RMC at least in part through the 12/15-LO pathway.

Discussion

The pathogenesis of DN involves hyperglycemia and growth factor–induced cellular hypertrophy, increased production of ECM proteins, and decreased production of matrix-degrading proteinases. However, much less is known about the role played by lipids that are released by growth factors. TGF-β plays a pivotal role in cellular growth and ECM production, and many of its actions in renal cells resemble those of high ambient glucose in the kidney. We recently demonstrated that the 12/15-LO pathway of arachidonate metabolism was enhanced in experimental DN in rats and correlated with expression of the ECM protein FN (27). 12/15-LO expression and products were also increased in HG-stimulated MC (27). Furthermore, LO products such as 12(S)-HETE could directly increase cellular hypertrophy and FN production in RMC (31). Thus, both 12/15-LO pathway products and TGF-β can mediate cellular growth, ECM production, and HG effects in MC. The present study provides the first evidence of an interaction and cross-talk between the actions of oxidized lipids of the 12/15-LO pathway and TGF-β in MC resulting in a potential feedback/feedforward amplifying loop in glomerulosclerosis.

We observed for the first time that TGF-β can increase the formation of the LO product 12(S)-HETE and also augment 12/15-LO protein and mRNA expression in MC and thus directly activate the 12/15-LO pathway. Evidence shows that other growth factors, such as Ang II and PDGF, can induce 12/15-LO expression and activity in MC and VSMC (29–32). Furthermore, Ang II–induced effects in MC such as hypertrophy and FN expression were mediated, at least in part, by 12/15-LO activation (31). These data indicate that oxidized bioactive lipids such as those generated by the 12/15-LO pathway may mediate the effects of TGF-β as well as other growth factors and thereby play a key role in the pathogenesis of DN.

We observed that the 12-LO product 12(S)-HETE can increase the protein and mRNA expression of TGF-β and also induce TGF-β promoter transcriptional activity. Furthermore, 12(S)-HETE increased phosphorylation of Smad2/3. This coincided temporally with the time that TGF-β expression was induced...
by 12(S)-HETE. The effects of 12(S)-HETE were specific because an isomer, 12(R)-HETE, which is not a LO product, did not alter TGF-β expression. 12(S)-HETE–induced increase in the transcriptional activity of TGF-β was comparable to the effects of HG; furthermore their effects were synergistic, suggesting that HG and 12(S)-HETE may operate through some similar as well as synergistic pathways. The specific molecular mechanisms and promoter elements involved in 12(S)-HETE–induced TGF-β expression are not yet clear and will be the focus of future studies.

Further evidence of TGF-β regulation by 12/15-LO was obtained by data showing that MMC overexpressing mouse 12/15-LO had increased levels of TGF-β mRNA as well as p-Smad2/3 compared with mock-transfected cells. These LO-overexpressing MMC also had elevated levels of FN mRNA (33). Reciprocally, MMC derived from LOKO mice had reduced levels of TGF-β relative to those from WT mice. This is supported by our recent data showing that MMC from these LOKO mice produced significantly lower levels of 12(S)-HETE and also markedly decreased FN expression (33). Overall, these results suggest that 12/15-LO pathway may contribute to renal dysfunction via activation of the TGF-β signaling pathway.

In this study, we showed for the first time that 12(S)-HETE can also induce the expression of another key TGF-β-regulated ECM protein, namely α1 chain of type 1 collagen [collagen α1(I)] that is a downstream target gene of Smad. It is interesting that 12(S)-HETE–induced collagen α1(I) and FN expression were blocked by a TGF-β–neutralizing antibody. We then examined whether the effect of TGF-β on collagen α1(I) and FN expression can be attenuated in 12/15-LO shRNA transfected MCs. The recently developed technique of RNA interference (RNAi) mediated by siRNA and shRNA is powerful because it can investigate specific functions of a target gene that cannot be studied easily with knockout mouse models (39–41). We noted that TGF-β–induced collagen α1(I) and FN expression were attenuated by the 12/15-LO shRNA in MC compared with scrambled shRNA transfected cells. These results indicate the operation of a loop mechanism wherein 12(S)-HETE can stimulate collagen α1(I) and FN mRNA expression through activation of TGF-β signaling, whereas TGF-β can stimulate collagen α1(I) and FN mRNA expression through 12/15-LO pathway. The former may be mediated by Smad, whereas MAPK may be involved in the latter because evidence shows that collagen is a key target of Smad whereas TGF-β–induced FN is Smad independent (13,17). This cross-talk can further amplify ECM protein expression.

To evaluate the interactive signaling mechanisms involved, we examined the effect of 12(S)-HETE and TGF-β on MAPK activation. Our recent data implicated p38 but not ERK1/2 MAPK activation in 12(S)-HETE–induced FN expression in MC (31). Because TGF-β and HG can also activate p38 MAPK in MC (42,43), we reasoned that p38 MAPK activation may be a key focal point of integration of signals from 12(S)-HETE, TGF-β, and possibly HG. In this study, we observed that 12(S)-HETE could directly activate p38 MAPK but not ERK1/2, whereas TGF-β could stimulate both p38 MAPK and ERK1/2 activation as reported earlier (17,43,44). Moreover, a specific chemical inhibitor of p38 MAPK, SB202190, blocked 12(S)-HETE–induced TGF-β, collagen α1(I), and FN expression. The ERK1/2 pathway–specific inhibitor PD98059 and p38 MAPK inhibitor SB202190 inhibited TGF-β–induced 12/15-LO, collagen α1(I), and FN expression. Thus, MAPK seem to play important roles in mediating TGF-β and 12/15-LO interaction and ECM accumulation.

To evaluate further the functional significance in DN, we examined the consequences of 12/15-LO blockade on HG-induced TGF-β mRNA expression by using a novel Rz targeted to cleave rat 12/15-LO and also tested the 12/15-LO shRNA. Rz are RNA enzymes that catalytically cleave specific RNA sequences, resulting in irreversible inactivation of the target RNA (45,46). We showed earlier that a Rz directed to cleave rat leukocyte-type 12/15-LO was effective in vitro in VSMC and in vivo in a rat model of neointimal thickening (47). Furthermore, a modified new generation “short” Rz to rat 12/15-LO could attenuate Ang II–induced FN expression in RMC (31). In this study, we showed that the “short” 12/15-Rz but not mRz could inhibit HG-induced TGF-β mRNA expression in RMC. Similarly, new 12/15-LO shRNA also inhibited HG-induced TGF-β mRNA expression. These results suggest that HG-induced TGF-β is mediated, at least in part, by the 12/15-LO pathway and also illustrate the utility of Rz and shRNA to reduce expression of genes related to the pathogenesis of DN.

Although we mainly used MC in these studies, 12/15-LO and TGF-β interactions may occur in other relevant renal cells, such as podocytes. This is supported by the recent report that increased podocyte 12/15-LO expression is also observed in experimental DN and in HG-stimulated cultured podocytes (48). In summary, our study demonstrates a novel interaction between 12/15-LO and TGF-β actions in mediating MC matrix deposition and glomerulosclerosis associated with DN. HG- or diabetes-induced TGF-β and 12/15-LO activation may influence each other to amplify downstream signaling pathways and gene expression. Thus, new strategies aimed at combined blockade of TGF-β as well as 12/15-LO may yield optimal therapeutic benefits.

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