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Differential behavior of mesangial cells derived from 12/15-lipoxygenase knockout mice relative to control mice[1]

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Differential behavior of mesangial cells derived from 12/15-lipoxygenase knockout mice relative to control mice.

Background. The 12/15-lipoxygenase (12/15-LO) enzyme has been implicated in the pathogenesis of diabetic nephropathy since lipoxygenase products induce cellular hypertrophy and extracellular matrix deposition in mesangial cells. In this study, in order to determine the potential in vivo functional role of 12/15-LO in kidney disease, we compared mouse mesangial cells (MMCs) derived from 12/15-LO knockout mice with those from genetic control wild-type mice.

Methods. MMCs were isolated from wild-type and 12/15-LO knockout mice. Cellular growth, activation of mitogen-activated protein kinases (MAPKs), transcription factors, superoxide levels, and fibronectin expression were compared in the two cell types.

Results. Levels of the 12/15-LO product and protein were lower in MMC from 12/15-LO knockout relative to wild-type. MMCs from 12/15-LO knockout mice grew slower than wild-type cells, and also showed lower rates of tritiated thymidine and leucine incorporation (21% and 15% of wild-type, respectively, $P < 0.001$). Levels of superoxide and the matrix protein fibronectin were also lower in 12/15-LO knockout mice cells. Serum and angiotensin II (Ang II)-stimulated activities of p38 or ERK1/2 MAPKs, and cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) transcription factor were lower in 12/15-LO knockout relative to wild-type cells. In addition, DNA binding and transcriptional activities of activated protein-1 (AP-1) and CREB were lower in 12/15-LO knockout cells. Furthermore, stable 12/15-LO overexpression in MMC led to reciprocal increase in p38 MAPK activation and fibronectin expression.

Conclusion. The differential activation of oxidant stress, specific signaling pathways, transcription factors, and growth and matrix genes may lead to reduced growth and growth factor responses in 12/15-LO knockout versus wild-type MMCs. These results provide ex vivo functional evidence for the first time that 12/15-LO activation plays a key role in mesangial cell responses associated with renal diseases such as diabetic nephropathy.

[1]**See Editorial by Kasinath, p. 1918.**

Key words: diabetic nephropathy, 12/15-lipoxygenase, mouse mesangial cells, mitogen-activated protein kinases, growth factor, transcription factor.

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Diabetic nephropathy is characterized by the thickening of glomerular basement membranes, increased cellular hypertrophy and deposition of extracellular matrix in mesangial areas [1, 2]. Studies indicate that these renal pathologies can be mediated by the action of hyperglycemia, advanced glycation end products, oxidant stress, several growth factors such as angiotensin II (Ang II), platelet-derived growth factor (PDGF), and transforming growth factor-beta (TGF-β) [3–8]. However, the molecular and cellular mechanisms involved in the pathogenesis of diabetic nephropathy are not very clear.

Lipoxygenases (LO) are a family of nonheme iron-containing enzymes that insert molecular oxygen into polyunsaturated fatty acids such as arachidonic and linoleic acids [9, 10]. They are classified as 5-, 8-, 12-, and 15-LOs according to the carbon atom of arachidonic acid at which the oxygen is inserted. 12-LO activation can lead to the formation of oxidized lipids such as 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] [9, 10]. Three major isoforms of 12-LO are platelet-type 12-LO, macrophage- or leukocyte-type 12-LO (12/15-LO), and epidermal-type 12-LO [9–11]. Human and rabbit 15-LOs as well as the leukocyte-type 12-LO have high homology and are classified as 12/15-LO since they can form both 12(S)-HETE and 15(S)-HETE from arachidonic acid and mainly 13(S)-hydroperoxyoctadecadienoic acid from linoleic acid [9–11]. The presence of leukocyte-type 12/15-LO has been demonstrated in various cells and tissues, including adrenal cells, vascular smooth muscle cells (VSMC), brain, and kidney [4, 12–16]. In mice, both leukocyte 12/15-LO as well as platelet 12-LO are ex-

pressed [17]. A leukocyte-type 12/15-LO has been identified and partially sequenced from rat glomeruli [15].

Evidence shows that 12/15-LO and their products of arachidonic acid metabolism such as 12(S)-HETE can mediate growth factor effects in cells such as VSMC and fibroblasts, as well as responses to vascular injury [18–23]. One study reported that urinary excretion of 12(S)-HETE was increased in diabetic patients as compared with control subjects [24]. Although the presence of 12/15-LO has been recognized in the kidney [4, 15], much less is known regarding the functional effects or the in vivo significance. We recently showed that 12/15-LO mRNA and protein are increased in glucose-stimulated mesangial cells and in experimental diabetic nephropathy [4]. Furthermore, the in vivo expression of the matrix protein fibronectin and TGF-β in the diabetic rat glomeruli was associated with increased glomerular 12/15-LO expression [4] thereby implicating the 12/15-LO pathway in the pathogenesis of diabetic nephropathy. Factors relevant to the pathogenesis of diabetic nephropathy such as high glucose, Ang II and PDGF can induce leukocyte-type 12/15-LO activity and expression in VSMC and in rat mesangial cells [4, 16, 19, 25]. The growth-promoting and matrix-inducing effects of Ang II in mesangial cells were blocked by pharmacologic 12-LO inhibitors as well as a novel 12-LO ribozyme [25]. Furthermore, 12(S)-HETE could directly induce cellular hypertrophy and expression of the extracellular matrix protein, fibronectin in rat mesangial cells and VSMC with similar potency as Ang II [18, 20, 25]. 12(S)-HETE-induced effects were mediated at least in part by activation of the p38 mitogen-activated protein kinase (p38 MAPK) and its target transcription factor, cyclic adenosine monophosphate (AMP) response element binding protein (CREB), which is related to matrix gene expression [20, 25]. A similar observation was recently made in adrenocortical cells [26].

Sun and Funk [27] have created mice that are deficient in leukocyte (macrophage) 12/15-LO. Cross-breeding studies showed that these mice could reduce the rates of atherosclerosis in the susceptible low density lipoprotein (LDL) receptor knockout as well as apolipoprotein E (Apo E) knockout mice [28–30]. This appeared to be due to reduced rates of macrophage LDL oxidation [30, 31]. Furthermore, these 12/15-LO knockout mice were resistant to the development of diabetes in response to low-dose streptozotocin [32]. While these results have established an in vivo role for 12/15-LO in the pathogenesis of atherosclerosis and islet dysfunction associated with type 1 diabetes, its in vivo role in the development of renal diseases such as diabetic nephropathy is not yet known. In the present study, in order to determine the potential in vivo functional role of 12/15-LO in mesangial cell growth related to kidney diseases such as glomerulosclerosis and nephropathy, and also to identify the molecular mechanisms involved, we compared the properties of mouse mesangial cells (MMCs) derived from 12/15-LO knockout mice with those obtained from genetic control wild-type mice ex vivo. We observed that cellular growth, matrix production, activation of oxidant stress, specific signaling pathways, and transcription factors were attenuated in the 12/15-LO knockout cells relative to wild-type cells. In addition, we also noted that stable overexpression of mouse 12/15-LO in a MMC cell line led to reciprocal increase in MAPK activation and fibronectin expression. Our results suggest that 12/15-LO activation can play a key role in mediating mesangial cell growth and matrix responses associated with the development of renal disease, particularly that associated with diabetes.

METHODS

Histologic evaluation of sections from wild-type and 12/15-LO knockout mouse kidney cortices

All animal studies were performed according to the guidelines of the National Institutes of Health and with an approved protocol from the City of Hope Research Animal Care Committee. Genetic control (wild-type, C57BL/6) and leukocyte 12/15-LO knockout mice on a C57BL/6 background (Strain name, B6.129S2-Alox15tm1Fun, Stock Number 002778) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Renal cortices from the kidneys of 8- to 10- week-old wild-type and 12/15-LO knockout mice were incubated overnight at 4°C in 4% paraformaldehyde and then embedded in paraffin. Paraffin-embedded sections from the two mice were evaluated by hematoxylin-eosin staining by standard methods.

Isolation and characterization of murine mesangial cells (MMCs)

Kidneys from eight-to ten-week-old male wild-type and 12/15-LO knockout mice were removed using sterile techniques. After washing in RPMI 1640 medium, the cortices were separated from medullas, pooled, minced, and the glomeruli isolated by differential sieving using standard methods [4, 33, 34]. Glomeruli were then treated with collagenase (Sigma Chemical Co., Inc., St. Louis, MO, USA) for 15 minutes, plated in T-25 culture flasks (Nunc, Rochester, NY, USA) in RPMI 1640 medium containing 15% fetal calf serum (FCS) (Omega Scientific, Co., Tarzana, CA, USA) and 20 ug/mL insulin (Sigma Chemical Co.) and then cultured at 37°C with 5% CO_2. Outgrowths of spindle-like mesangial cells were seen after 7 to 10 days. Cells were grown for a further 48 hours in the presence of 50 mmol/L D-valine, replacing L-valine in the RPMI 1640 to exclude fibroblast growth. After 48 hours, L-valine-containing normal medium was replaced. For characterization of the mesangial pheno-

type, the MMCs were grown on glass chamber slides, fixed and stained with monoclonal Thy1.1 antibody. Cells between 5 to 12 passages were used for all studies.

12(S)-HETE assay

Serum-depleted cells were lysed, deacetylated, and extracted as described earlier [16]. 12(S)-HETE levels in the extracts were determined using a specific radioimmunoassay described earlier [16]. This assay is specific for 12(S)-HETE and does not cross-react with the stereoisomer 12(R)-HETE.

Growth curves

Wild-type and 12/15-LO knockout MMCs were plated at 5000 cells per dish in 60 mm culture dishes in growth medium containing 10% FCS. Cell numbers were noted daily on a Coulter counter (Coulter Z™ Series, Beckman Coulter, Fullerton, CA, USA) after trypsinization.

Superoxide measurement by confocal microscopy

MMCs cultured on chamber slides were serum-depleted and treated with agonists for 30 minutes. They were then washed with phosphate-buffered saline (PBS) and incubated with the superoxide probe dihydroethidium (DHE) 10 umol/L (Molecular Probes, Inc., Eugene, OR, USA) at 37°C for 10minutes. After incubation, cells were washed to remove extracellular dye and imaged with a laser confocal microscope (excitation 488 nm/emission 514 nm). The generation of superoxide is indicated by the appearance of red fluorescence. All images were collected using a 512 × 512 pixel format and archived for subsequent analysis to determine relative fluorescence intensity as described [35].

Immunoblot analysis

Cells were lysed as described earlier [25] and the lysates fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were transferred to nitrocellulose in 25 mmol/L Tris-HCl, 192 mmol/L glycine, and 10% methanol for 1½ hours at constant current of 100 mA. Membranes were incubated in 20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, 0.2% vol/vol Tween 20 with 5% wt/vol nonfat milk at room temperature and incubated overnight at 4°C with antibodies to either 12/15-LO [4, 19], phospho-specific MAPKs, total MAPKs, phospho- and total CREB (Cell Signaling, Beverly, MA, USA), or cellular fibronectin (EDA sequence) (Chemicon, Temecula, CA, USA). After further washing in Tris/NaCl/Tween 20, membranes were incubated for 1 hour with horseradish-linked secondary antibody, and immunoreactive proteins detected by Supersignal chemiluminescence reagent (Pierce, Rockford, IL, USA). Immunoblots were scanned using GS-800 densitometer and protein bands quantified with Quantitation One software (Bio-Rad Laboratories).

Incorporation of [^{3}H]-thymidine and [^{3}H]-leucine

Serum-depleted MMCs were incubated for 24 hours with [^{3}H]-thymidine or [^{3}H]-leucine and Ang II or FCS at the indicated concentrations. Radioactivity in trichloroacetic acid–insoluble protein precipitates from cells was determined as described earlier [25].

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

After stimulation with Ang II or FCS at the indicated concentrations, total RNA was extracted and relative multiplex RT-PCRs were performed using18S RNA as internal control in each sample as described earlier [25]. After normalization to 18S RNA, the results were expressed as fold expression over control unstimulated cells. Mouse fibronectin mRNA was amplified using primers, 5′-GCACAACAGACCACCAAACTCG-3′ (forward) and 5′-CTGAAGTCACTTCTCGGGGTGC-3′ (reverse). Primers for c-*fos* and 18S RNA were from Ambion Inc. (Austin, TX, USA).

Fibronectin expression by immunofluorescence

Confluent MMCs on glass slides were fixed with 2% paraformaldehyde in PBS for 15 minutes. Cells were permeabilized with 0.2% Triton-X 100 in PBS and washed. To reduce nonspecific binding, cells were treated with 5% goat serum in PBS and incubated with fibronectin antibody (1:40), followed by incubation with rhodamine-conjugated secondary antibody (1:200) and viewed by fluorescence microscopy. Cells were also stained with the Hoechst 33342 (Molecular Probes, Inc.) to detect nuclei.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared as described previously [20]. Briefly, after serum depletion, MMCs were stimulated with Ang II (0.1 umol/L) or 10% FCS, washed with cold PBS (pH 7.6), scraped into 1 mL PBS, and spun down at 4°C. Cell pellets were resuspended in nuclear extraction buffer A (NEA), 10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and protease inhibitors and then lysed in NEA containing 0.1% NP-40. Cell lysates were centrifuged and nuclei resuspended in NEC [10 mmol/L HEPES, pH 7.9, 0.42 mol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L dithiothreitol (DTT), 25% glycerol, and protease inhibitors). After 30 minutes' incubation on ice, they were centrifuged at 4°C for 15 minutes and supernatants used as nuclear extracts. DNA binding of nuclear proteins was performed as described earlier [20, 25, 36]. Labeled synthetic oligonucleotides containing consensus-binding

sequences for transcription factors CREB or activator protein-1 (AP-1) were used as probes for EMSA. As control for DNA binding, we used oligonucleotides containing consensus-binding sequences for the transcription factor SP-1. Gels were dried, protein-DNA complexes visualized on a phosphorimager, and the radioactivity of complexes quantitated using Imagequant software (Molecular Dynamics, Sunnyvale, CA, USA).

Transient transfections and reporter gene assays

MMCs from both wild type and 12/15-LO knockout mice were plated at a density of 1×10^4 cells per well in 24-well dishes. Next day, they were transiently transfected with either pAP-1-Luc (100 ng/well) or pCRE-Luc (200 ng/well) (Stratagene, La Jolla, CA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Cells were allowed to recover overnight and then transferred to serum depletion medium [RPMI-1640 containing 0.2% bovine serum albumin (BSA)]. Next day, they were stimulated with FCS (10%) for 6 hours and luciferase activities in the cell lysates assayed as described previously [20].

Stable transfection of a MMC cell line with mouse 12/15-LO

Stable mouse 12/15-LO overexpression was performed in an immortalized MMC cell line [34, 37] (a generous gift from Dr. K. Sharma, Thomas Jefferson University, Philadelphia, PA, USA). The MMC cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS containing 20 mmol/L HEPES, pH 7.4, 10 mmol/L glucose, penicillin, and streptomycin at 37°C in 5% CO_2 and 95% air. To generate mouse leukocyte type 12/15-LO overexpressing cells, MMCs were plated at a density of 3×10^6 cells in a 60 mm dish. The cells were then transfected with either mouse leukocyte 12/15-LO cDNA in pcDNA3.1 vector (generous gift from Dr. Colin Funk, University of Pennsylvania, Philadelphia, PA) or control pcDNA3.1 vector (Invitrogen) using Lipofectamine 2000 reagent according to manufacturer's instructions. The empty control vector pcDNA 3.1 without the insert was used as the mock-transfected control. After 48 hours, the transfected cells were split 1:10 in 100 mm dishes in medium containing selection agent G418 (400 ug/mL). This medium was changed every 3 days until G418-resistant colonies appeared. Individual resistant clones were isolated 2 to 3 weeks later and expanded into cell lines. Clones were checked for 12/15-LO overexpression by Western blotting and 12(S)-HETE levels. Stable transfectants were maintained in complete medium containing 10% FCS and 400 ug/mL G418.

Data analyses

Data are expressed as mean ± SEM of multiple experiments. Paired Student *t* tests were used to compare two groups, or analysis of variance (ANOVA) with Dunnett post test for multiple groups using PRISM software (Graph Pad, San Diego, CA, USA). Statistical significance was detected at the 0.05 level.

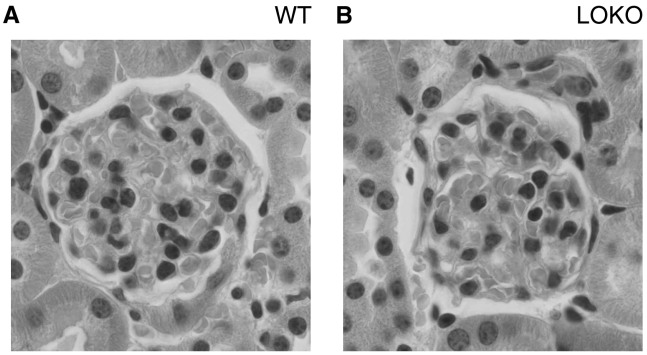

Fig. 1. Renal glomeruli from wild-type (WT) and 12/15-lipoxygenase knockout (LOKO) mice. Glomeruli from 8- to 10-week-old male WT (*A*) and 12/15-LO knockout mice (*B*) mice showing similar morphology. Hematoxylin-eosin stain. Magnification ×400.

RESULTS

Morphology of glomerular sections from wild-type and 12/15-LO knockout mice

Hematoxylin-eosin staining of renal cortical sections from wild-type and 12/15-LO knockout mice are seen in Figure 1. They indicate that the glomeruli of 12/15-LO knockout mice appear normal and that there are no visible morphologic differences among the mesangial cells in vivo in the two mice types.

Lipoxygenase activity and expression in MMC from 12/15-LO knockout mice versus wild-type mice

MMCs were isolated from wild-type and 12/15-LO knockout mice and cultured in medium containing 10% serum. We first compared basal, Ang II (0.1 umol/L), and FCS (10%)-induced levels of cell-associated 12/15-LO product, 12(S)-HETE, in wild-type versus 12/15-LO knockout MMCs, by a specific radioimmunoassay that is specific to 12(S)-HETE [16]. Figure 2A shows that basal levels of 12(S)-HETE were significantly lower in 12/15-LO knockout cells relative to wild-type. Furthermore, while Ang II significantly increased 12(S)-HETE levels in the wild-type MMC, it had no effect in the 12/15-LO knockout cells (Fig. 2A). Figure 2B shows that MMCs from 12/15-LO knockout mice also had significantly lower FCS-induced levels of cell associated 12(S)-HETE than MMCs from wild-type mice.

We then evaluated the level of expression of leukocyte-type 12/15-LO protein in the two cell types. Serum-depleted MMCs were stimulated with Ang II (0.1 umol/L) or FCS (10%) for 24 hours, and cell lysates subjected to immunoblotting with a 12/15-LO–specific

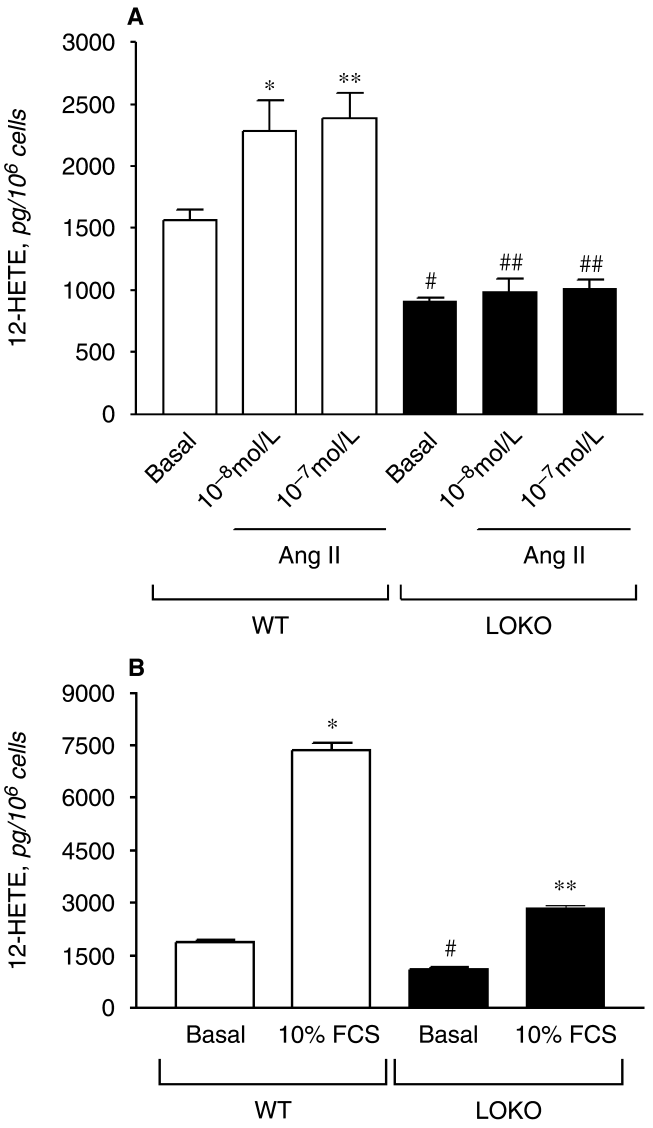

Fig. 2. Murine mesangial cells (MMCs) from 12/15-lipoxygenase (LO) knockout (12/15-LOKO) mice have reduced 12(S)-HETE levels. Serum-depleted cells were left alone or treated with angiotensin II (Ang II) (*A*) or fetal calf serum (FCS) (*B*) at concentrations indicated. Cell-associated 12(S)-HETE levels were extracted and quantitated by a specific radioimmunoassay. Data represent mean ± SEM of three independent experiments. (A) $*P < 0.05$ vs. wild-type (WT) basal; $**P < 0.001$ vs. wild-type basal; $\#P < 0.05$ vs. wild-type basal; $\#\#P < 0.001$ vs. wild-type Ang II. (B) $*P < 0.001$ vs. wild-type basal; $**P < 0.01$ vs. 12/15-LO knockout basal and wild-type FCS; $\#P < 0.05$ vs. wild-type basal.

peptide antibody [4, 19]. Figure 3 shows that, in contrast to wild-type cells, 12/15-LO knockout cells did not have a ~72 kD 12-LO protein. Furthermore, wild-type MMCs treated with Ang II and FCS displayed increased expression of 12/15-LO compared to basal as we have shown earlier [16, 25]. Authentic porcine leukocyte 12-LO (Oxford Biomedical Research, Inc., Oxford, MI, USA) is shown in the far right lane. The same blot stripped and reprobed with actin as internal control (lower panel) indicates equal protein loading in all the lanes.

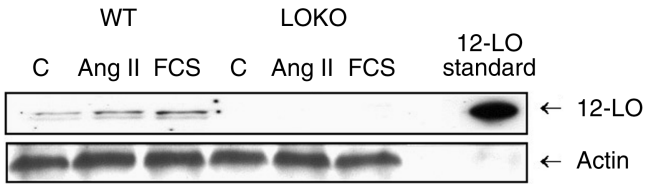

Fig. 3. Expression of 12/15-lipoxygenase (12/15-LO) protein is decreased in murine mesangial cells (MMCs) from 12/15-LO knockout mice. Serum-depleted MMCs were stimulated with angiotensin II (Ang II) (0.1 umol/L) or fetal calf serum (FCS) (10%) for 24 hours and cell lysates analyzed for 12/15-LO expression by immunoblotting with a peptide antibody against porcine leukocyte 12-LO (1:1000) that recognizes mouse 12/15-LO. Authentic porcine leukocyte 12-LO is shown in the far right lane. The blot was stripped and reprobed with an antibody to actin as internal control for protein loading (lower panel).

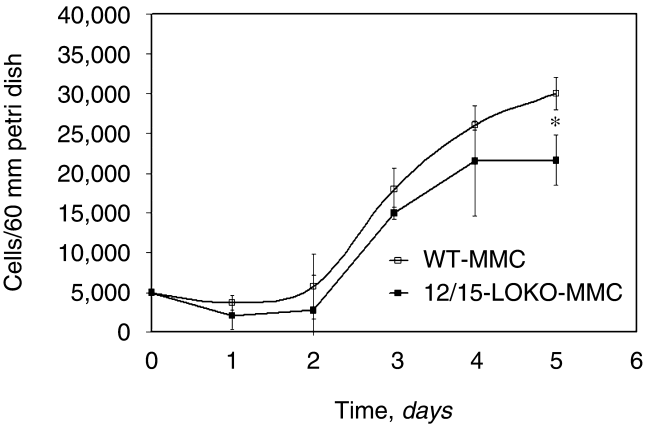

Fig. 4. Growth pattern of mouse mesangial cells MMCs) from wild-type (WT) versus 12/15-liopxygenase (LO) knockout (12/15-LOKO) mice. Wild-type and 12/15-LO knockout MMCs were plated at 5000 cells per 60 mm dish and cell numbers counted daily (after trypsinization) using a Coulter counter. Results shown are representative of data from five MMC preparations. $*P < 0.05$ vs. 12/15-lipoxygenase knockout at day 5.

Cell proliferation

In order to determine whether 12/15-LO deficiency altered MMC proliferation rates, wild-type and 12/15-LO knockout MMCs were plated in equal numbers in growth medium and cell numbers counted at regular intervals. As shown in Figure 4, the MMCs from the 12/15-LO knockout mice grew at a 20% to 25% slower rate than those from the wild-type mice. At day 5, the reduced growth of 12/15-LO knockout cells was statistically significant ($P < 0.05$, $N = 5$).

Incorporation of [^{3}H]-thymidine and [^{3}H]-leucine

We next compared the rates of tritiated thymidine and leucine incorporation into the cells to evaluate whether the 12/15-LO knockout cells display decreased DNA and protein syntheses, respectively. As shown in Figure 5, MMCs from 12/15-LO knockout mice had significantly lower basal and Ang II-induced incorporation of [^{3}H]-thymidine (Fig. 5A) as well as [^{3}H]-leucine incorporation

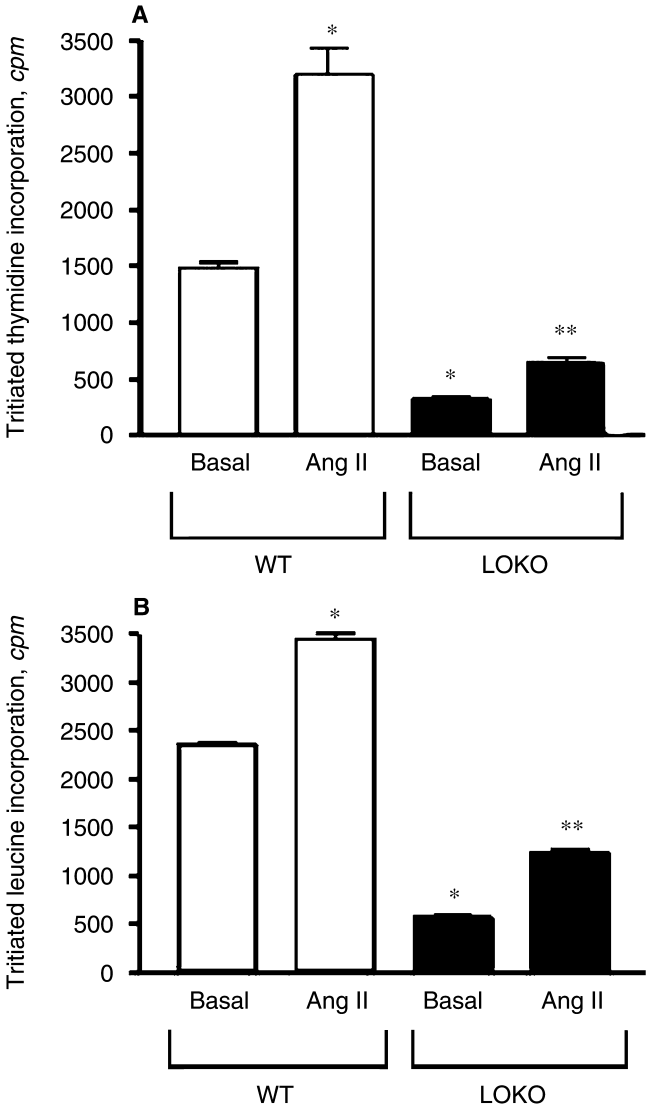

Fig. 5. [^{3}H]-thymidine and [^{3}H]-leucine incorporation are reduced in murine mesangial cells (MMCs) from 12/15-liopxygenase (LO) knockout (12/15-LOKO) mice relative to wild-type (WT). Serum-starved wild-type and 12-LO knockout MMCs were incubated overnight with [^{3}H]-thymidine (*A*) or [^{3}H]-leucine (*B*) with or without angiotensin II (Ang II) (10^{-7} M) and radioactivity in trichloroacetic acid–insoluble protein precipitates was determined. Data represent mean ± SEM from three independent experiments. *$P < 0.001$ vs. wild-type basal; **$P < 0.01$ vs. 12/15-LO knockout basal and wild-type Ang II.

(Fig. 5B). Although Ang II increased these effects in both wild-type and 12/15-LO knockout cells, the magnitude in the latter was markedly reduced. These results suggest that lipoxygenase activation can play important roles in mesangial cell growth and hypertrophy.

Superoxide production as an indicator of oxidant stress

In order to examine key signals that may be involved in the altered growth responses, we first tested superoxide anion production in the two cell types as an indicator of

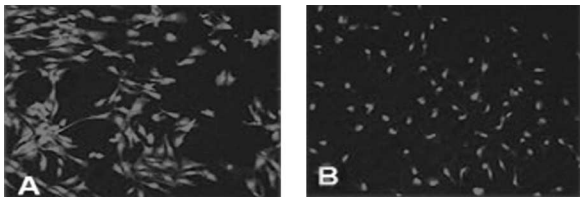

Fig. 6. Superoxide production is lower in 12/15-liopxygenase (LO) knockout (12/15-LOKO) cells relative to wild-type (WT). Serum-depleted murine mesangial cell (MMC) monolayers on 4-well chamber slides were incubated for 30 minutes, washed, and treated with 10 μmol/L dihydroethidium (DHE) for 10 minutes. Then the cells were washed to remove extracellular dye, mounted, and fluorescence detected using a confocal microscope.

oxidant stress. Lipoxygenase activation has been shown to lead to superoxide anion production [38]. Reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion have been linked to altered cellular growth via intracellular signaling events such protein kinase C and MAPK activation. We therefore examined superoxide production with DHE using confocal microscopy. Results in Figure 6 show that basal superoxide production was greatly reduced in 12/15-LO knockout mice MMC relative to wild-type (wild-type 54.98 ± 12.94 relative fluorescence intensity units versus 12/15-LO knockout 14.44 ± 0.48).

Activation of MAPKs

Since the growth effects of lipoxygenase products and growth factors have been linked to the activation of MAPKs [20, 25], we hypothesized that MAPK activation would be attenuated in the 12/15-LO knockout cells. Serum-depleted MMCs were treated with Ang II (0.1 umol/L) for 5, 10, and 30 minutes and with FCS (10%) for 5 minutes and the cell lysates analyzed by immunoblotting with phospho-specific ERK1/2 and p38 MAPK antibodies that recognize only the activated kinases. As shown in Figure 7A, p38 MAPK activation could be seen within 5 minutes after stimulation with Ang II and FCS, especially in the wild-type cells. Basal phospho- p38 MAPK levels were lower in the 12/15-LO knockout MMCs compared to wild-type MMCs. Furthermore, while Ang II at 5 minutes could significantly increase p-p38 MAPK levels in the wild-type cells, it had no effect in the 12/15-LO knockout cells (Fig. 7). The stimulatory effects of FCS were also greatly attenuated in the 12/15-LO knockout cells relative to wild-type (Fig. 7). The lower panel in Figure 7A shows that levels of total p38 MAPK were not altered under these conditions. Thus growth factor-induced p38 MAPK activation was greatly reduced in the 12/15-LO knockout cells. This is consistent with our earlier data showing that 12(S)-HETE and other lipoxygenase products are potent inducers of p38 MAPK activation and that this can play

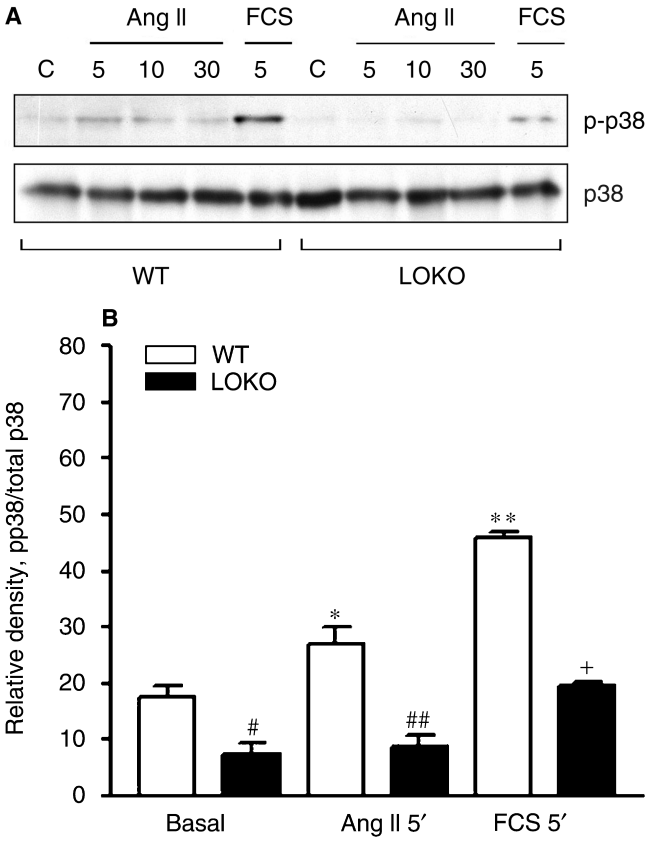

Fig. 7. Angiotensin II (Ang II) and serum-induced p38 mitogen-activated protein kinase (MAPK) activation are reduced in murine mesangial cells (MMCs) derived from 12/15-lipoxygenase (LO) knockout (12/15-LOKO) mice. Serum-depleted MMCs were stimulated with Ang II (0.1 umol/L) for 5, 10, and 30 minutes or with 10% fetal calf serum (FCS) for 5 minutes. Cell lysates were analyzed by immunoblotting with the phosphospecific p38 MAPK antibody (*A*, upper panel). The blots were stripped and then probed with p38 MAPK antibody (lower panel). Bar graph (*B*) shows mean ± SEM data from three to five experiments. $*P < 0.01$ vs. wild-type basal; $**P < 0.001$ vs. wild-type basal; $\#P < 0.05$ vs. wild-type basal; $\#\#P < 0.001$ vs. wild-type Ang II; $+P < 0.001$ vs. wild-type FCS ($N = 5$).

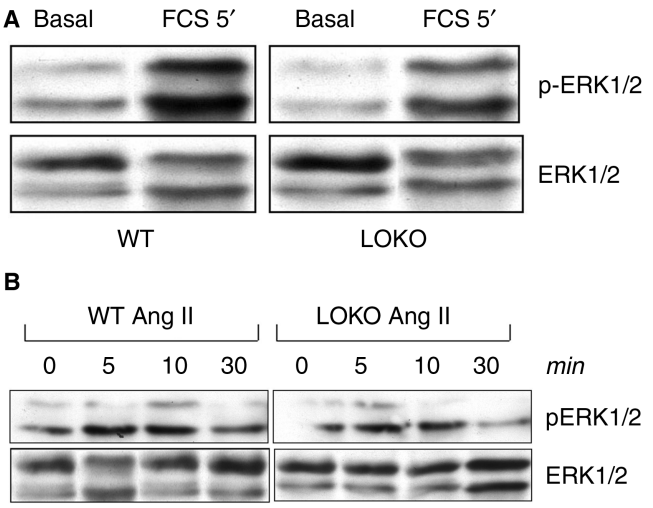

Fig. 8. Comparison of ERK1/2 mitogen-activated protein kinase (MAPK) activation in wild-type (WT) vs. 12/15-lipoxygenase (LO) knockout (12/15-LOKO) cells. Experiments were performed with fetal calf serum (FCS) and angiotensin II (Ang II) treatments as described in Fig. 7 and cell lysates probed with an antibody to phospho-ERK1/2 (*A* and *B*, upper panels). The blots were stripped and probed with and antibody to ERK1/2 (lower panels). Data shown are representative of three to four experiments.

a key role in cellular hypertrophy, matrix, and inflammatory gene induction [20, 25, 36]. Furthermore, p38 MAPK activation was augmented in rat VSMC and cardiac fibroblasts overexpressing 12/15-LO [20, 21].

We also examined the activation of ERK1/2 MAPK. Figure 8A shows that, similar to p38 MAPK, basal and FCS-induced levels of p-ERK1/2 were clearly lower in the 12/15-LO knockout MMCs relative to wild-type. However, Figure 8B shows that Ang II-induced levels of p-ERK1/2 were not lower in 12/15-LO knockout MMCs. Total ERK1/2 is seen in the lower panels of Figure 8.

Comparison of CREB phosphorylation

The significance of MAPK activation is that it can lead to the phosphorylation and activation of key transcription factors involved in cellular growth and hypertrophy. Our recent study demonstrated that 12(S)-HETE could directly lead to the activation of the key transcription factor, CREB via p38MAPK activation [20, 25, 26]. Furthermore, CREB activation appeared to mediate the expression of the key matrix protein, fibronectin [20, 25]. Hence, in the next step, we compared CREB activation in wild-type versus 12/15-LO knockout cells. CREB transcriptional activity is increased by phosphorylation at Ser 133 mediated by MAPKs such as p38 and ERK1/2 [39, 40]. Figure 9A shows that both basal and FCS-stimulated levels of phospho-CREB (p-CREB) were reduced in the 12/15-LO knockout cells relative to wild-type cells. Figure 9B shows that p-CREB activation could be seen within 5 to 10 minutes after stimulation with Ang II. But this was greatly attenuated in the 12/15-LO knockout cells. In contrast, the same blots stripped and probed with an antibody to CREB shows that levels of total CREB were not altered (lower panels of Fig. 9). These results indicate that basal, FCS, and Ang II-induced CREB activation are lower in MMC from 12/15-LO knockout mice.

Comparison of DNA binding activities of key transcription factors in MMC from wild-type and 12/15-LO knockout mice using EMSA

Since MAPK activation and CREB phosphorylation were attenuated in 12/15-LO knockout cells, we evaluated whether the DNA binding activities of the transcription factors CREB, as well as AP-1, another key target of MAPK, such as ERK1/2, are altered. These transcription factors play key roles in the expression of genes required

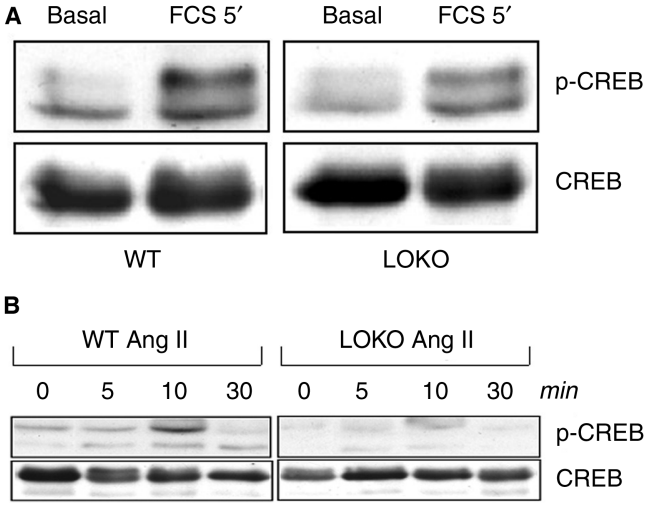

Fig. 9. Comparison of cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) activation in wild-type (WT) versus 12/15-lipoxygenase (LO) knockout (12/15-LOKO) cells. Experiments were performed as described in Fig. 8 and cell lysates probed with an antibody to phospho-CREB (*A* and *B*, upper panels). The blots were stripped and probed with an antibody to CREB (lower panels). Data shown are representative of three experiments.

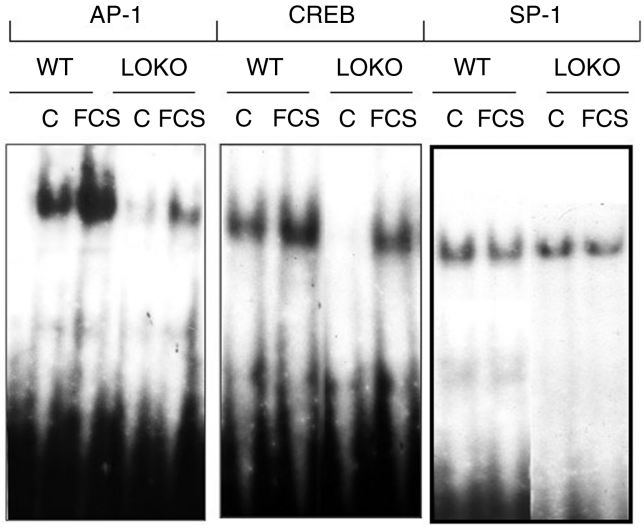

Fig. 10. Activator protein-1 (AP-1) and cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) transcription factor DNA-binding activities are reduced in murine mesangial cells (MMCs) derived from 12/15-lipoxygenase (LO) knockout (12/15-LOKO) mice. Nuclear extracts were prepared from control and 10% FCS-treated cells and analyzed by gel shift assays for DNA binding activity of the transcription factors AP-1, CREB, and SP-1 as described in the **Methods** section.

for cell growth and production of matrix proteins, including fibronectin in response to growth factors [41–43]. Serum-depleted MMCs were left untreated or stimulated with FCS (10%) for 1 hour. Nuclear extracts were prepared and analyzed for DNA binding activity by gel shift assays using ^{32}P-labeled oligonucleotides containing consensus DNA binding sequences for AP-1, CREB, and a constitutively active transcription factor, SP-1. Results shown in Figure 10 indicate that both basal and serum-induced DNA binding activities of AP-1 and CREB were clearly reduced in the 12/15-LO knockout cells compared to wild-type. However, SP-1 DNA binding activity was similar in these nuclear extracts, indicating that the differences in DNA binding activities were specific to AP-1 and CREB. Interestingly, there was no difference in Ang II-induced DNA binding of AP-1 and CREB in the two cells types (results not shown).

Comparison of the transcriptional activities of AP-1 and CREB in wild-type versus 12/15-LO knockout cells

In order to determine whether the observed differences in DNA binding activities translate to attenuated transcriptional activities in the 12/15-LO knockout cells, we compared basal and FCS-stimulated AP-1 and CREB transcriptional activities using luciferase reporter gene assays with plasmids containing these specific transcription factor binding sites fused to the luciferase gene (Stratagene, La Jolla, CA, USA). Thus, MMCs from both wild-type and 12/15-LO knockout mice were transiently transfected with either pAP-1-Luc (100 ng/well) or pCRE-Luc (200 ng/well) as described in the **Methods** section. They were then stimulated with FCS (10%) for 6 hours and luciferase activities in the cell determined. Figure 11A shows that basal AP-1 activity was significantly lower in 12/15-LO knockout cells relative to wild-type cells. Furthermore, while serum led to significant increase in AP-1 transcriptional activity in the wild-type cells, it was without much effect in the 12/15-LO knockout cells. Figure 11B shows that, in a similar fashion, basal and FCS-induced CREB transcriptional activities were greatly attenuated in the 12/15-LO knockout cells relative to the wild-type cells. These results demonstrate that the transcriptional activities of these two key nuclear DNA binding factors, AP-1 and CREB, are greatly attenuated in the MMCs from 12/15-LO knockout mice.

Relative expression of the immediate early response (IE) gene, c-fos, and the matrix protein, fibronectin, in wild-type and 12/15-LO knockout cells

Since the transcriptional activities of AP-1 and CREB were attenuated in the 12/15-LO knockout cells, we next determined whether this translates to reduced transcription of key target genes of these transcription factors that are relevant to cellular growth and glomerulosclerosis. Activation of MAPKs and their target transcription factors such as AP-1 can lead to the activation of the IE gene, c-*fos,* which is involved in cellular growth and is one of the most extensively investigated members of the IE gene family. We compared the expression of *c-fos* by

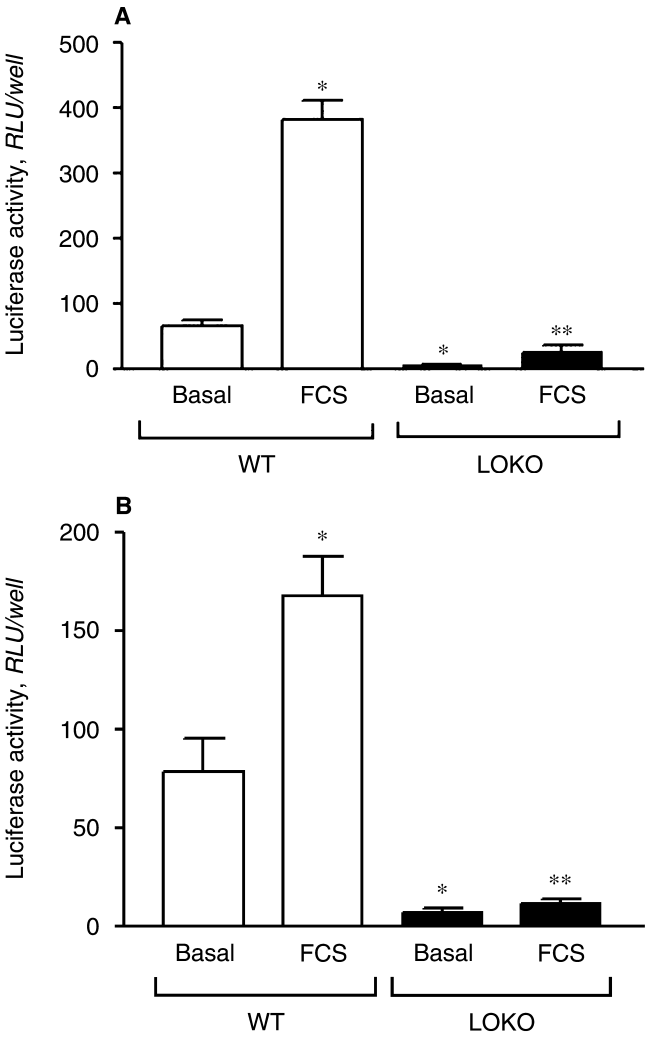

Fig. 11. Activator protein-1 (AP-1) and cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) transcriptional activities are reduced in murine mesangial cells (MMCs) derived from 12/15-lipoxygenase (LO) knockout (12/15-LOKO) mice. MMCs from wild-type or 12/15-LO knockout mice were transiently transfected with either pAP-1-Luc plasmid (100 ng/well) (*A*) or p-CRE-Luc plasmid (200 ng/well) (*B*) as described in the **Methods** section. They were then stimulated with fetal calf serum (FCS) (10%) for 6 hours and luciferase activities in the cell lysates assayed. Results shown are mean ± SEM from two experiments run in triplicate. $^{*}P < 0.05$ vs. wild-type basal; $^{**}P < 0.001$ vs. wild-type FCS.

relative RT-PCR using 18S as an internal control in the wild-type and 12/15-LO knockout cells. Figure 12 shows that FCS-induced *c-fos* mRNA expression was evident by 15 minutes in both wild-type and 12/15-LO knockout cells and remained visible up to 60 minutes after stimulation. However, the effects of FCS at both 15 minutes and 30 minutes were clearly greater in the wild-type cells relative to 12/15-LO knockout cells. Thus, the reduced induction of IE genes such as *c-fos* may be one of the mechanisms for the reduced growth effects in the 12/15-LO knockout cells. The slightly stronger *c-fos* mRNA level at 60 minutes may indicate that induction may be delayed in 12/15-LO knockout mice but could last longer. The implication of this is not clear and it is likely that other IE genes are also altered.

In the next step, we compared the expression of fibronectin mRNA and protein in the two cell types. Several lines of evidence indicate that increased deposition of extracellular matrix proteins such as fibronectin plays a key role in the pathogenesis of glomerulosclerosis and diabetic nephropathy. We recently showed that both Ang II and 12(S)-HETE increased fibronectin mRNA expression in rat mesangial cells via CREB and p38 MAPK [25]. We therefore hypothesized that fibronectin levels may be attenuated in the 12/15-LO knockout cells. We tested this by three methods: first, immunoblotting with a fibronectin antibody, second, immunostaining with a FN antibody, and finally, relative multiplex RT-PCR for fibronectin mRNA with 18S RNA as an internal control. The Western immunoblot in Figure 13A shows that basal fibronectin protein levels were clearly lower in 12/15-LO knockout MMCs with the 12/15-LO knockout cells showing little immunoreactivity. Similar results are seen in the immunofluorescent staining data in Figure 13B which shows that the 12/15-LO knockout cells have much less of this extracellular matrix protein compared to the wild-type, while the Hoechst staining indicated equal nuclei in both frames. Figure 13C shows that basal levels of fibronectin mRNA were also lower in 12/15-LO knockout MMCs. Furthermore, while both Ang II and FCS could increase fibronectin mRNA expression in the wild-type cells, they had no stimulatory effects in the 12/15-LO knockout cells (Fig. 13C). These results suggest that lipoxygenase activation plays a key role in matrix protein production in rat mesangial cells. We had noted earlier that although FCS-induced DNA binding of AP-1 and CREB were reduced in the 12/15-LO knockout cells, Ang II-induced effects were not. Thus, the attenuated responses of Ang II on fibronectin mRNA in 12/15-LO knockout cells may involve additional mechanisms such as reduced CREB phosphorylation as noted in Figure 9, or other unknown nuclear events.

Effects of stable overexpression of mouse 12/15-LO in a MMC line

Since 12/15-LO–deficient MMC had reduced MAPK activation and fibronectin expression, we hypothesized that 12/15-LO overexpression may lead to increased MAPK activation and fibronectin expression. For these studies, we overexpressed 12/15-LO in an immortalized MMC cell line that has been shown to retain properties of mesangial cells [34, 37]. These cells were transfected with either mouse 12/15-LO cDNA or control pcDNA3 vectors. Figure 14A shows one clone with high levels of 12/15-LO relative to the mock-transfected clone (pcDNA). Authentic 12-LO is shown in the far end.

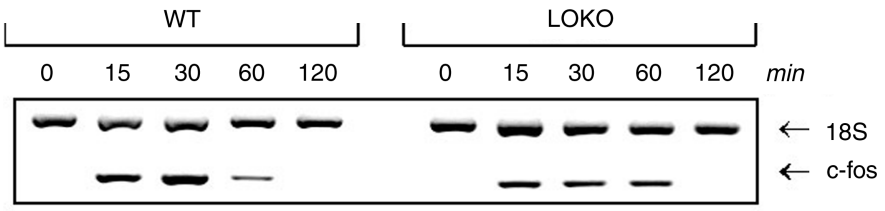

Fig. 12. Serum-stimulated expression of the IE gene, *c-fos*, is reduced in murine mesangial cells (MMCs) from 12/15-lipoxygenase (LO) knockout (12/15-LOKO) mice. Fetal calf serum (FCS) stimulated *c-fos* mRNA expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) using mouse *c-fos* gene-specific primers and 18S RNA primers. The data shown are representative of two similar experiments.

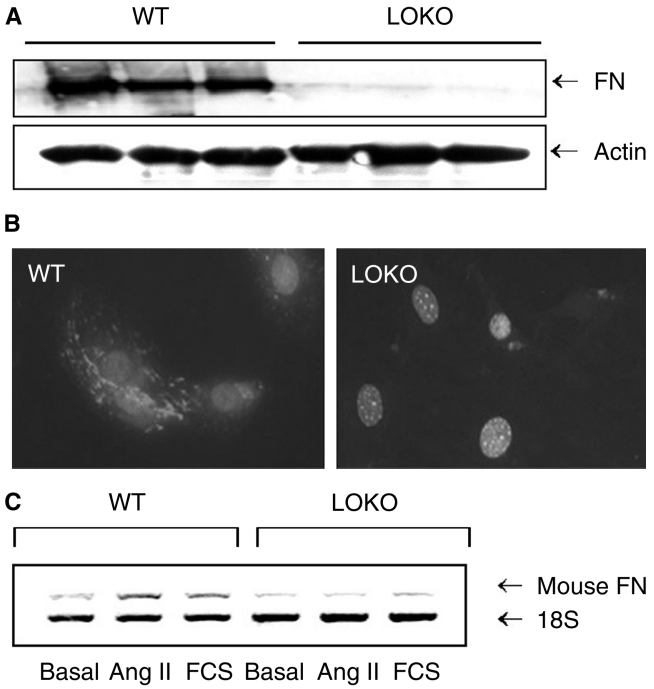

Fig. 13. Expression of fibronectin (FN) is reduced inmurine mesangial cells (MMCs) derived from 12/15-lipoxygenase (LO) knockout (12/15-LOKO) mice. (*A*) MMC were assayed for fibronectin expression by immunoblotting with fibronectin (FN) antibody (1:1000). (*B*) Fibronectin expression by immunofluorescence using the fibronectin primary antibody (1:40) followed by incubation with rhodamine-conjugated secondary antibody (1:200). Cells were also counterstained with Hoechst dye to detect nuclei. (*C*) MMC were serum depleted for 48 hours and then stimulated with angiotensin II (Ang II) (0.1 umol/L) or fetal calf serum (FCS) (10%) for 24 hours. Expression of mouse fibronectin mRNA was determined by reverse transcription-polymerase chain reaction (RT-PCR) using 18S RNA as an internal control.

Figure 14B shows that levels of phospho-p38 were also clearly higher in the 12/15-LO–overexpressing cells relative to control cells, indicating increased p38 MAPK activity. Furthermore, Figure 14C shows that these cells also had greater levels of fibronectin mRNA expression. These results indicate that 12/15-LO overepxression appears to increase these key parameters associated with renal dysfunction. This supports earlier data showing increased glomerular 12/15-LO and fibronectin expression, as well as p38 MAPK activity in a rat model of diabetic nephropathy [4, 46].

DISCUSSION

While several factors have been implicated in the pathogenesis of diabetic nephropathy, the cellular and molecular mechanisms involved are still not fully clear. We recently demonstrated that 12/15-LO expression and activity were enhanced in experimental diabetic nephropathy in rats and correlated with expression of the extracellular matrix protein fibronectin [4]. 12/15-LO expression and products were also increased in glucose- and Ang II-stimulated mesangial cells [4, 25]. This pathway also seemed to mediate the hypertrophic effects of Ang II [25]. Furthermore, lipoxygenase products such as 12(S)-HETE could directly increase cellular hypertrophy and fibronectin production in rat mesangial cells [25]. In the present study, we took advantage of the availability of the 12/15-LO knockout mice to evaluate the potential in vivo functional significance of this pathway in mesangial cells. We compared basal and growth factor-stimulated oxidant stress, MAPK and transcription factor activation, cellular growth, IE gene, and matrix fibronectin production ex vivo in MMC derived from 12/15-LO knockout mice versus wild-type control mice. Our present results demonstrate the significance of the 12/15-LO pathway in leading to cellular growth and matrix production relevant to glomerulosclerosis and diabetic nephropathy.

The MMCs from the 12/15-LO knockout mice showed absence of the leukocyte-type 12/15-LO. Basal levels of the lipoxygenase product, 12(S)-HETE, were greatly attenuated, while the stimulatory effect of Ang II on 12(S)-HETE production was abrogated. Funk [10], Chen et al [17], Sun and Funk [27], and Johnson, Brass, and Funk [44] have demonstrated that mice express both the platelet- as well as leukocyte-type 12/15-LO and they also successfully created mice in which each of these isoforms have been specifically disrupted. Since the 12/15-LO knockout mice in which the leukocyte 12/15-LO has been disrupted still have the platelet isoform, it is likely that the residual 12(S)-HETE could potentially arise from the platelet isoform. However, there is no specific report of the presence of a platelet 12-LO in MMCs and more work is needed to determine the source of the low background levels of 12(S)-HETE in the 12/15-LO knockout cells.

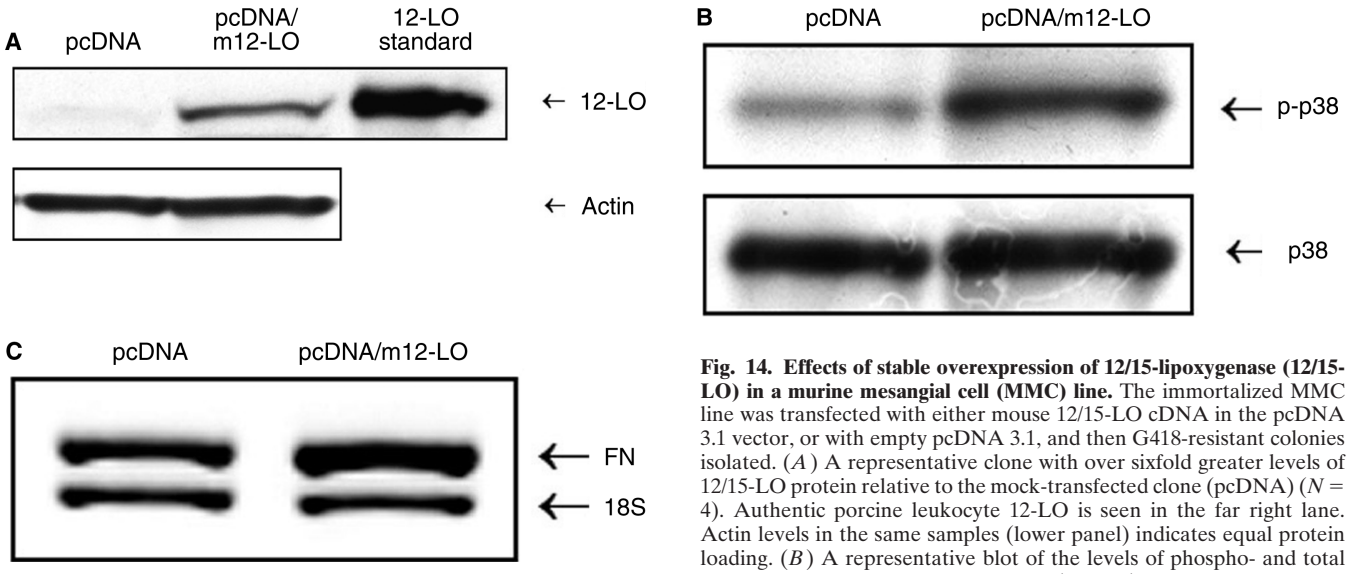

Fig. 14. Effects of stable overexpression of 12/15-lipoxygenase (12/15-LO) in a murine mesangial cell (MMC) line. The immortalized MMC line was transfected with either mouse 12/15-LO cDNA in the pcDNA 3.1 vector, or with empty pcDNA 3.1, and then G418-resistant colonies isolated. (*A*) A representative clone with over sixfold greater levels of 12/15-LO protein relative to the mock-transfected clone (pcDNA) ($N = 4$). Authentic porcine leukocyte 12-LO is seen in the far right lane. Actin levels in the same samples (lower panel) indicates equal protein loading. (*B*) A representative blot of the levels of phospho- and total p38 mitogen-activated protein kinase (MAPK) in the two cell types. (*C*) Differential fibronectin mRNA levels in the two clones with 18S RNA as internal control.

MMCs derived from the 12/15-LO knockout cells grew at a slower rate and also had reduced basal and growth factor Ang II-induced DNA and protein syntheses. This indicates that the 12/15-LO knockout MMCs have decreased growth responses when compared to the MMCs from wild-type mice. At this stage, it is not clear whether there are differences in the number of Ang II receptors in the two cell types. We also noted that there was decreased production of superoxide in the 12/15-LO knockout cells relative to the wild-type cells which is consistent with data that lipoxygenase activation can lead to increased superoxide and oxidant stress [38].

Earlier studies by us and others have indicated that 12/15-LO products such as 12(S)-HETE and 13(S)-HPODE can induce cellular growth and inflammatory gene expression via activation of MAPKs such as ERK1/2 and p38 MAPKs [20, 25, 26, 36, 45, 46]. This was further corroborated in the present study where we noted that basal and growth factor-stimulated activation of p38 and ERK1/2 MAPKs were greatly attenuated in the 12/15-LO knockout cells relative to wild-type cells. Furthermore, basal and FCS-induced DNA binding activities of the transcription factors AP-1 and CREB, which are key targets of ERK1/2 and p38 MAPKs, were attenuated in the MMC derived from lipoxygenase-deficient mice compared to the wild-type mice. These effects were functionally relevant since the transcriptional activation of AP-1 and CREB were also attenuated. Furthermore, levels of *c-fos* mRNA, a key gene controlled by AP-1, and expression of the mRNA and protein for the matrix protein fibronectin, which is controlled by CREB [42, 43], were both greatly reduced in 12/15-LO knockout cells. Interestingly, while Ang II-induced p38 MAPK and CREB activation were diminished in the 12/15-LO knockout cells, Ang II-induced ERK1/2 activation was not significantly different. These results are again consistent with the in vitro data that 12(S)-HETE leads to fibronectin expression via activation of p38 MAPK and CREB [20, 25]. Furthermore, p38 MAPK and CREB activities and expression were found to be increased in mesangial cells under high glucose conditions in vitro, and in early diabetic glomeruli in vivo in animal models of diabetic nephropathy [4, 46, 47]. In addition, we noted that stable overexpression of mouse 12/15-LO cDNA in a MMC cell line could increase p38 MAPK activity and fibronectin mRNA expression. These results are consistent with our earlier data that lipoxygenase products such as 12(S)-HETE can directly increase fibronectin expression and p38 MAPK activity in mesangial cells [25] and that 12/15-LO expression is increased in a rat model of diabetic nephropathy with parallel increases in fibronectin expression and p38 MAPK activity [4, 46].

Recently, there has been heightened interest in 12/15-LO due to key data from animal models implicating this pathway in the pathogenesis of atherosclerosis, restenosis, and hypertension [22, 23, 28–30, 48, 49]. Pharmacologic 12/15-LO inhibitors as well as a rat 12-LO ribozyme could block neointimal thickening in injured rat arteries [22, 23]. Increased 12/15-LO expression was observed in a swine model of diabetes-induced accelerated atherosclerosis [50]. Convincing evidence for the proatherogenic role of 12/15-LO comes from data showing that cross-breeding of 12/15-LO knockout mice with atherosclerosis prone Apo E−/− or LDLR−/− mice could

greatly reduce atherosclerosis development in the latter two mice models [28–31]. Interestingly, very recent data indicate that VSMCs derived from these 12/15-LO knockout mice display attenuated responses to growth factors relative to those from their wild-type controls [51].

In the present study, we used the 12/15-LO knockout mice to demonstrate another pathologic role for this enzyme in mesangial cells functions relevant to diabetic nephropathy. Our observations of reduced growth and matrix responses in the mesangial cells from 12/15-LO knockout mice relative to wild-type mice suggest additional effects of this enzyme in the kidney. Clinical evidence for this is obtained from data showing that urinary 12(S)-HETE excretion is markedly increased in diabetic patients with or without microalbuminuria compared to healthy controls [24]. Studies have also shown that lipoxygenase activation may play a role in renovascular hypertension [48, 49, 52]. Lipoxygenase activation has been shown to consume nitric oxide [53], which has key protective effects in the vascular and renal systems. This could be an additional mechanism for the pathologic role of the 12/15-LO pathway in the kidney. Apart from MAPKs, 12(S)-HETE has also been shown to activate protein kinase C [54, 55] that is known to be a major factor underlying several diabetic complications [56]. Thus, the 12/15-LO pathway could potentially mediate the glomerular changes of diabetic nephropathy through numerous mechanistic pathways and we have been able to demonstrate relevance by utilizing cells from 12/15-LO knockout mice. While we have mainly used mesangial cells in these studies, it is highly likely that altered 12/15-LO expression in other relevant renal cells such as podocytes may also contribute to cellular effects mediating diabetic nephropathy. This is supported by a recent report demonstrating increased 12/15-LO expression in podocytes in response to high glucose treatment [abstract; Adler et al, *J Am Soc Nephrol* 12:380A, 2001]. In summary, our studies show that renal 12/15-LO may play an important role in mesangial cell growth, oxidant stress, and extracellular matrix gene expression relevant to pathologic conditions such as diabetic nephropathy.

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