Lipoxins Inhibit Akt/PKB Activation and Cell Cycle Progression in Human Mesangial Cells

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Lipoxins (LX) are endogenously produced eicosanoids with a spectrum of bioactions that suggest anti-inflammatory, pro-resolution roles for these agents. Mesangial cell (MC) proliferation plays a pivotal role in the pathophysiology of glomerular inflammation and is coupled to sclerosis and tubulointerstitial fibrosis. We have previously reported that LXA₄ acts through a specific G-protein-coupled-receptor (GPCR) to modulate MC proliferation in response to the proinflammatory mediators LTD₄ and platelet-derived growth factor (PDGF). Further investigations revealed that these effects were mediated by modulation of receptor tyrosine kinase activity. Here we have explored the underlying mechanisms and report inhibition of growth factor (PDGF; epithelial growth factor) activation of Akt/PKB by LXA₄. LXA₄ (10 nmol/L) modulates PDGF-induced (10 ng/ml, 24 hours) decrements in the levels of cyclin kinase inhibitors p21^{Cip1} and p27^{Kip1}. PDGF-induced increases in CDK2-cyclin E complex formation are also inhibited by LXA₄. The potential of LXA₄ as an anti-inflammatory therapeutic is compromised by its degradation; this has been circumvented by synthesis of stable analogs. We report that 15-(R/S)-methyl-LXA₄ and 16-phenoxy-LXA₄ mimic the native compound with respect to modulation of cell proliferation and PDGF-induced changes in cell cycle proteins. *In vivo*, MC proliferation in response to PDGF is associated with TGFβ₁ production and the subsequent development of renal fibrosis. Here we demonstrate that prolonged (24 to 48 hours) exposure to PDGF is associated with autocrine TGFβ₁ production, which is significantly reduced by LXA₄. In aggregate these data demonstrate that LX inhibit PDGF stimulated proliferation via modulation of the PI-3-kinase pathway preventing mitogen-elicited G₁-S phase progression and suggest the therapeutic potential of LX as anti-fibrotic agents. (*Am J Pathol* 2004, 164:937–946)

Lipoxins (LX) are endogenously produced eicosanoids with potent anti-inflammatory bioactions.¹ LX are typically generated by transcellular metabolism initiated by the sequential actions of 15- and 5-, or 5- and 12-lipoxygenases on arachidonic acid, depending on the cellular context.² In a cytokine-primed milieu, aspirin-induced acetylation of COX-2 shifts its activity from an endoperoxide to a lipoxygenase, thereby promoting synthesis of 15-epi-LX [aspirin-triggered lipoxins (ATL)].¹ It has been proposed that some of the desirable effects of aspirin independent of its anti-thrombotic actions may be attributed to the biosynthesis of anti-inflammatory lipid mediators such as ATLs.² It is increasingly appreciated that there are two distinct phases of lipid mediator production in inflammation, an initial phase characterized by the production of proinflammatory mediators essential to effective host defense, which is superseded by the production of anti-inflammatory mediators such as LX during the resolution phase.³ LXA₄ and LXB₄ are the principal LX formed in mammals. In particular, LXA₄ has been shown to exert potent anti-inflammatory actions, modulating leukocyte trafficking and phagocytic clearance of apoptotic cells.^{1,3} LX biosynthesis has been demonstrated in many inflammatory conditions such as the glomerulonephritides (GN),⁴ human pleural disease,³ and in experimental models where the potential for cell-cell interactions is significant.⁵ LXA₄ may exert its anti-inflammatory effects through signals generated by binding to a high-affinity, G-protein-coupled LXA₄ receptor (ALXR).² LX are rapidly metabolized, the major routes of degradation being dehydrogenation at C-15 and possibly ω-oxidation at C-20.² To circumvent such metabolic inactivation, stable synthetic analogs have been developed that are modified at C-15, C-16, and/or C-20.⁶ These compounds retain the biological activity of native LXs and have been shown to bind with higher affinity to the ALXR, resulting in greater potency.^{2,6} The enhanced stability and improved efficacy of these analogs following local and systemic administration in models of inflammation and ischemia-reperfusion suggests significant therapeutic potential.⁴

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Mesangial cells (MC) are modified smooth muscle cells that play a pivotal role in renal physiology by regulating circulation and glomerular structural integrity. Mesangial cell proliferation and/or matrix accumulation characterizes many forms of GN and other progressive renal diseases including diabetic nephropathy. MC proliferation can be induced by several mitogens, including platelet-derived growth factor (PDGF) isoforms,⁷ epidermal growth factor (EGF)⁸ and eicosanoids such as the cysteinyl leukotriene D₄.⁹ PDGF has been widely implicated in the etiology of GN, triggering MC proliferation, migration, contraction, and synthesis of other cytokines (eg, TGF β ₁ and IL-1). In this context, blockade of PDGF bioactions with anti-PDGF antibody¹⁰ aptamers,¹¹ soluble receptors,¹² or inhibitors of receptor activation¹³ have been proposed as therapeutic strategies in proliferative GN. Such blockade of PDGF activity inhibits mesangio-proliferative changes, scarring and interstitial fibrosis.¹⁴

The mitogenic actions of PDGF in MC are mediated via the PDGFR, a member of the receptor tyrosine kinase (RTK) family. Predominant among the PDGF isoforms that are mitogenic for MC is PDGF B which acts via the PDGFR β .⁷ Activation of the intrinsic tyrosine kinase activity of the receptor facilitates recruitment of several SH2 domain-containing molecules and associated proteins including the p85 subunit of PI-3-kinase, RasGAP and PLC γ 1.¹⁵ Our previous investigations have indicated that LXA₄ inhibits MC proliferation in response to mitogens such as LTD₄⁹ and PDGF.¹⁶ These potential anti-inflammatory, pro-resolution bioactions of LX involve complex cross-talk between distinct GPCR and receptor tyrosine kinases.¹⁶ We have shown that LX modulate PI-3-kinase activation⁹ and recruitment of the p85 subunit of PI-3-kinase to the activated PDGFR.¹⁶

Here we have investigated the mechanisms underlying LX inhibition of MC proliferation and whether stable synthetic LX analogs can mimic the effects of the native compound in this regard. We report that 15-(*R/S*)-methyl-LXA₄ and 16- phenoxy-LXA₄ significantly inhibit PDGF and EGF-stimulated MC proliferation. We demonstrate that LXA₄ modulates PDGF-induced decrements in the levels of p21^{Cip1} and p27^{Kip1} and promotes nuclear retention of these CKI. Importantly, LXA₄ significantly modulated autocrine production of TGF β ₁ by PDGF-stimulated MC. MC proliferation in response to PDGF and EGF was coupled to activation of the Ser/Thr kinase, Akt/PKB (Akt), a downstream target of PI-3-kinase. Interestingly, inhibition of PI-3-kinase with LY294002 mimicked the effects of LXA₄ with respect to nuclear retention of p27^{Kip1} in PDGF-stimulated MC. Our data suggest that LXs modulate PDGF-induced proliferation by attenuation of Akt activation and prevention of G₁-S progression.

Materials and Methods

Materials

LXA₄ and LTD₄ were obtained from Biomol (Plymouth Meeting, PA). Human recombinant PDGF-BB and EGF were purchased from Upstate Biotechnology (Milton Key-

nes, UK). TGF β ₁ and anti-TGF β ₁ polyclonal antibody were from BD Biosciences (Oxford, UK). AG1296 and AG1478 were acquired from Calbiochem (Nottingham, UK). Transfer membranes were from Millipore (Bedford, MA). All other reagents were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. Stable synthetic LX analogs were a generous gift from Dr. Nicos Petasis, University of Southern California, Los Angeles, CA.

Cell Culture

Human kidneys were obtained from excess nephrectomy specimens according to the Mater Misericordiae University Hospital ethical guidelines. Renal glomeruli were isolated by differential sieving and mesangial cells were obtained and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μ g/ml) (all purchased from Gibco BRL, Paisley, Scotland). Isolated cells retained the phenotypic characteristics of mesangial cells, including stellate morphology, stained positive for vimentin and α -smooth muscle actin expression, and were negative for factor VIII and cytokeratin excluding endothelial and epithelial cell contamination, respectively.¹⁷ The CHOK1 cell line stably expressing ALXR and a control cell line were established and cultured as previously described.¹⁶

Cell Proliferation Assay

For analysis of proliferation, primary cultures of mesangial cells and CHOK1 cells were grown to approximately 70% confluence on 24-well plates before serum restriction in 0.2% FCS RPMI-1640 for 48 hours (MC) or serum deprivation in 0% FCS Ham's F12 medium for 24 hours (CHOK1 cells). After this period, cells were stimulated with various agents in triplicate wells for indicated times (44 hours for MC or 20 hours for CHOK1 cells) as detailed in Figure legends. Proliferation of cells was measured by determining [³H]-thymidine incorporation as follows: 1 μ Ci [³H]-thymidine (90 to 120 Ci/mmol; NEN, Cambridge, UK) was added to each well and incubated for 4 hours. Cells were washed twice in DPBS, solubilized in 0.2% sodium dodecyl sulfate (SDS) and counts per minute (cpm) were measured in 10 ml of scintillant (σ -Fluor). Data provided are from 4 to 5 independent experiments, as indicated.

Western Blot Analysis

Mesangial cells were serum restricted in 0.2% FCS RPMI 1640 for 48 hours and exposed to various agents for indicated times. Lysates were harvested in RIPA lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 5 mmol/L ethylene diaminetetraacetic acid, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 5 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, 1 μ mol/L leupeptin, 0.3 μ mol/L aprotinin). The lysates were clarified by centrifugation at 12,000 $\times$ *g* for 10 minutes and protein concentration in the supernatant was measured by Bradford protein assay. For Western blot

analysis, 30 μg of MC protein extract was loaded onto each lane, separated under reducing conditions on an SDS-polyacrylamide gel electrophoresis (PAGE) gel, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) by electroblotting. To reduce non-specific antibody binding, the membranes were first blocked with 5% nonfat dried milk for 1 hour at room temperature. This was followed by an overnight incubation at 4°C with antibodies to either cyclin A (Upstate Biotech, Lake Placid, NY), cyclin E (BD Biosciences), CDK2, p21^{Cip1} or p27^{Kip1} (all Santa Cruz Biotechnology, Heidelberg, Germany). Controls included omitting the primary antibody and/or replacing the primary antibody with rabbit or mouse serum. Membranes were incubated with a horse-radish-peroxidase-conjugated secondary antibody for 1 hour at room temperature and were visualized by chemiluminescence. To check for equal loading, membranes were either stained with Ponceau-S staining solution or were stripped and reprobed for β -actin (Sigma). For analysis of Akt phosphorylation, membranes were blocked with 5% bovine serum albumin (BSA) and probed with antibodies to either phospho-Akt (Ser⁴⁷³) or Akt protein (both New England Biosciences, Hertfordshire, UK).

Analysis of Cyclin E-CDK2 Complex Formation

MC lysate (300 μg) from each condition was precleared with Protein-G agarose beads (Santa Cruz) for 1 hour at 4°C before incubation with antibody to CDK2 (Santa Cruz, 1:500 dilution) overnight at 4°C with constant rocking. Protein-G agarose beads (10 μl) were then added to each immunoprecipitation and incubated for 2 hours at 4°C with constant rocking. Immunocomplexes were washed three times in fresh RIPA lysis buffer, denatured in 5X reducing sample buffer and boiled for 5 minutes to elute protein off beads. Samples were electrophoresed on SDS-PAGE gels, transferred to PVDF membranes, and probed for either cyclin E or CDK2 (loading control).

Quantitation of TGF β_1 Production

Quiescent MC were treated as indicated. At 24-, 48-, and 72-hour time points, medium was removed and assayed for TGF β_1 release by ELISA (R&D systems, Abingdon, UK) as per manufacturer's protocol. TGF β_1 produced was expressed as picogram TGF β_1 per microgram of cellular protein.

Cellular Fractionation

Using differential centrifugation, nuclear and cytosolic fractions from stimulated cells were harvested using a Nuclear Extract kit (Activemotif, Rixensart, Belgium). Briefly, cell lysates were fractionated at 14,000 $\times g$ for 30 seconds. Protein concentration in the pellet and soluble fractions was measured by Bradford protein assay. Cytosolic fractions were concentrated using 10,000 MW cut-off filters (Millipore) to maximize protein yield. Nuclear-cytoplasmic translocation of p27^{Kip1} was assayed by immunoblotting of cytosolic lysates and densitometric analysis.

Immunocytochemistry

MC were cultured in 4-well chamber slides (Nalge Nunc, Naperville, IL), rendered quiescent and stimulated as indicated. After stimulation, cells were washed with PBS, fixed with 2% paraformaldehyde (10 minutes) and permeabilized with 0.1% Triton X-100 (15 minutes). Fixed cells were then treated with blocking solution (5% BSA) for 1 hour. Localization of p27^{Kip1} was determined using anti-p27^{Kip1} polyclonal antibody (1:500). After washes, samples were treated with Oregon Green-conjugated anti-rabbit IgG (1:200). For DNA staining, samples were incubated with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) dye (1 $\mu\text{g}/\text{ml}$, 10 minutes). Cells were viewed and recorded in phase contrast (10 $\times$ and 40 $\times$ magnification) and corresponding fields using an Axiovert 200 fluorescent microscope (Carl Zeiss, Jena, Germany).

Results

15-(R/S)-LXA₄ and 16-Phenoxy-LXA₄ Inhibit Mitogen-Induced Proliferation of MCs

Serum, PDGF, and EGF induced maximal DNA synthesis in primary human MC by 48 hours poststimulation. The mitogenic response of MCs to PDGF-BB and EGF was dose-dependent, with an apparent maximal concentration of 100 ng/ml and 250 ng/ml and an EC₅₀ of 8 ng/ml and 44 ng/ml, respectively. In subsequent experiments, PDGF-BB and EGF were applied at 10 ng/ml and 50 ng/ml concentrations, respectively, which consistently induced a minimum of a 2.5-fold increase in MC DNA synthesis. Incubation of MCs with increasing concentrations of LXA₄ did not induce MC DNA synthesis at any concentration tested (10⁻¹² M to 10⁻⁸ M). Preincubation of quiescent MC with LXA₄ (1 nmol/L) significantly inhibited mitogenesis induced by PDGF (10 ng/ml), as assessed by [³H]-thymidine uptake (Figure 1A). (The effect of LXA₄ was found to be dose-dependent [10 pM to 1 $\mu\text{mol}/\text{L}$, data not shown] with an EC₅₀ of approximately 10 nmol/L LXA₄, in contrast to other LX responses,⁹ the dose:response was not bell-shaped). This effect was replicated by 16-phenoxy-LXA₄ (10 pM), a stable synthetic analog of LXA₄, and 15-(R/S)-LXA₄ (10 pM), a stable synthetic analog of ATL (Figure 1A). These data indicate that stable LX analog can act as mimetics of endogenous and aspirin-triggered LXA₄ in the context of counter regulation of mitogenic signals. AG1296, the tyrophostin inhibitor of PDGF receptor tyrosine kinase activity was included as a negative control in these experiments.¹⁸

To investigate whether the effect was specific to PDGF-induced proliferation we stimulated cells with LX analogs before treatment with both EGF and serum. As Figure 1B depicts, this anti-proliferative effect was replicated on EGF (50 ng/ml)-stimulated MC proliferation ($n = 2$). The reduction in DNA synthesis by LX on EGF-treated MC was less than the reduction seen in PDGF-stimulated MC (25% reduction vs. 48% reduction, respectively). Analogous to data with AG1296, the inhibitor of EGF receptor tyrosine kinase activity, AG1478, inhibited EGF-stimulated MC proliferation.

Figure 1. Inhibition of PDGF and EGF-stimulated MC DNA synthesis by lipoxins. **A** and **B**: Subconfluent human MCs were serum restricted in 0.2% FCS medium for 48 hours before stimulation with LXA₄ (1 nmol/L), 15-(R/S)-LXA₄ (10 pM), 16-phenoxyl-LXA₄ (10 pM), AG1296 (10 μmol/L) (**A**), AG1478 (100 nmol/L) (**B**), for 60 minutes before stimulation with PDGF-BB (10 ng/ml) (**A**) and EGF (50 ng/ml) (**B**). Cell proliferation was measured by [³H]-thymidine incorporation after 48 hours. Vehicle (EtOH <0.1%) and 10% FCS were used as negative and positive controls, respectively. Results are expressed as fold/basal cell proliferation. Data represent the mean ± SD of four (**A**) and two (**B**) independent experiments performed in triplicate. ** *P* < 0.01 vs. vehicle, * *P* < 0.05 vs. PDGF alone (unpaired Student's *t*-test). **C**: Inhibition of serum-stimulated CHO K1-ALXR proliferation by LXA₄ and stable synthetic analogs, effect not seen in vector control cell line. CHO K1 cells (± ALXR) were preincubated with LXA₄ (1 nmol/L), 15-(R/S)-LXA₄ (10 pM), 16-phenoxyl-LXA₄ (10 pM), for 60 minutes before stimulation with serum-containing media. Cell proliferation was measured by [³H]-thymidine incorporation after 48 hours. Results are expressed as percentage cell proliferation. Data are mean ± SEM of four independent experiments performed in triplicate. * *P* < 0.05 vs. 10% serum alone (unpaired Student's *t*-test).

The antiproliferative effect of LXA₄ analogs was also observed in serum-stimulated CHO K1 cells stably expressing the lipoxin A₄ receptor (ALXR) but not in control

cells (Figure 1C). Preincubation of quiescent CHO K1 ± ALXR with LXA₄ (1 nmol/L, 60') significantly (*P* < 0.05, unpaired Student's *t*-test) inhibited mitogenesis (approximately 20% reduction) induced by serum, an effect replicated by 15-(R/S)-methyl-LXA₄ (10 pM) and 16-phenoxyl-LXA₄ (10 pM). Further investigation of the antiproliferative effects of LX using an assay based on reduction of a tetrazolium component (MTT) to an insoluble formazan product indicate that LXA₄ inhibition of serum-stimulated proliferation does not involve promotion of apoptosis (data not shown).

LXA₄ Modulates Mitogen-Induced Activation of Akt

We have previously observed a reduction in phosphorylation of the PDGFR in MC by LXA₄.¹⁶ This, in conjunction with data showing LXA₄-induced inhibition of LTD₄-stimulated PI-3-kinase activation⁹ and p85 subunit recruitment to the PDGFR,¹⁶ suggested that the PI3-kinase pathway may be a locus of the inhibitory effect of LXA₄ in MC. As indicated in Figure 2, both PDGF (10 ng/ml) (**A**) and EGF (50 ng/ml) (**B**) stimulated activation of Akt via phosphorylation at Ser⁴⁷³. Both PDGF- and EGF-stimulated activation was inhibited by preincubation of MC with LXA₄ (10 nmol/L, 30 minutes and 60 minutes) and by a specific inhibitor of PI-3 K activity, LY294002 (3 μmol/L). The LXA₄-dependent inhibition was not mimicked by two other GPCR-coupled agonists, LTD₄ (10 nmol/L) (**A**) and 5-HT (10 μmol/L) (**B**) which have been previously shown to be involved in cross-talk with PDGF and EGF RTK, respectively, in mesangial cells.^{9,19} Expression of the receptors for both of these GPCR agonists has been previously demonstrated in MC.^{16,20} The observation that LXA₄ inhibition of PDGF and EGF-stimulated proliferation is coupled to modulation of Akt phosphorylation is noteworthy given that expression of dominant-negative Akt in rat mesangial cells inhibits mitogenic responses to PDGF.²¹

PDGF Decreases CKI p21^{Cip1} and p27^{Kip1} Levels: Modulation by LXA₄

The levels of G₁ phase CKI p21^{Cip1} and p27^{Kip1} in quiescent and proliferating MC were determined by western blot analysis (Figure 3A). Quiescent MCs were stimulated with LXA₄ (10 nmol/L, 60 minutes) or vehicle before addition of PDGF-BB (10 ng/ml, 24 hours). Detectable levels of p21^{Cip1} and p27^{Kip1} were expressed in quiescent MCs and no change in their levels was observed over a 24-hour period in vehicle or LXA₄-treated MC (data not shown). However, incubation with PDGF caused a decrease in detectable levels of both p21^{Cip1} and p27^{Kip1} at 24 hours poststimulation (Figure 3A). These decreases were significantly abrogated by preincubation with LXA₄ (10 nmol/L, 60 minutes) or by co-stimulation with TGFβ₁. TGFβ₁ has previously been shown to act in an anti-mitotic manner in PDGF-stimulated MC, acting on G₁-phase cell cycle proteins.²² In MC, TGFβ₁-treated cells showed no effect on levels of p21^{Cip1} but showed an increase in

Figure 2. Akt phosphorylation is modulated by LX₄. Subconfluent cultures of MC were made quiescent in 0.2% FCS medium for 48 hours. MC were then incubated with LX₄ (10 nmol/L, 30', 60'), LY29002 (3 μ mol/L, 30'), LTD₄ (10 nmol/L, 30') (A), 5-HT (10 μ mol/L, 30') (B) before stimulation with PDGF-BB (10 ng/ml, 5') (A) or EGF (50 ng/ml, 5') (B). Forty microns of protein were electrophoresed and transferred onto PVDF membrane and immunoblotted for P-Akt (Ser⁴⁷³) or Akt. Results are depicted graphically and represent the mean $\pm$ SD for three independent experiments. Values given are fold/basal relative to vehicle-treated cells. * $P < 0.001$ relative to vehicle, # $P < 0.05$ relative to PDGF-stimulated cells.

levels of p27^{Kip1} at 24 and 48 hours (data not shown), which is in agreement with previous data.²³ Consistent with our data showing inhibition of PDGF-stimulated MC proliferation by the stable synthetic LX analogs (Figure 1), we found that 15-(R/S)-LXA₄ and 16-phenoxy-LXA₄ replicated the effect of LX₄ on cell cycle protein expression. As Figure 3B depicts, the PDGF-induced reduction in levels of p21^{Cip1} and p27^{Kip1} at 24 hours was inhibited by preincubations with 15-(R/S)-LXA₄ (10 pM) or 16-phenoxy-LXA₄ (10 pM).

PDGF Stimulates G1-S Phase Transition: Cyclin E-CDK2 Complex Formation Is Attenuated by LX₄

The levels of G₁ associated proteins, CDK2 and cyclin E were determined by Western blot (Figure 4). PDGF stimulation resulted in increased levels of cyclin E at 24 hours and CDK2 at 6 and 24 hours (A). These increases were

Figure 3. LX regulate PDGF-induced reduction in protein levels of G1 phase. CKI p21^{Cip1} and p27^{Kip1} in human MC. A: Subconfluent cultures of human MC were serum restricted in 0.2% FCS medium for 48 hours. Quiescent MC were then stimulated with LX₄ (10 nmol/L, 60') or vehicle as indicated, before addition of PDGF-BB (10 ng/ml). PDGF-treated MC were co-incubated with TGF β ₁ (10 ng/ml) to act as a negative control. MCs were harvested and protein was extracted at 6- and 24-hour intervals poststimulation. Thirty microns protein per lane was resolved by SDS-PAGE and levels of p21^{Cip1} and p27^{Kip1} were determined by Western blot. β -actin protein was examined as a loading control. Results are depicted graphically and represent the mean $\pm$ SD of three independent experiments. Values given are fold/basal relative to vehicle-treated cells. * $P < 0.01$ relative to vehicle, # $P < 0.05$ relative to PDGF-stimulated cells. B: LX₄ stable analogs mimic effect of native LX₄ on PDGF-induced reduction in p21^{Cip1} and p27^{Kip1} protein levels. Quiescent MCs were preincubated with LX₄ (10 nmol/L), 15-(R/S)-methyl-LXA₄ (10 pM), 16-phenoxy-LXA₄ (10 pM) for 60 minutes before treatment with PDGF-BB (10 ng/ml) for 24 hours. Results are depicted graphically and represent the mean $\pm$ SD of two independent experiments. Values given are fold/basal relative to vehicle-treated cells.

significantly abrogated by pre-incubation with LX₄. Vehicle-treated MC or MC stimulated with LX₄ (10 nmol/L) alone at 6 and 24 hours showed no alteration in protein levels of either cyclin E or CDK2 (data not shown). Additionally, cyclin A levels were increased by PDGF stimulation at 24 hours (data not shown). However, levels of cyclin A were not attenuated by LX₄ or TGF β ₁.

Due to the observation that LX₄ significantly inhibited PDGF-stimulated levels of CDK2 and cyclin E, we examined the effect of LX₄ on mitogen-induced cyclin E

Figure 4. LXA₄ regulates PDGF-induced protein levels of G₁ phase CDK2 and cyclin E and CDK2-cyclin E complex formation in human MC. **A:** Subconfluent cultures of human MC were serum-restricted in 0.2% FCS medium for 48 hours. Quiescent MC were then stimulated with LXA₄ (10 nmol/L, 60') or vehicle as indicated, before addition of PDGF-BB (10 ng/ml). PDGF-treated MC were co-incubated with TGFβ₁ (10 ng/ml) to act as a negative control. MCs were harvested and protein was extracted at 6- and 24-hour intervals after stimulation. Thirty microns of protein per lane were size-fractionated by SDS-PAGE and protein expression of cyclin E and CDK2 was determined by Western blot. Expression of β-actin protein was examined as a loading control. Results are depicted graphically and represent the mean ± SD for three independent experiments. Values given are fold/basal relative to vehicle-treated cells. * *P* < 0.01 relative to vehicle, # *P* < 0.05 relative to PDGF-stimulated cells. **B:** The protein from the above conditions was immunoprecipitated with polyclonal antibody to CDK2. The immunoprecipitated lysate was divided in half, separated on an SDS-PAGE, and a Western blot analysis was performed with an antibody to CDK2 and cyclin E. Results are depicted graphically and represent the mean ± SD of two independent experiments. Values given are fold/basal relative to vehicle-treated cells.

association with CDK2 to form an active complex, an important step for progression of cells from G₁ into S phase.²⁴ As depicted in Figure 4B, PDGF induced association of cyclin E and CDK2 at 24 hours. Preincubation of PDGF-stimulated MCs with LXA₄ (10 nmol/L, 60 minutes) inhibits the formation of this complex. To act as a control in this experiment, PDGF-stimulated MCs co-stimulated with TGFβ₁ (10 ng/ml) showed a modulation of assembly of cyclin E-CDK2 complexes at 24 hours, as previously reported.²³ Incubation of unstimulated control cells with LXA₄ or TGFβ₁ had no effect on cyclin E-CDK2 association (data not shown).

PDGF-Stimulated Production of TGFβ₁ Is Blocked by LXA₄

It has previously been shown that PDGF stimulates production of TGFβ₁ from MC.²⁵ Consistent with this, an ELISA for levels of secreted TGFβ₁ on samples of cell-free medium from PDGF-stimulated MC indicated a significant (*P* < 0.001) increase in autocrine production of TGFβ₁ in a time-dependent manner (24 to 48 hours, Figure 5A; data are expressed as picograms of TGFβ₁ produced per microgram of protein present and are consistent with values reported by others²⁵). TGFβ₁ release reached a peak of 2.3 ± 0.2 pg/μg (mean ± SEM) at 48 hours poststimulation with PDGF (10 ng/ml). LXA₄ (10 nmol/L) reduced the level of autocrine production of TGFβ₁ at 24 and 48 hours to basal levels. Extending the timecourse to 72 hours, we also observed a significant decrease (84% reduction) in PDGF-stimulated TGFβ₁ production (data not shown).

TGFβ₁ has previously been shown to promote MC cell cycle arrest via up-regulation of p27^{Kip1}.²⁴ Consistent with enhanced TGFβ₁ production from PDGF-treated cells (Figure 5A), we observed a reversal of the initial diminution in p27^{Kip1} levels at 48 hours poststimulation with PDGF (Figure 5B). In agreement with our hypothesis that this effect may be mediated through PDGF-stimulated autocrine production of TGFβ₁, we observe that this effect is blocked with an inhibitory TGFβ₁ antibody (Figure 5C).²⁶

Akt Mediates the Subcellular Localization of p27^{Kip1}: Modulation by LXA₄

We have investigated the subcellular location of the CKI p27^{Kip1} in growth factor-stimulated cells. Cells were either processed for indirect immunofluorescence or fractionated to enrich for nuclear and cytoplasmic proteins. Figure 6A shows a representative example of immunofluorescence. In quiescent (vehicle-treated) cells, p27^{Kip1} localized to the nucleus (90% of cells, counted in two separate fields, 10× magnification, 50 colonies/field, two independent observers). Nuclear staining was confirmed by staining with DAPI stain (data not shown). On stimulation with PDGF an increase in the levels of cytoplasmic p27^{Kip1} was observed, this effect was inhibited by preincubation with LXA₄. PI-3-kinase and subsequent Akt phosphorylation has been proposed to regulate stability of p21^{Cip1} and p27^{Kip1} by promoting nuclear to cytoplasmic translocation and subsequent degradation.²⁷⁻²⁹ We observe that in MC exposed to the PI-3-kinase inhibitor LY294002, PDGF did not promote cytoplasmic (6A) or cytosolic translocation of p27^{Kip1} (6B). Our observations are consistent with a role for p27^{Kip1} export from the nucleus in regulating proliferation.

Discussion

MC proliferation plays a key role in glomerular inflammation. Proliferative responses to a variety of stimuli are

associated with matrix accumulation and the develop-

Figure 5. PDGF-stimulated TGFβ₁ production is attenuated by LXA₄. **A:** Quantitative analysis of TGFβ₁ ELISA performed on supernatants of cells at indicated time points. Results are expressed as picograms per microns of MC protein. Data are mean ± SD of seven independent experiments (**P* < 0.001 vs. vehicle, # *P* < 0.05 vs. PDGF alone, unpaired Student's *t*-test). **B:** Subconfluent cultures of human MC were serum restricted in 0.2% FCS medium for 48 hours. Quiescent MC were then stimulated with LXA₄ (10 nmol/L, 60') or vehicle as indicated, before addition of PDGF-BB (10 ng/ml). MCs were harvested and protein was extracted at 6-, 24-, and 48-hour intervals after stimulation. Thirty microns of protein per lane was size-fractionated by SDS-PAGE and protein expression of p27^{Kip1} was determined by western blot. Expression of β-actin protein was examined as a quantity loading control. **C:** Western blot analysis at 48 hours poststimulation with above conditions. MCs were stimulated with vehicle, LXA₄ (10 nmol/L), PDGF-BB (10 ng/ml) for 48 hours, or pretreated with LXA₄ (10 nmol/L) or TGFβ₁ monoclonal antibody (1 nmol/L) for 60 minutes before stimulation with PDGF-BB for 48 hours. Results of **B** and **C** are depicted graphically and represent the mean ± SD of three independent experiments. Values given are fold/basal relative to vehicle-treated cells. * *P* < 0.01 relative to vehicle, ** *P* < 0.05 relative to vehicle, # *P* < 0.05 relative to PDGF-stimulated cells (24 hours), ## *P* < 0.05 relative to PDGF-stimulated cells (48 hours).

ment of glomerulosclerosis, which may lead to interstitial fibrosis, the final common pathway of progressive renal disease.³⁰ In this context it is noteworthy that modulation of MC proliferation is considered an attractive target for therapeutic intervention. PDGF-BB, -AB and -DD, ligands for the PDGFRβ, evoke proliferation of MC.¹² A key role for these agents produced either locally or systemically has been demonstrated in renal inflammation.¹⁴ Several therapeutic strategies have been proposed to limit PDGF-induced renal damage, including inhibition of either PDGF ligand activity with anti-PDGF antibody,¹⁰ or PDGF receptor activation by the tyrosine kinase inhibitor STI 571.¹³

LX have been proposed as important, endogenously produced pro-resolution agents in host defense and inflammation.¹ In the current study we have investigated the mechanisms underlying LX inhibition of mesangial cell proliferation in response to various agents including PDGF and EGF. We report that, similar to the endogenously produced LXA₄, the stable synthetic analog 16-phenoxyl-LXA₄ and the stable analog of ATL, 15-(*R/S*)-methyl-LXA₄, can modulate MC proliferation. Evidence that these effects are mediated through the previously described ALXR are provided by the observation that serum-stimulated proliferation of CHOK1 cells stably expressing the ALXR was attenuated by both 16-phenoxyl-LXA₄ and 15-(*R/S*)-methyl-LXA₄.

We have investigated the effects of LX on levels of cell cycle proteins. The modulation of proliferation by LX might reflect arrest at G₀-G₁ or G₁-S phase transitions of the cell cycle. Under appropriate stimuli, cells exit quiescence (G₀) and enter the cycle at early G₁ phase. In late G₁ phase, cells pass through the "restriction point" marking the commitment point after which they are no longer sensitive to extracellular signals. Traversing the G₁-S phase is coupled to DNA synthesis, followed by entry into G₂ and finally mitosis occurs in M phase.³¹ Progression through the cycle is dependent on the complex interaction of multiple regulatory proteins. The mechanisms of regulation of the G₁ phase cell cycle involve the interaction of cyclin-CDK complexes with CDK inhibitors (CKI).³² In MC, a role for decreases in the levels of CKI has been demonstrated in response to PDGF.²³ These CKI play an important role regulating proliferation. Both p21^{Cip1} and p27^{Kip1} contain binding domains for CDK which may interfere with the ability of CDK to form active complexes with cyclins.³³ Furthermore, p21^{Cip1} can also bind to PCNA, an auxiliary protein to DNA polymerase, either directly or in a quaternary complex with PCNA, cyclins, and CDK.³⁴ Here, we demonstrate that LX attenuate PDGF-induced decrements in p21^{Cip1} and p27^{Kip1}. Furthermore, the decreases in p21^{Cip1} and p27^{Kip1} levels that we observe in PDGF-stimulated cells are coupled to increased levels of cyclin E and establishment of cyclin E-CDK2 complexes, indicative of progression through G₁-S. LXA₄ inhibited this effect, an observation consistent with maintaining levels of CKIs. It is noteworthy that Shankland and colleagues³⁵ have proposed that the resolution of glomerular inflammation may be associated with a return of depleted p27^{Kip1} levels. We have previously reported that LX directly attenuated PDGF and EGF

Figure 6. PDGF increases cytosolic levels of p27^{Kip1}. Influence of LXa₄ and LY294002. **A:** Subcellular localization of p27^{Kip1} detected by immunocytochemistry. Quiescent MCs on coverslips were incubated with LXa₄ (10 nmol/L), LY294002 (3 μ mol/L) or vehicle for 30 minutes before stimulation with PDGF (10 ng/ml). At 24 hours, fixed cells were stained with polyclonal antibody against p27^{Kip1} (green) or phase contrast. **B:** Cytosolic transition of p27^{Kip1}. Quiescent MCs were incubated with LXa₄ (10 nmol/L), LY294002 (3 μ mol/L) or vehicle for 30 minutes before stimulation with PDGF (10 ng/ml). After 24 hours, cytosolic and nuclear fractions were extracted. Twenty-five microns of protein per lane were size-fractionated by SDS-PAGE and cytoplasmic levels of p27^{Kip1} were determined by Western blot. Results are depicted graphically and represent the mean $\pm$ SD of three independent experiments. Values given are fold/basal relative to vehicle-treated cells. * $P < 0.05$ relative to vehicle, # $P < 0.05$ relative to PDGF-stimulated cells.

receptor activation.¹⁶ The underlying mechanisms are not clear and may involve altered phosphatase activity or endocytic trafficking of the RTKs in response to LX.

We have investigated signaling events downstream of receptor activation and report LX modulation of Akt activation in response to PDGF and EGF. Akt is a target of PI-3-kinase where binding of D3-phosphorylated inositides to the PH domain of Akt stimulates phosphorylation of the protein.³⁶ The precise role of Akt in regulating proliferation is unclear; however, recent data suggest that Akt-mediated phosphorylation of p27^{Kip1} may facilitate its translocation from the nucleus to the cytoplasm where the phosphorylated protein is a target for proteasomal degradation.^{27–29} Using an analogous mechanism to that proposed for p27^{Kip1}, Akt can also promote p21^{Cip1} degradation via MDM2 phosphorylation of p53.³⁷ Translocation of CKIs from the nucleus removes repression of cyclin E-CDK2 activity and the cell cycle proceeds through G₁-S. There is also evidence for the importance of a trimeric complex with CDK in regulating ubiquitination of p27^{Kip1}. The elevated levels of the cyclin E-CDK2 complex observed in proliferating MC may promote proteasomal degradation of CKIs.³⁸ Consistent with a role for p27^{Kip1} export from the nucleus in regulating proliferation are our observations using immunostaining. Under basal (quiescent) conditions p27^{Kip1} is observed as a discrete focus in the nucleus. However, on stimulation with PDGF

this becomes cytoplasmic. These data are corroborated by immunoblotting of cytosolic fractions showing increased levels of p27^{Kip1}. In addition to the proposed role of Akt in promoting degradation of p27^{Kip1} in MC, it may also exert an effect on transcription through phosphorylation-dependent inhibition of the forkhead family of transcription factors.³⁹ Controversy exists over whether p21^{Cip1} and p27^{Kip1} follow similar patterns of activation and down-regulation in MC and in models of glomerular disease.⁴⁰ We observe similar down-regulation patterns of both CKI in response to PDGF and maintenance of levels of both CKI in response to preincubations with LX. We propose a model where PDGF stimulation of human mesangial cells is coupled to PI-3-kinase and Akt activation, resulting in phosphorylation of p27^{Kip1} and its translocation from the nucleus, removing the repression on cyclin E-CDK2 activity (see Figure 7). In cells treated with LX, inhibition of PDGF receptor phosphorylation is coupled to an attenuation of downstream PI-3-kinase activity and subsequent Akt phosphorylation. In this scenario, p27^{Kip1} remains within the nucleus where it exerts its inhibitory effects on the activity of cyclin E-CDK2 complexes. After 48-hour stimulation with PDGF we observed a recovery of p27^{Kip1} to basal levels. We propose that this may be due to autocrine production of TGF β ₁. TGF β ₁ is known to prevent PDGF induced decreases in p27^{Kip1} levels.²² Recent evidence demonstrates that the growth-

Figure 7. Proposed mechanism of LX modulation of PDGF-stimulated MC proliferation. PDGF stimulation of PDGFR β in human mesangial cells is coupled to PI-3-kinase and Akt activation, resulting in phosphorylation of p27^{Kip1} and its translocation from the nucleus, removing the repression on cyclin E-CDK2 activity. In cells treated with LX, inhibition of PDGF receptor phosphorylation is coupled to an attenuation of subsequent Akt phosphorylation and downstream events.

arrest and hypertrophic effects of TGF β ₁ are dissociated.⁴¹ This may be important in the context of LX, suggesting that mimicry of TGF β ₁ with respect to maintenance of p21^{Cip1} and p27^{Kip1} levels does not implicate LX as profibrotic indeed, LX significantly attenuated PDGF induced TGF β ₁ production.

The results presented here are of interest in the context of the bioactions of non-steroidal anti-inflammatory drugs (NSAIDs).⁴² Whereas the nonselective NSAID aspirin can provoke the generation of 15-epi-LX, thereby acting to promote the resolution of inflammation,^{43,44} the action of COX-2-specific inhibitors in impeding 15(R)-HETE production may compromise the pro-resolution potential of endogenously produced eicosanoids. Our data elaborate on the potential of LX and ATLs as agents that are not only anti-inflammatory and pro-resolution but may also act as anti-fibrotic agents.

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