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Tubular Cell Phenotype in HIV-Associated Nephropathy: Role of Phospholipid Lysophosphatidic Acid

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Abstract

Collapsing glomerulopathy and microcysts are characteristic histological features of HIV-associated nephropathy (HIVAN). We have previously reported the role of epithelial mesenchymal transition (EMT) in the development of glomerular and tubular cell phenotypes in HIVAN. Since persistent tubular cell activation of NFκB has been reported in HIVAN, we now hypothesize that HIV may be contributing to tubular cell phenotype via lysophosphatidic acid (LPA) mediated downstream signaling. Interestingly, LPA and its receptors have also been implicated in the tubular interstitial cell fibrosis (TIF) and cyst formation in autosomal dominant polycystic kidney disease (PKD). Primary human proximal tubular cells (HRPTCs) were transduced with either empty vector (EV/HRPTCs), HIV (HIV/HRPTCs) or treated with LPA (LPA/HRPTC). Immunoelectrophoresis of HIV/HRPTCs and LPA/HRPTCs displayed enhanced expression of pro-fibrotic markers: a) fibronectin (2.25 fold), b) connective tissue growth factor (CTGF; 4.8 fold), c) α-smooth muscle actin (α-SMA; 12 fold), and d) collagen I (5.7 fold). HIV enhanced tubular cell phosphorylation of ILK-1, FAK, PI3K, Akt, ERKs and P38 MAPK. HIV increased tubular cell transcriptional binding activity of NF-κB; whereas, a LPA biosynthesis inhibitor (AACOCF3), a DAG Kinase inhibitor, a LPA receptor blocker (Ki16425), a NF-κB inhibitor (PDTC) and NFκB-siRNA not only displayed down regulation of a NFκB activity but also showed attenuated expression of profibrotic/EMT genes in HIV milieu. These findings suggest that LPA could be contributing to HIV-induced tubular cell phenotype via NFκB activation in HIVAN.

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Competing interests

The authors declare that they have no competing interests.

Author's Contributions

KRA, performed biochemical experiments, while PR helped KRA in the experiments; AM and MH analyzed the results and helped in writing the manuscript. KRA and PCS conceived the experimental plan, analyzed the results and wrote the manuscript.

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Graphical abstract

Keywords

Lysophosphatidic acid (LPA); p-38 Kinase; Microcysts; Nuclear factor κ B (NF κ B); Connective tissue growth factor (CTGF); Collagen-I; HIV associated Nephropathy (HIVAN). Epithelial Mesenchymal Transition (EMT)

Introduction

Renal tubular epithelial cell infection and microcyst formation have been reported in HIV associated nephropathy (HIVAN). It has been suggested that HIV-1 gene transcript increases as tubules dilate and the epithelium becomes flattened and atrophic [1–3]. To date, the fate of the segments that become infected by HIV-1 and the ones which develop microcysts in HIVAN is not clearly understood. The molecular mechanism by which the viral gene expression leads to microcyst formation is far from clear. We have earlier demonstrated the role of epithelial mesenchymal transition for proliferative phenotype for both glomerular and tubular cells in HIVAN (4, 5).

Renal fibrosis is characterized by glomerulosclerosis and tubulointerstitial fibrosis (TIF) with accumulation of extracellular matrix (collagens and fibronectin) and infiltration of macrophages and lymphocytes [3–4, 6]. During the last decade, connective tissue growth factor (CTGF) has been implicated to have a dominant role either directly or indirectly in mediating tubular cell injury [4–6]. EMT plays a critical role in renal fibrosis. Tightly bound epithelial cells get detached following loss of cell polarity and get converted to mesenchymal cells expressing mesenchymal proteins and develop migratory potential. In kidney and lung, collagen and CTGF are excessively expressed. CTGF is a secreted matricellular protein which plays an important role in pathogenesis of chronic fibrotic diseases.

Recent reports demonstrate significant involvement of phospholipids in the development of fibrosis including platelet activating factor (PAF), phosphatidyl choline, and lysophosphatic acid (LPA) [7–8]. Cystic fluid in patients of Polycystic Kidney Disease (PKD) has been shown to have increased levels of LPA [9]. LPA seeps to vascular as well as interstitial spaces and promotes cyst enlargement. LPA is known to regulate several cellular processes including cell motility, proliferation, survival, and differentiation [9–10]. LPA acts via specific G-protein coupled receptors (LPA1 to LPA5) [10]. However, the metabolic origin of LPA remains to be elucidated. Based on biosynthetic pathways, several enzymes involved seem to be activated such as phospholipases A1/A2, lysophospholipase D/autotoxin (ATX), glycerol phosphate acyl transferase, or monoacyl glycerol kinase (MAGK)-all leading to increased LPA synthesis [11].

In the present study, we have demonstrated that HIV-transduced/LPA treated-HRPTCs display an increased expression of molecular markers of profibrotic/EMT phenotype; moreover, HIV-and LPA-induced downstream signaling events are ILK-FAK dependent and result in NF κ B activation and gene transcription.

Methods

Cells and viruses

Human renal proximal tubular cells (HRPTCs) were obtained from ScienCell Research Labs (Carlsbad, CA, USA). HRPTCS were cultured using EpiCM medium (ScienCell). Cells were maintained at 37°C, 5% CO₂ in a humidified incubator. LPA was purchased from Avanti Polar Lipids (Alabaster, AL). LPA was prepared in PBS containing 0.1% BSA (vol/vol) and sonicated before use. HRPTCS, Control (C/HRPTCs) and Empty vector (EV/HRPTC) or treated with HIV/LPA [(HIV/HRPTC)/LPA/HRPTCs] wherever indicated were used in the study.

Production of pseudotyped retroviral supernatant

Replication-defective viral supernatants were prepared as published previously [3]. Briefly, green fluorescence protein (GFP) reporter gene (from pEGFP-C1; Clontech, Palo Alto, CA) was substituted in place of *gag/pol* genes in HIV-1 proviral construct pNL4-3. This parental construct (pNL4-3: G/P-GFP) was used to produce VSV.G pseudotyped viruses to provide pleiotropism and high-titer virus stocks. Infectious viral supernatants were produced by the

transient transfection of 293T cells using effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions. The HIV-1 *gag/pol* and VSV.G envelope genes were provided in *trans* using pCMV R8.91 and pMD.G plasmids, (gifts by Dr. Didier Trono, Salk Institute, La Jolla, CA). Viral stocks ranging from 10^5 to 10^6 GEU/ml were obtained.

Transfection

HRPTCs were transfected using lipofectamine plus reagent according to the manufacturer's protocol with a total of 1 µg/well of plasmid DNA. Twenty-four hours later, the cells were treated with HIV or LPA (24 hr), followed by further incubation at 37°C. For NFκB-luciferase activity, HRPTCs were transfected with NFκB-luciferase reporter plasmid and/or using p65 DN plasmid with pCMV-β-gal by Lipofectamine Plus. pcDNA3 was used to normalize all groups to equal amounts of DNA Luciferase (Promega, Madison, WI) further normalizing with β-galactosidase activity. NFκB-luciferase, DN-p65 plasmids were kindly provided by Dr. George Rawadi (Institute Pasteur, Laboratoire des Mycoplasmes, Paris, France) (12). The expression vector for flag-IKKα was a gift from Dr Zheng-Gang Liu (National Institutes of Health, Bethesda, MD).

Silencing of NFκB—HRPTCs were transfected with 25–50 nM NFκB small interfering (Si) RNA (Santa-Cruz Biotechnology; Santa Cruz, CA) with Siport Neofax transfection reagent and left in optiMEM medium for 24–48h and the cells were transferred back to HRPTC medium an hour before transfection with NL4-3 GFP.

Immunodetection by Western blot

HRPTCs, HIV/HRPTCs, and EV/HRPTCs were incubated in medium for 3 days. Cells were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 1× protease inhibitor cocktail I (Calbiochem, EMD Biosciences, Gibbstan, NJ), 1 mM PMSF, and 0.2 mM sodium orthovanadate. Protein concentration was determined using the Biorad Protein Assay (Pierce, Rockford, IL). Protein lysates (20 µg) were separated on 12% polyacrylamide gels (PAGE, Bio-Rad, Hercules, CA) and transferred onto a nitrocellulose membrane using Bio-Rad miniblott apparatus. Nitrocellulose membranes were then subjected to immunostaining with primary antibodies against CTGF, TGF-β, fibronectin, vimentin, α-SMA and SNAIL (Santa Cruz Biotechnology, Dallas, TX, USA), NFκB pathway proteins (phosphospecific, Cell Signaling, Danvers, MA), p-ILK1, and p-FAK (EMD Millipore, Billerica, MA, USA), and subsequently with horseradish peroxidase-labeled appropriate secondary antibodies (Biorad, Hercules, CA). The blots were developed using a chemiluminescence detection kit (ThermoScientific, Rockford, IL, USA) and exposed to X-ray film (Eastman Kodak, Rochester, NY). Equal protein loading was confirmed by stripping and reprobed the same blots immunoblotting for β-actin protein. For quantification, the immunoblots were scanned, and densitometry was performed by Image J analysis; values were normalized to β-actin expression and expressed as fold increase when compared to control values as shown.

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

Nuclear extracts from control and experimental cells (1×10^7) were prepared as described previously [12–14]. Aliquots (1µg) were used for the electrophoretic mobility shift assay

using the NF κ B DNA-binding protein detection system kit (Affymetrix). Briefly, the protein-binding biotinylated DNA probes (NF κ B) were incubated with nuclear extracts prepared from control and experimental cells according to the manufacturer's protocol (Panomics, Redwood City, CA). The DNA-protein binding reactions were performed at room temperature for 10 min in 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol plus 1 μ g of poly (dI-dC), 5% (v/v) glycerol, and ~10 ng of biotinylated NF κ B probe. Protein DNA complexes were resolved from protein-free DNA on 6 % polyacrylamide gels (Invitrogen) at 4° C in 50 mM Tris, pH 8.3, 2 mM EDTA. DNA-protein complexes and rest of the gel contents were transferred to Biodyne B membrane (Pall, Ann Arbor, MI) for 60 min at 300 mA. The membranes now containing the DNA-protein complexes were UV cross linked and chemiluminescent detection of biotinylated DNA was performed using the Panomics EMSA kit.

Results

HIV/HRPTCs and LPA/HRPTCs show higher expression of fibronectin, CTGF, collagen I, α -SMA, and vimentin

HIV expressing tubular cells and LPA-treated tubular cells displayed similar molecular phenotype (Fig. 1A). LPA inhibitors and a LPA receptor inhibitor (Ki16425) attenuated the expression of profibrotic/EMT molecular markers. HIV enhanced tubular cell fibronectin by an average 2.3 (50–100 U) fold, whereas LPA enhanced tubular cell fibronectin expression by an average 1.7 (1 μ M–5 μ M) fold. Similarly, HIV enhanced tubular CTGF expression by an average 4.85 fold and LPA increased tubular cell expression of CTGF by an average 5.15 fold at two different concentrations. HIV increased tubular cell collagen-I expression by an average of 5.95 fold and LPA enhanced collagen-I expression by an average 5.75 fold. HIV enhanced tubular cell α -SMA expression by an average 12 fold and LPA enhanced tubular cell expression of α -SMA by an average 5.9 fold. Similarly, both HIV and LPA enhanced tubular cell expression of vimentin 1.85 and 1.6 folds at two different concentrations, respectively (Fig 1.B). HRPTCs were preincubated with either a LPA receptor blocker (Ki16425 1, 10 μ M), a PLA2 inhibitor, AAOCOCF3 (1 and 10 μ M), or a DAG kinase inhibitor (50, 150 nM) for 4 h prior to treatment with HIV or with LPA. After 72h, cells were harvested and protein blots were probed for fibronectin, collagen I, vimentin, α -SMA, SNAIL, and CTGF. HIV and LPA enhanced tubular cell expression of fibronectin, collagen I, α -SMA, SNAIL or CTGF by several fold (Fig. 2A) On the other hand, a LPA synthesis blockers and a LPA receptor inhibitor overtly attenuated the expression of fibronectin, α -SMA, SNAIL and CTGF (Approx 80%); however, decrease in tubular cell expression of collagen I and vimentin was quite modest in response to LPA inhibitors.

Both LPA and HIV enhanced tubular cell expression of p-ILK-1, p-FAK, p-PI3K, p-Akt, and pP38 MAPK, p-ERK, leading to p65 phosphorylation (NF κ B)

Several reports show the involvement of *Integrin Linked Kinase* (ILK), Focal Adhesion Kinase (FAK) pathways leading to the NF κ B activation [16]. The activation of ILK-1/FAK pathways has also been associated with EMT [17–18]. Immunoblots from LPA/HIV stimulated HRPTCs displayed enhanced expression of p-ILK, p-FAK, p-PI3K, p-p38, ERK, p-AKT ranging from 5 to 10 fold (Fig. 3). We observed several fold increases in the

phosphorylation status of these upstream kinases (ILK, FAK as well PI3K) by 6h while the downstream kinases displayed further persistent activation up to 3 days (p-Akt, ERK as well p-p38) (Fig. 3). We observed similar increases (5–10 fold) in the phosphorylated status of p65 (the NF κ B component) by day 1 and persistent increased levels up to day 5. HIV/HRPTCs or LPA/HRPTCs showed significant increases in the activation of both upstream as well downstream kinases leading to NF κ B activation (as shown in p-p65 phosphorylation status) and in the expression of the profibrotic/EMT genes such as fibronectin, CTGF, collagen-I, α -SMA and vimentin.

HIV enhanced transcriptional binding activity of NF- κ B

Lysates from HIV/HRPTCs (Fig. 3) were probed for molecular markers of canonical NF κ B pathway. According to current understanding, activation and phosphorylation of p65 involves activation of upstream IKK $\alpha/\beta/\gamma$ complex of proteins by upstream regulators often leading to phosphorylation of α/β subunits of the IKK complex, which subsequently phosphorylates the I κ B α –bound to p65-p50 subunit complex [12, 19–20]. Phosphorylation of I κ B complex enables to dissociate itself from p-65-p50 subunits, which otherwise are sequestered in the cytoplasm bound to I κ B- α . Detached p65-p50 subunit complex now migrates to the nucleus and then binds to κ B (kappa B) elements of the promoter regions of the transcribed genes. Phosphorylated p65 (p-p65), is an indication of transcriptionally active NF κ B complex. Immunoblot analysis of the lysates from HIV treated cells, demonstrated an increase in I κ B- α/β phosphorylation and upregulation of I κ B- α phosphorylation, thus leading to p-p65 phosphorylation (results not shown).

Persistent NF- κ B activation in renal epithelial cells has been reported in a mouse model of HIVAN [21]; however, the role of either HIV or LPA leading to the activation of profibrotic/EMT molecules involving the activation of NF κ B pathways in HIVAN has not been examined so far. We studied the effect of these various inhibitors of the LPA biosynthesis pathways (see Fig. 2A) on NF κ B activation via EMSA (Fig. 4). Both HIV and LPA increased the NF κ B binding activity in HIV/HRPTCs [Fig. 4A]. We had earlier reported sphingomyelinase (generation of ceramide) is redox sensitive and *vice versa* [14] and in conjunction with studies more recently that, HIV induced altered cellular redox [3], treatment of HRPTCs with either sphingomyelinase or with hydrogen peroxide led to a robust activation of NF κ B binding activity. Interestingly pretreatment with various inhibitors, both LPA blockers and a LPA receptor inhibitor (Ki16425) reduced NF κ B binding activity in HRPTCs [Fig. 4A]. This activation of NF κ B was also blocked by PDTC (an antioxidant as well as NF κ B blocker). To confirm the involvement of the canonical NF κ B pathway by p65 Rel A, we examined the effect of PDTC, and a p65 DN construct on NF κ B reporter activity (Fig. 4B). HRPTCs were transiently transfected with NF κ B-luciferase followed by treatment with or without PDTC in HIV milieu. After 4 h, the cell lysates were processed for luciferase activity. PDTC significantly inhibited the HIV induced NF κ B Luciferase activity in transfected HRPTCs. Control cells showed minimal basal activity. Further cells co-transfected with an expression vector of IKK- α , along with NF κ B luciferase reporter, were treated with HIV/PDTC. Luciferase activity in cells transiently expressing IKK α was further increased in comparison to control NF κ B reporter activity in HRPTCs, suggesting that LPA acts upstream of IKK α in the NF κ B pathway. The inhibitory

effect of PDTC in NF κ B luciferase reporter activity in HRPTC is due to its effect on NF κ B pathway as well as in part could be due to the down regulation of upstream events mainly by reducing an increase in altered cellular redox. Finally, in order to test if blocking NF κ B related events can result in down regulation of elevated gene expression, HRPTCs were transfected with p65 NF κ B siRNA or treated with PDTC, before stimulation with HIV (Fig. 4C). Results showed reduced expression of TGF- β , CTGF, fibronectin, and collagen-I, α -SMA and SNAIL. p65 siRNA/HRPTCs also displayed reduced expression of p65 (NF κ B). [Fig. 4]. These results confirm that NF κ B either directly or indirectly regulates HIV induced altered gene expression in HRPTCs.

Discussion

The present study demonstrates involvement of LPA in HIV mediated up regulation of profibrotic/EMT markers. We delineated the mechanisms involved in HIV activated and LPA mediated EMT/profibrotic gene expression in the involvement of FAK/ILK pathways and PI-3 kinase, ERK, Pkb/Akt activation of canonical NF- κ B pathway. Several experimental studies support the hypothesis that HIV induces upregulation of these various profibrotic/EMT mediators via LPA as well NF κ B activation. First, by use of LPA biosynthetic pathway inhibitors and LPA receptor blockers. Second, by inhibition of NF κ B activity by using a NF κ B blocker (PDTC) as well as silencing of NF κ B in tubular cells; both the interventions reduced the LPA/HIV-induced downstream signaling, and expression of profibrotic mediators (Fig. 5). These findings indicate that HIV and LPA activate several common signaling intermediary molecules (FAK/ILK pathway involving Pkb/Akt, PI-3 kinase, ERK and p38 kinases) leading to the activation of FAK/ILK and canonical NF κ B pathways. Our current study highlights a crucial regulatory role of the NF κ B, demonstrating 1) enhanced binding activity of NF κ B by either HIV or LPA treatment of HRPTCs, 2) reduction of NF κ B binding activity by LPA synthesis blockers and LPA receptor inhibitors, 3) down regulation of profibrotic/EMT gene expression by LPA synthesis blockers and LPA receptor inhibitors, 4) participation of various signaling intermediates such as FAK/ILK involving activation of downstream signaling molecules such as PI-3 K, Pkb/Akt, ERK, and p38 kinase, 5) reduction in NF κ B binding by cell signaling inhibitors, 6) reduction in the expression of tubular cell profibrotic protein markers by NF κ B inhibitor (PDTC) and silencing of NF κ B and 7) enhanced expression of NF κ B Luciferase activity, that can be significantly reduced by PDTC, and co-expression of dominant negative -p65 (DN-p65). All these observations suggest the participation of canonical NF κ B pathway which was confirmed by demonstration that over expression of IKK α causes significant increases in NF κ B Luc activity; however, this was effectively reduced in cells pretreated with PDTC or by co-expressing DN-P65. Our findings support the hypothesis that a) LPA plays a critical role in HIV mediated profibrotic and EMT events which may be contributing to microcyst formation in HIV milieu, b) HIV through LPA is involved in signaling events that activate FAK/ILK, PI-3-Kinase, Pkb/Akt, ERK, p38 kinases and involvement of canonical NF κ B activation cascades thereby leading to the enhanced transcription of these profibrotic proteins. Several recent reports suggest a cross talk between TGF- β pathways leading to an increase in ILK-1 activity and induction of EMT [17]. Although the current emphasis and focus is on TGF- β lead pathways, the present study demonstrates that HIV/LPA mediates

their effects via LPA receptor stimulated mechanisms which may or may not have a crosstalk with TGF β stimulated pathways. It reflects degeneracy in mechanisms related to growth and survival. CTGF stimulated pathways may function downstream or in parallel to TGF- β led pathways in leading the cells towards loss of their phenotype [22]. Several recent reports suggest a cross talk between TGF- β pathways leading to an increase in ILK-1 activity and an induction of EMT [17]. *Focal adhesion kinase* (FAK a downstream ILK linked signal transducer) yet another important molecule has also been associated with TGF- β -induced renal tubular EMT [18]. Overexpression of ILK correlated to aberrant expression in EMT markers such as SNAIL, in oral squamous cell carcinoma [23]. ILK has been implicated in high glucose-induced renal tubular cell injury [24]. Small interfering RNA targets ILK inhibited cancer metastasis [25]. Thus, ILK seems to be a key molecule in EMT and tubular cell injury.

Peptide mediators PDGF- β as well CTGF activate kidney pericytes and fibroblasts causing proliferation, collagen synthesis and fibrosis (26). These peptides are produced in abundance by tubules when they regenerate during reperfusion and ischemic injury. And unlike the normal proximal tubules, these tubular cells without differentiated features seem to express vimentin, an intermediary filament protein expressed by surviving cells as they dedifferentiate and proliferate after injury.

In summary, we have shown that both LPA and HIV lead tubular cells to express proteins related to pro-proliferative phenotype including SNAIL and vimentin. Several other reports suggest that promoter of NF κ B site may act as a positive regulatory element for the transcription of vimentin [21–22]. Interestingly, NF- κ B suppresses the expression of E-cadherin and desmoplakin and induces the expression of vimentin [27–28]. Similarly, together with Snail1, the p65 subunit of NF- κ B enhances fibronectin gene 29–30].

We conclude that HIV promotes tubular cell expression of EMT/profibrotic markers via LPA, and by upregulation of FAK/ILK, PI-3 kinase cascades involving Akt, p38 and ERK kinases, and thereby causing an activation of canonical NF κ B pathway.

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Abbreviations

HRPTC	Human renal proximal tubular cells
PL A 1/2	Phospholipase A 1/2
LysoPLD	Lysophospholipase D
PDTC	Pyrrolidine Dithiocarbamate
DAG	Di-acyl Glycerol
CKD	chronic kidney disease

ESRD	End stage renal disease (ESRD)
ECM	Extracellular matrix
TIF	Tubular interstitial fibrosis

References

1. Rao TK. Renal complications in HIV disease. *Med Clin North Am.* 1996 Nov; 80(6):1437–51. [PubMed: 8941230]
2. Ross MJ, Bruggeman LA, Wilson PD, Klotman PE. Microcyst formation and HIV-1 gene expression occur in multiple nephron segments in HIV-associated nephropathy. *J Am Soc Nephrol.* 2001 Dec; 12(12):2645–51. [PubMed: 11729233]
3. Salhan D, Pathak S, Husain M, Tandon P, Kumar D, Malhotra A, Meggs LG, Singhal PC. HIV gene expression deactivates redox-sensitive stress response program in mouse tubular cells both in vitro and in vivo. *Am J Physiol Renal Physiol.* 2012 Jan 1; 302(1):F129–40. [PubMed: 21993884]
4. Yadav A, Vallabu S, Kumar D, Ding G, Charney DN, Chander PN, Singhal PC. HIVAN phenotype: consequence of epithelial mesenchymal transdifferentiation. *Am J Physiol Renal Physiol.* 2010 Mar; 298(3):F734–44. [PubMed: 20015943]
5. Kumar D, Salhan D, Magoon S, Torri DD, Sayeneni S, Sagar A, Bandhlish A, Malhotra A, Chander PN, Singhal PC. Adverse host factors exacerbate occult HIV-associated nephropathy. *Am J Pathol.* 2011 Oct; 179(4):1681–92. [PubMed: 21871425]
6. Yokoi H, Mukoyama M, Sugawara A, Mori K, Nagae T, Makino H, Suganami T, Yahata K, Fujinaga Y, Tanaka I, Nakao K. Role of connective tissue growth factor in fibronectin expression and tubulointerstitial fibrosis. *Am J Physiol Renal Physiol.* 2002 May; 282(5):F933–42. [PubMed: 11934704]
7. Gonzalez JP, Klein J, Valet P, Grès S, Salant D, Bascands JL, Saulnier-Blache JS, Schanstra JP. Lysophosphatidic acid and renal fibrosis. *Biochim Biophys Acta.* 2008 Sep; 1781(9):582–7. [PubMed: 18455518]
8. Natoli TA, Smith LA, Rogers KA, Wang B, Komarnitsky S, Budman Y, Belenky A, Bukanov NO, Dackowski WR, Husson H, Russo RJ, Shayman JA, Ledbetter SR, Leonard JP, Ibraghimov-Beskrovnaia O. Inhibition of glucosylceramide accumulation results in effective blockade of polycystic kidney disease in mouse model. *Nat Med.* 2010 Jul; 16(7):788–92. [PubMed: 20562878]
9. Blazer-Yost BL, Blacklock BJ, Flaig S, Bacallao RL, VH. Lysophosphatidic acid is a modulator of cyst growth in autosomal dominant polycystic kidney diseases. *Cell Physiol Biochem.* 2011; 28(6):1255–64. [PubMed: 22179013]
10. Yang M, Zhong WW, Srivastava N, Slavin A, Yang J, Hoey T, SG. An G protein-coupled lysophosphatidic acid receptors stimulate proliferation of colon cancer cells through the {beta}-catenin pathway. *Proc Natl Acad Sci U S A.* 2005 Apr 26; 102(17):6027–32. [PubMed: 15837931]
11. Ye X, Chun J. Lysophosphatidic acid (LPA) signaling in vertebrate reproduction. *Trends Endocrinol Metab.* 2010 Jan; 21(1):17–24. Review. 10.1016/j.tem.2009.08.003 [PubMed: 19836970]
12. Ayasolla KR, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) attenuates the expression of LPS- and Abeta peptide-induced inflammatory mediators in astroglia. *J Neuroinflammation.* 2005 Sep 20; 21. [PubMed: 16174294]
13. Ayasolla K, Khan M, Singh AK, Singh I. Inflammatory mediator and beta-amyloid (25–35)-induced ceramide generation and iNOS expression are inhibited by vitamin. *Free Radic Biol Med.* 2004 Aug 1; 37(3):325–38. [PubMed: 15223066]
14. Ayasolla KR, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) attenuates the expression of LPS- and Abeta peptide-induced inflammatory mediators in astroglia. *J Neuroinflammation.* 2005 Sep 20; 21. [PubMed: 16174294]
15. Rai P, Plagov A, Kumar D, Pathak S, Ayasolla KR, Chawla AK, Mathieson PW, Saleem MA, Husain M, Malhotra A, Singhal PC. Rapamycin-induced modulation of HIV gene transcription attenuates progression of HIVA. *Exp Mol Pathol.* 2013 Feb; 94(1):255–61. [PubMed: 23010541]

16. Kiefel H, Bondong S, Pfeifer M, Schirmer U, Erbe-Hoffmann N, Schäfer H, Sebens S, Altevogt P. EMT-associated up-regulation of L1CAM provides insights into L1CAM-mediated integrin signalling and NF- κ B activation. *Carcinogenesis*. 2012 Oct; 33(10):1919–29. [PubMed: 22764136]
17. Serrano I, McDonald PC, Lock FE, Dedhar S. Role of the integrin-linked kinase (ILK)/Rictor complex in TGF β -1-induced epithelial-mesenchymal transition (EMT). *Oncogene*. 2013 Jan 3; 32(1):50–60. [PubMed: 22310280]
18. Cicchini C, Laudadio I, Citarella F, Corazzari M, Steindler C, Conigliaro A, Fantoni A, Amicone L, Tripodi M. TGF β -induced EMT requires focal adhesion kinase (FAK) signaling. *Exp Cell Res*. 2008 Jan 1; 314(1):143–52. [PubMed: 17949712]
19. Diaz-Meco MT, Moscat J. The atypical PKCs in inflammation: NF- κ B and beyond. *Immunol Rev*. 2012 Mar; 246(1):154–67. [PubMed: 22435553]
20. Sengul S, Zwizinski C, Simon EE, Kapasi A, Singhal PC, PCV. Endocytosis of light chains induces cytokines through activation of NF- κ B in human proximal tubule cell. *Kidney Int*. 2002 Dec; 62(6):1977–88. [PubMed: 12427122]
21. Martinka S, Bruggeman LA. Persistent NF- κ B activation in renal epithelial cells in a mouse model of HIV-associated nephropathy. *Am J Physiol Renal Physiol*. 2006 Mar; 290(3):F657–65. Epub 2005 Oct 4. [PubMed: 16204413]
22. Zhang C, Meng X, Zhu Z, Yang X, Deng A. Role of connective tissue growth factor in renal tubular epithelial-myofibroblast transdifferentiation and extracellular matrix accumulation in vitro. *Life Sci*. 2004 Jun 4; 75(3):367–79. [PubMed: 15135656]
23. Zhao D, Tang XF, Yang K, Liu JY, Ma XR. Over-expression of integrin-linked kinase correlates with aberrant expression of Snail, E-cadherin and N-cadherin in oral squamous cell carcinoma: implications in tumor progression and metastasis. *Clin Exp Metastasis*. 2012 Dec; 29(8):957–69. [PubMed: 22638656]
24. Peng L, Yang JC, Ning C, Zhang J, Xiao X, He D, Wang X, Li Z, Fu SJ. Ning Rhein inhibits integrin-linked kinase expression and regulates matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio in high glucose-induced epithelial-mesenchymal transition of renal tubular cell. *Biol Pharm Bull*. 2012; 35(10):1676–85. [PubMed: 23037158]
25. Xing Y, Qi J, Deng S, Wang C, Zhang L, Chen J. Small interfering RNA targeting ILK inhibits metastasis in human tongue cancer cells through repression of epithelial-to-mesenchymal transition. *Exp Cell Res*. 2013 May 23; :S0014–4827.
26. Geng H, Lan R, Singha PK, Gilchrist A, Weinreb PH, Violette SM, Weinberg JM, Saikumar P, Venkatachalam MA. Lysophosphatidic acid increases proximal tubule cell secretion of profibrotic cytokines PDGF-B and CTGF through LPA2- and G α q-mediated Rho and α v β 6 integrin-dependent activation of TGF- β . *Am J Pathol*. 2012 Oct; 181(4):1236–49. [PubMed: 22885106]
27. Zhang K, Zhao J, Liu X, Yan B, Chen D, Gao Y, Hu X, Liu S, Zhang DC. Activation of NF- κ B upregulates Snail and consequent repression of E-cadherin in cholangiocarcinoma cell invasion. *Hepatogastroenterology*. 2011 Jan-Feb; 58(105):1–7. [PubMed: 21510277]
28. Kuphal S, Poser I, Jobin C, Hellerbrand C, AK. Bosserhoff Loss of E-cadherin leads to upregulation of NF- κ B activity in malignant melanoma. *Oncogene*. 2004 Nov 4; 23(52):8509–19. [PubMed: 15378016]
29. Stanisavljevic J, Porta-de-la-Riva M, Batlle R, de Herreros AG, Baulida J. The p53 subunit of NF- κ B and PARP1 assist Snail1 in activating fibronectin transcription. *J Cell Sci*. 2011 Dec 15; 124(Pt 24):4161–71. [PubMed: 22223884]
30. Julien S, Puig I, Caretti E, Bonaventure J, Nelles L, Roy Fvan, Dargemont C, Herreros AGde, Bellacosa A, Larue L. Activation of NF- κ B by Akt upregulates Snail expression and induces epithelial-mesenchyme transition. *Oncogene*. 2007 Nov 22; 26(53):7445–56. [PubMed: 17563753]

Highlights

- HIV and LPA treated tubular cells displayed profibrotic/EMT molecular phenotype
- HIV and LPA treated tubular cells showed phosphorylation of ILK-1, FAK, PI3K, Akt, ERKs and P38 MAPK
- HIV and LPA treated tubular cells displayed activation of NF- κ B
- LPA blockers, kinase inhibitors, siRNA of NF- κ B inhibited these tubular cell effects of HIV and LPA

Fig. 1.

A. HIV/LPA induced upregulation of EMT/fibrosis markers fibronectin, Connective Tissue Growth factor (CTGF), Collagen I, α-SMA and Vimentin HRPTCS were treated with HIV/LPA for 72 h followed by cell lysate preparation and immunoblot analysis together with Control (CN) and Empty vector (EV). Quantified protein bands reported here as fold change, was calculated by densitometry with normalization to βactin expression and are shown in the Table (B).

Fig. 2. LPA synthesis blockers (AACOCF3 and DAG kinase inhibitor) as well LPAR inhibitor (Ki16425) attenuated HIV/LPA- induced EMT/profibrotic gene expression. Cellular lysates were prepared following pretreatment in the presence or absence of inhibitors Protein blots showing the expression of various profibrotic proteins are displayed.

Fig. 3.

A and C. Cell lysates were prepared following the indicated treatment, in the presence of phosphatase inhibitors. Immunoblots were prepared from the cell lysates. Protein level expression at various time points are shown. Fold increases in phospho-protein expression, normalized to actin expression, are represented as a curve graph (B and D).

Fig. 4. Both HIV and LPA induced higher binding activity of NFκB

A. Nuclei from HRPTCs were extracted following various treatments (as indicated) and EMSA was performed. HIV or LPA added in indicated concentrations. Sphingomyelinase (SMase) 100 mUnits/ml or Hydrogen peroxide (HP-0.5 mM) were loaded in lanes 8 and 10 respectively. Use of cold unlabeled probe, in the binding assay, suggests the specific binding activity of the fluorescent probe. Treatment with LPA synthesis blockers (as indicated) followed by HIV treatment, reduces NFκB binding activity. Lane 10 depicts addition of cold oligomer to the reaction mix, 10 min prior to adding labeled fluorescent probe

C. PDTC (50 μM) and DN NFκB inhibit tubular cell NFκB-Luciferase in HIV milieu. HRPTCs were transiently transfected with either NFκB-luciferase construct (0.5 μg/well, CMV-β-gal (0.5 μg/well), 0.5 μg/well DN-p65, or with IKK-α. After overnight incubation, the transfected cells were treated with PDTC for 4h before transducing cells with HIV for 4h. Cells were lysed and processed for luciferase and β-gal activities. Luciferase activity was normalized with respect to β-gal activity and expressed relative to activity of the control. Data are mean SD of of three different experimental values.

*P< 0.005 compared to relative control and # P< 0.001 compared with HIV treatment alone.

D. PDTC(+ 25 μM, ++ 50 μM) as well as NFκB siRNA inhibited HIV-induced expression of TGF-β, CTGF, Fibronectin, Collagen-I, α-SMA and SNAIL.

HRPTCs were pre-incubated with PDTC (25 or 50 μM) or transfected with NFκB siRNA prior to stimulation with HIV and were incubated for 72h. Cellular lysates were prepared and protein blots were probed for different protein expression.

Fig. 5. Schematic diagram showing HIV induced alteration in biological membrane leading to LPA biosynthesis, and activating various kinase cascade and to NFκB activation and EMT related gene modulation. Inhibitors that block LPA pathway, signal transduction process and or NFκB activation could effectively block this pathway of EMT gene expression/modulation.