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Lysophosphatidic acid and renal fibrosis

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Summary

The development of fibrosis involves a multitude of events and molecules. Until now the majority of these molecules were found to be proteins or peptides. But recent data show significant involvement of the phospholipid lysophosphatidic acid (LPA) in the development of pulmonary, liver and renal fibrosis. The latest data on the role of LPA and the G-protein-coupled LPA₁ receptor in the development of renal fibrosis will be discussed. LPA₁ receptor-activation was found to be associated with increased vascular leakage and increased fibroblast recruitment in pulmonary fibrosis. Furthermore, in renal fibrosis LPA₁ receptor-activation stimulates macrophage recruitment and connective tissue growth factor expression. The observations make this receptor an interesting alternative and new therapeutic target in fibrotic diseases.

Renal fibrosis

Renal fibrosis is the principal process involved in the progression of chronic kidney disease (CKD) to end-stage renal disease (ESRD). As the incidence of ESRD continues to increase throughout the world, research to better understand the development of renal fibrosis has intensified [1, 2]. The development of renal fibrosis involves the progressive appearance of glomerulosclerosis, tubulointerstitial fibrosis (TIF) and changes in renal vasculature (loss of glomerular and peritubular capillaries). On a molecular level, fibrosis can be defined as excessive accumulation of extracellular matrix (ECM), such as collagens and fibronectins. The presence of TIF, compared to glomerular sclerosis, has been strongly correlated to evolution to ESRD [1, 3]. The first step in the development of TIF is inflammation associated with infiltration of macrophages, lymphocytes, and an increase in cytokine and chemokine secretion (**Figure 1**). This inflammatory state induces a disequilibrium between apoptosis and proliferation of tubular cells, as well as accumulation of myofibroblasts. Myofibroblasts arise from epithelial mesenchymal transition, from proliferation/activation of the few resident fibroblasts or from infiltrated cells [1, 4]. These myofibroblasts are the main cell type responsible for the secretion of the ECM. As these events occur, the quantity of fibrotic tissue increases, causing a steady decline of renal function until eventually the kidney is no longer able to function and organ failure occurs. In the past a number of mediators of TIF have been identified including chemokines, cytokines and growth factors [5]. Among these, transforming growth factor (TGF) β is thought to be the most fibrogenic, directly or indirectly, through the action of connective tissue growth factor (CTGF) [6].

As most renal disease converts sooner or later to renal fibrosis, treatment aiming to slowdown, halt, or even better, reverse renal fibrosis will have an enormous

impact in renal disease. The multitude of events and factors [5] involved in the development of renal fibrosis is reflected by the increasing number of experimental reports showing the potential anti-fibrotic effect of a number of strategies and compounds [2, 3]. However, the only currently clinically available drugs which have shown to slow down the progression towards ESRD are the inhibitors of the renin angiotensin system (RAS, [1]). Unfortunately, even when treated with RAS inhibitors CKD continues to progress. Alternative molecules or therapies are thus necessary [2, 3].

Phospholipids and Fibrosis

For the majority of organs and tissues the development of fibrosis involves a multitude of events and factors [7], similar to what described above for the kidney. Until now, the majority of these molecules were found to be proteins or peptides (profibrotic cytokines, chemokines, metalloproteinases etc... [7]). But more recent data show significant involvement of phospholipids in wound healing [8] and in the development of fibrosis. These phospholipids include platelet activating factor (PAF), phosphatidyl choline and lysophosphatidic acid (LPA). Involvement of these three phospholipids in the development of fibrosis in various organs will be presented below, with a particular emphasis on LPA.

In vitro studies show PAF as an important actor in the development of renal tubulointerstitial fibrosis. PAF induces the production of the ECM-components collagen type I et IV and fibronectin by rat fibroblasts and tubular cells [9]. PAF also induces the production of the profibrotic cytokine TGF β [10]. *In vivo*, genetic ablation or pharmacological blockade of the PAF receptor induces a significant decrease of folic acid-induced renal fibrosis in rodent models [11]. Furthermore, PAF is also

involved in pulmonary fibrosis as administration of a PAF receptor antagonist attenuates bleomycin-induced pulmonary fibrosis [12].

Other phospholipids are associated with the progression of pulmonary fibrosis. Different studies describe an increase of phospholipid concentrations in the bronchoalveolar lavage fluid and in particular phosphatidylcholine (PC) in rabbits and rodents injected with bleomycin [13, 14]. The production of PC under these conditions might be beneficial as it was shown that dilinoleoylphosphatidylcholine *in vitro* and *in vivo*, induces a significant reduction in the expression of genes coding for ECM molecules and inflammatory mediators during bile duct ligation-induced liver fibrosis [15].

LPA and fibrosis

LPA is a growth factor-like phospholipid known to regulate several cellular processes including cell motility, cell proliferation, cell survival, and cellular differentiation. LPA acts on specific G-protein coupled receptors (LPA₁ to LPA₅) [16]. Pharmacological tools to study the involvement of these different LPA receptors have been limited. Currently, the most frequently used pharmacological agent is Ki16425 that has been shown to block LPA₁- and LPA₃-receptor subtypes both *in vitro* [17] and *in vivo* [18]. Mice invalidated for the LPA receptor subtype 1, 2 and 3 are also available [19-21]. Among the different bioactive phospholipids, lysophosphatidic acid (LPA) has been associated with the etiology of a growing number of disorders [22]. Furthermore, a number of studies suggest a role for LPA in the development of fibrosis (see the discussion below).

A number of muscular dystrophies are characterized by a progressive weakness and wasting of musculature, and by extensive fibrosis. It has been shown

that LPA-treatment of cultured myoblasts induced significant expression of connective tissue growth factor (CTGF). CTGF subsequently induces collagen, fibronectin and integrin expression and induces dedifferentiation of these myoblasts [23]. This example is introducing the unambiguous link between LPA and CTGF; it has been shown that treatment of a variety of cell types with LPA induces reproducible and high level induction of CTGF [24-32], although not necessarily in cells directly involved in the fibrotic process. CTGF is an important profibrotic cytokine, signaling down-stream and in parallel with TGF β [33]. In this context CTGF expression by gingival epithelial cells, which are involved in the development of gingival fibromatosis, was found to be exacerbated by LPA treatment [34].

LPA might also be associated with the progression of liver fibrosis. *In vitro*, LPA induces stellate cell and hepatocyte proliferation [35]. These activated cells are the main cell type responsible for the accumulation of ECM in the liver [36]. Furthermore, LPA plasma levels raise during CCl₄-induced liver fibrosis in rodents, or in hepatitis C virus-induced liver fibrosis in human [37, 38].

In addition it was recently shown, in an elegant study of Tager et al., that LPA is an important protagonist in the evolution of pulmonary fibrosis [39]. They first showed that LPA is detected in bronchoalveolar lavage fluid (BAL) and that its concentration is significantly increased in BAL of bleomycin-challenged mice. In the next step it was shown that genetic ablation (LPA₁ receptor knockout mice) or pharmacological knockdown (Ki16425) of the LPA₁ receptor reduced bleomycin-induced pulmonary fibrosis and increased animal survival. Complementary studies further demonstrated that profibrotic effects of LPA₁ receptor stimulation might be explained by LPA₁ receptor-mediated vascular leakage and increased fibroblast recruitment, both profibrotic events. Finally, in human pulmonary fibrosis it was shown that LPA and

the LPA₁ receptor also play an important role. The LPA₁ receptor was the LPA receptor most highly expressed on fibroblast obtained from patients with idiopathic pulmonary fibrosis (IPF). Furthermore, BAL obtained from IPF patients induced chemotaxis of human foetal lung fibroblasts that was blocked by the LPA₁ receptor antagonist Ki16425 [39]. Taken together these studies show that the LPA₁ receptor is a potential target in treatment of IPF. Unfortunately the role of CTGF in this pulmonary profibrotic action of LPA was not studied [39].

Finally, we have recently identified a role for LPA and the LPA₁ receptor in the development of renal fibrosis which will be discussed in detail below after a more general state of the art of the knowledge of LPA and its role in renal pathophysiology.

LPA and renal pathophysiology

In vivo: Only little information is available on the *in vivo* involvement of LPA in renal disease. LPA was shown to attenuate lesions induced by ischemia/reperfusion (I/R) in mice by inhibition of caspase-dependent apoptosis in tubular cells and reduced complement activation and neutrophil recruitment [40]. However, this protective role of LPA in I/R injury has been contested since the use of the LPA₃ receptor antagonist (VPC12249) induced a significant decrease in, and the use of a LPA analogue (OMPT) enhanced, the lesions induced by I/R [41]. Further studies are necessary to better define the role of LPA and its receptors in renal I/R injury. Other studies on patients with chronic renal failure have shown the presence of increased LPA concentrations in plasma [42, 43]. These studies showed that in 18 dialyzed patients with renal failure, plasma LPA levels were 3-fold higher in dialyzed patients than in controls (respectively $1,41 \pm 0,16$ nmol/mL and $0,54 \pm 0,08$ nmol/mL). These data suggest an abnormal LPA metabolism with renal failure, but the cause and

consequences of elevated LPA levels under these conditions remains to be clarified [42]. As the renal expression of LPA receptors is relatively high (especially LPA_{1/2} and LPA₃ receptors, [31, 41]), this organ might be targeted by these high plasma LPA concentrations.

In vitro: Separately from these *in vivo* experiments, the expression of LPA receptors and the effects of LPA treatment on a variety of renal cell types were studied. It seems that, *in vitro*, LPA can induce biological effects on the majority of the kidney cell types: mesangial cells, renal tubular cells or renal fibroblasts.

- *Mesangial cells* (For review [44]): Mesangial cells are an important cell type in the glomerulus, which is the filtration unit of the kidney. Mesangial cell activation (exemplified by mesangial profibrotic chemokine and cytokine secretion) and proliferation was identified in a number of renal pathologies [1]. LPA induces mesangial cell proliferation *via* mitogen-activated protein (MAP) kinase activation, contractility mediated by intra-cellular calcium mobilization, cyclic adenosine monophosphate (cAMP) accumulation, and prostaglandin E2 synthesis [45-47]. LPA can also be a mesangial cell survival factor, effects that are mediated by the Pi3k/Akt pathway [48]. Finally, LPA induces CTGF production by human mesangial cells [49]. With this large variety of biological responses on mesangial cells, it is most likely that LPA is an important actor in glomerular pathologies.

- *Proximal tubular cells*: LPA induces tubular cell proliferation mediated by Pi3k/MAP kinase pathway activation and can inhibit serum starvation-induced apoptosis *via* the Pi3k/Akt pathway in primary culture of human tubular cells [45, 50]. LPA is also able to induce intra-cellular calcium mobilization in opossum kidney proximal tubule cells [51]. This increase might be the onset of NHE3 channel translocation and activation in apical membrane of tubular cells, consequently inducing Na⁺⁺ absorption [52, 53].

Finally, it was shown that primary culture human proximal tubular cells express the LPA₁ receptor [54].

- *Renal fibroblasts*: LPA can induce CTGF expression and secretion, which is mediated by small GTPase Rho activation [29].

LPA and renal fibrosis

Both *in vitro* and *in vivo*, LPA can mediate a number of processes involved in fibrosis and renal pathology. However, clear evidence for the involvement of LPA in the development of renal fibrosis has never been demonstrated. Incrimination of LPA and/or one of its receptors could lead to the proposition of new therapeutic agents for the treatment of renal fibrosis that are currently scarce [2]. We have therefore studied LPA and its receptors in an animal model of renal fibrosis: unilateral ureteral obstruction (UUO). This model mimics in an accelerated manner the development of renal fibrosis including renal inflammation, fibroblast activation and accumulation of extracellular matrix in the tubulointerstitium (**Figure 1**, [4]). The results of this study are summarized in **Figure 2** and described below (Details of this study can be found in [31]).

Confirming a previous report [41], we found that renal LPA receptors are expressed under basal conditions with an expression order of LPA₂>LPA₃=LPA₁>>LPA₄ (**Figure 2a**). UUO significantly induced LPA₁ receptor expression while the expression of the three other LPA receptors remained stable, except for a small, but significant decrease, in expression of the LPA₃ receptor. This was paralleled by renal LPA production (3.3 fold increase) in conditioned media from kidney explants (**Figure 2b**). Contro-lateral kidneys exhibited no significant changes in LPA release and LPA receptors expression. This shows that a prerequisite for an

action of LPA in fibrosis is met: production of a ligand (LPA) and induction of one of its receptors (the LPA₁ receptor). In order to determine whether this induction plays role in the development of renal fibrosis, UUO-induced renal TIF was compared between mice invalidated for the LPA₁ receptor (LPA₁ (-/-)) and wild type mice (LPA₁(+/+))[19, 55]. Interestingly, the development of renal fibrosis was significantly attenuated in LPA₁(-/-) mice (**Figure 2c**). This genetic invalidation was confirmed upon the use of the LPA₁ receptor antagonist Ki16425 [17, 18]. UUO mice treated with this antagonist closely resembled the LPA₁ (-/-) mice (**Figure 2c**). These observations clearly demonstrated the crucial involvement of LPA and its receptor LPA₁ in the etiology of kidney fibrosis [31]. However, the contribution of the different renal cells in this profibrotic effect was less clear.

Since UUO-induced fibrosis is essentially interstitial, without visible glomerular lesions [56, 57], the glomerular (mesangial cell) LPA₁ receptor is likely not involved in the effects of LPA on UUO-induced TIF. The other cell types that can be potential targets of LPA in the development of UUO-induced renal fibrosis include tubular- and inflammatory-cells and interstitial fibroblasts. Since it was already known that LPA can participate in intraperitoneal accumulation of monocyte/macrophages [58, 59] and that LPA can induce expression of the pro-fibrotic cytokine CTGF in primary culture human fibroblasts [29], we focused the remainder of our studies on the *in vitro* effects of LPA treatment on tubular cells. In addition, it has been shown that primary culture human proximal tubular cells express the LPA₁ receptor [54]. LPA treatment of a mouse epithelial renal cell line MCT [60] induced a rapid increase of the expression of profibrotic cytokine CTGF (**Figure 2d**). CTGF plays a crucial role in UUO-induced TIF [61, 62], and is involved in the pro-fibrotic activity of TGFβ [6]. This induction was almost completely suppressed by co-treatment with the LPA-receptor antagonist

Ki16425 (**Figure 2d**). Similar observations were previously made in renal fibroblasts and mesangial cells [25, 28, 29], where the action of LPA on CTGF was shown to be mediated by the small GTPase rhoA and the down-stream kinase ROCK [28]. Interestingly, treatments with ROCK-inhibitors have been described to attenuate UUU-induced renal TIF [63], similar to what we observed in LPA₁(-/-)- and in Ki16425-treated mice. Altogether, these observations strongly suggested that the pro-fibrotic activity of LPA in kidney could result from a direct action of LPA on kidney cells involving induction of CTGF (**Figure 2**, [31]).

The metabolic origin of renal LPA remains to be determined. Several enzymes, including phospholipases A1/A2, lysophospholipase D/autotaxin (ATX), glycerol-phosphate acyltransferase, or monoacylglycerol kinase (MAGK), can possibly lead to renal LPA synthesis [64]. The expression and/or the activity of one of these enzymes might be increased in the kidney as an adaptive response to chronic kidney injury induced by UUU. Preliminary data from our laboratory suggest that neither ATX nor MAGK are responsible for the increased synthesis of LPA associated with renal fibrosis, since their expression is rapidly and strongly down-regulated during UUU (data not shown). In rat, UUU was shown to increase the activity of a phosphoethanolamine-specific PLA2 [65]. The involvement of this enzyme in LPA synthesis in the obstructed kidney remains to be explored.

LPA receptor expression in other models of renal fibrosis

Although the UUU model of renal fibrosis mimics the different stages of the development of renal fibrosis, the rapidity by which these lesions are installed does not exactly correspond to the slow progression of human renal disease. The murine model of nephrotoxic serum (NTS) nephritis described by Lloyd CM et al [66], is

characterized by a rapid progressive glomerulonephritis followed by the slow appearance of TIF leading after several weeks to progressive renal failure [67]. This model more closely mimics the slow progression of human renal disease. We show here for the first time modification of LPA receptor expression in this chronic model (**Figure 3**). As previously reported [67], NTS-induced TIF is characterized by an increased expression of fibrosis markers (macrophage infiltration and collagen expression, **Figure 3a**). Interestingly, renal expression of the LPA₁ receptor was significantly increased one and 6 weeks after NTS injection when compared to control mice (respectively $3,3 \pm 1$ and $5,9 \pm 1,3$ fold of control) (**Figure 3b**). In contrast, the expression of the other LPA receptors was not modified. This suggest, as shown in the UUO model of accelerated renal fibrosis, that LPA and its receptors can play role in the development renal fibrosis originating from glomerulonephritis. An interesting aspect of this model is the fact that this disease is originating from glomerular inflammation. As we discussed above, LPA is inducing important biological effects on glomerular mesangial cells *in vitro* [45-49]. Therefore blockade of the effects of LPA in this model might modify the progression of disease in both the glomerular- and tubular-compartment. Further studies comparable to those performed with the UUO model [31] of renal fibrosis will be necessary to better understand the role of LPA and its receptors in CKD.

Conclusion

Using both genetically engineered animals and pharmacological tools a number of laboratories have recently shown that LPA and its LPA₁ receptor can play an important role in the development of fibrosis. Mechanisms of the profibrotic action of LPA and the LPA₁ receptor in these different tissues involve stimulation of fibroblast migration, increased vascular permeability and CTGF secretion by a number of cells;

all events known to be involved in the fibrotic process [7]. These results suggest that the LPA₁ receptor could become a promising new therapeutic target in fibrosis.

In the kidney, TGF β seems to be only moderately involved in the anti-fibrotic effect of LPA₁ receptor blockade. This cytokine is one of the most potent pro-fibrotic factors involved in the (renal) fibrotic process. Since TGF β has many other effects [68], its blockage is not a realistic therapeutic option to reduce renal fibrosis. More recently, it has been proposed that CTGF blockade could represent a promising antifibrotic therapy [61]. Therefore, lowering CTGF production by pharmacological LPA₁ receptor blockade might be an interesting opportunity in the treatment of renal fibrosis.

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Figure legends

Figure 1: Overview of the different stages in the development of renal fibrosis:

The development of fibrosis in CKD is comparable between the different renal compartments [1]. For clarity the development of only renal TIF is shown. The first step in the development of TIF is renal inflammation. This is leading to infiltration of macrophages, lymphocytes, and to increased cytokine and chemokine secretion. This inflammatory state induces a disequilibrium between apoptosis and proliferation of tubular cells, as well as accumulation of myofibroblasts. These myofibroblasts are the main cell type responsible for the secretion of the ECM. As CKD progresses, ECM deposition becomes massive and uncontrolled apoptosis of tubular cells leads to tubular atrophy. This figure was adapted from [4], with permission.

Figure 2: Overview of the profibrotic effect of LPA and the LPA₁ receptor in unilateral obstruction (UUO)-induced renal fibrosis:

The kidney is composed of filtration units called nephrons. We propose that the antifibrotic effect of LPA₁ receptor blockade involves reduction of CTGF secretion by proximal tubular cells which are located in the tubular section of the nephron. **(a)** LPA₁ receptor expression is increased by UUO reaching a maximum after 8 days of obstruction, the day of sacrifice. The expression of the 3 other LPA receptors was not modified, except a slight decrease in the expression of the LPA₃ receptor. **(b)** In parallel, UUO also increases renal LPA production, starting as early as 3 days after obstruction. **(c)** UUO-induced renal fibrosis was significantly attenuated by LPA₁ receptor ablation and LPA₁ receptor antagonist (Ki16425) treatment as shown by immunohistochemical analysis and quantification of collagen type III expression. **(d)** *In vitro*, on tubular

cells, LPA treatment induces the expression and release of the pro-fibrotic cytokine, Connective Tissue growth Factor (CTGF). CTGF secretion induced by LPA seems to be LPA₁ receptor dependant, as CTGF secretion is blocked by the LPA₁ receptor antagonist (Ki16425). See details in [31]. Abbreviations: Cont, control non-UUO mice; UUO, unilateral ureteral obstruction; WT, wild type mice; KOLPA1, LPA₁(-/-) mice; WT+Ki16425, wild type mice treated with the LPA₁ receptor antagonist Ki16425.

Figure 3: LPA receptor expression in a chronic model of renal fibrosis induced by nephrotoxic serum injection (NTS): (a) Inflammation and fibrosis increases 6 weeks after NTS injection. Mice were sacrificed at 0 and 6 weeks after NTS injection and kidneys were analyzed for F4/80 (macrophage) and collagen III (fibrosis) expression using immunohistochemistry. The histograms represent computer-assisted analysis of 10 different microscopic fields of a kidney slice (as described in [31]), obtained from 5 different mice. (b) mRNA expression analysis of LPA receptors. NTS induces expression of the LPA₁ receptor only. Mice are injected with NTS (time 0). Mice are euthanized at the time of NTS injection and one or six weeks later (n=5). LPA receptor expression was quantified by real time PCR. Comparisons with time 0 were analyzed by the Student-t-test, *, p<0,05.

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