Mitogenic action of lysophosphatidic acid in proximal tubular epithelial cells obtained from voided human urine

Naonori KUMAGAI, Chiyoko N. INOUE, Yoshiaki KONDO and Kazuie IINUMA
Department of Pediatrics, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

ABSTRACT

Focal tubular cell multiplication at sites on an injured nephron is a critical event in the recovery phase following acute tubular necrosis. During this process, numerous viable tubular cells exfoliate and are shed into the urine. Lysophosphatidic acid (LPA) is generated in the plasma membrane of injured cells and acts as an intercellular mediator of various biological processes, including inflammation, proliferation and repair. In the present study, exfoliated proximal tubule (PT) cells were isolated from human urine and the mitotic effects of LPA were investigated as a model of repair and proliferation following renal injury. LPA stimulated a 23.5% increase in DNA synthesis, a 29.4% increase in cell number and an 86.6% decrease in cAMP content. All of these responses were pertussis toxin sensitive, indicating the involvement of Gi-type G-proteins in LPA signalling. Conversely, the LPA-induced DNA synthesis and the decrease in intracellular cAMP content were insensitive to wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), suggesting a mitogenic response via PI3K-independent mechanisms. Furthermore, we detected specific mRNA transcripts for the recently cloned human LPA-receptors, endothelial differentiation gene (Edg)-2 and Edg-4 (Edg-2 > Edg-4) by reverse transcription-PCR in PT cells. Our data suggest that LPA may behave as a local growth factor in PT cells following tubular injury.

INTRODUCTION

Proliferation of renal tubular epithelial cells is observed with a very low turnover rate under normal conditions. On the other hand, the kidney shows remarkable tubular regeneration under various pathological circumstances, including acute renal failure, acute tubular necrosis and diabetic nephropathy [1,2]. In such cases, histopathological studies have shown that the regeneration of tubular epithelial cells occurs focally, and that the growing cells often project into the tubular lumen [1]. These characteristic pathological features are commonly observed, regardless of the kind of insult, e.g. ischaemic or toxic. However, the mechanisms of focal proliferation of tubular epithelial cells in renal injury have not been identified. In addition, the growth factors mediating this process have not yet been discovered. Since tubular epithelial cell proliferation occurs in regions where tubular injuries are present, it has been hypothesized that local growth factors, which are produced in the proximity of, or within, damaged cells and released in response to cellular dysfunction or injury, may mediate the repair of the nephron by acting in an autocrine and/or paracrine fashion [1].

Key words: exfoliation, growth factor, lysophospholipid, repair.
Abbreviations: DMEM/F12, Dulbecco's modified Eagle's/F12 medium; Edg, endothelial differentiation gene; EGF, epidermal growth factor; EIA, enzyme immunoassay; FBS, fetal-bovine serum; IBMX, 3-isobutyl-1-methylxanthine; IGF, insulin-like growth factor; LPA, lysophosphatidic acid; PI3K, phosphatidylinositol 3-kinase; PT, proximal tubule; PTX, pertussis toxin; RT, reverse transcription.
Correspondence: Dr C. N. Inoue (e-mail cnagano@ped.med.tohoku.ac.jp).
Human kidney tubular epithelial cells that are shed into urine can be propagated in vitro. This in vitro culture system has been shown previously to be useful in defining pathological and biological alterations in renal cell functions in ischaemic or toxic injury, as well as in disorders of the kidney [3,4,5]. The majority of viable and culturable epithelial cells in human urine are derived from the proximal tubule (PT) [3,4,5]. We have reported previously that PT cells isolated from human urine and cultured in vitro still retained the ability to form tight junctions with brush border γ-GTP activity and trans-epithelial water movement. In addition, they could be used in physiological studies defining the differentiated tubular functions, including Na+/H+ exchanger-3 activity [5]. All of the properties of cultured PT cells, including the capability for proliferation and differentiation, and the integrity of these cells in a confluent monolayer, appear to be relevant in the regenerative process of epithelial cells during the recovery phase in an injured nephron. Therefore we believe that this experimental model could be a tool for investigation of the growth mechanisms of tubular epithelial cells in response to tubular injury in vivo.

Recently, lysophosphatidic acid (LPA), which can be produced in membranes of activated or injured cells by enzymic cleavage of stored glycerophospholipids, has been found to act as a lipid mediator possessing a broad spectrum of biological activities [6]. When added exogenously to cells in vitro, LPA promotes mitogenesis, prevents apoptotic cell death and induces cell contraction/relaxation, tumorigenicity and cell-shape changes [6]. LPA also stimulates cell motility and migration, which lends further support for its role in the regulation of epithelial cell wound healing [7]. Since the local synthesis of LPA in the plasma membrane of injured cells and its potential actions [8] indicate that it may function as a local growth factor, we speculated that LPA could be involved in the mitogenesis of PT cells during the restitution of renal tubular structure.

In the present study, we aimed to determine whether LPA could stimulate PT-cell proliferation in primary cultures which were established from voided human urine, and, if so, which subtype of LPA receptor was involved.

**MATERIALS AND METHODS**

**Materials**

1-α-Lysophosphatidic acid, ITS premix, BSA (fraction V), tri-iodothyronine (T₃), dexamethasone and wortmannin were obtained from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was purchased from Summit Biotechnology (Collins, CO, U.S.A.). Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12), minimal essential medium, other culture supplements and TRIZOL™ were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Type I-C collagen was obtained from Nittazetarin (Osaka, Japan), pertussis toxin (PTX) was from the Seikagaku Corporation (Tokyo, Japan), and all other reagents were purchased from Wako Pure Chemicals Co. (Tokyo, Japan) or Sigma.

**Primary culture of PT cells from voided human urine**

With informed consent, urine samples were collected by the clean-catch method from hospitalized 7–9-year-old children suffering from severe chronic glomerulonephritis, including IgA nephropathy. The voided urine samples (50–100 ml) were centrifuged at 250 g for 7 min at room temperature, the pellet was washed twice with minimal essential medium supplemented with 20% (v/v) FBS, and the cells isolated from the pellet were cultivated in growth medium on collagen (type IC)-coated culture dishes (3.5-cm diam.; Iwaki, Chiba, Japan) in a 5% CO₂ atmosphere at 37°C. The growth medium consisted of DMEM/F12 supplemented with 10% FBS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 10⁻⁶ M dexamethasone, 5 x 10⁻⁸ M T₃, 5 mM nicotinamide, 100 units/ml penicillin and 100 μg/ml streptomycin [5]. When the cells had reached subconfluent cell density, they were digested with 0.05% trypsin/0.02% EDTA, and the dispersed cell suspension was equally divided into new culture dishes for further cultivation until approx. 80% confluency was reached. Exclusive PT origin of the propagated epithelial cells was confirmed by histochemical γ-GTP staining [9]. Before each experiment, the PT cells were serum starved for 2–3 days in DMEM/F12 supplemented with 0.1% BSA and 5 mM nicotinamide without antibiotics. Clonal culture of PT cells for the detection of specific mRNA transcripts for LPA receptors was performed as described previously [5].

**Determination of PT cell proliferation**

For PT cell growth studies, quiescent PT cells in 96-well culture dishes were stimulated with various concentrations of LPA (0–100 μM) in DMEM/F12 supplemented with 0.5% FBS and 0.1% BSA for 2–4 days. By adding a small amount of FBS, we could maintain cell viability and attachment to culture dishes for at least 4 days. The cells were observed daily under a microscope and the studies were terminated when the cells reached confluent cell density. After trypsinization of adherent PT cells using 0.05% trypsin/0.02% EDTA, an aliquot of the cell suspension was mixed with an equal volume of 0.08% (w/v) Trypan Blue in Hanks balanced salt solution, and the fraction of cells which excluded Trypan Blue was assessed visually. The viable cell number was calculated by multiplying the total cell number by the fraction of cells excluding Trypan Blue.
For the quantification of DNA synthesis, quiescent PT cells in 96-well culture dishes were exposed to LPA (30 μM) in the presence or absence of wortmannin (10 nM) for 24 h and labelled with 1.5 μCi/ml [3H]-thymidine (Amersham, Tokyo, Japan). The cells were harvested after rinsing with PBS, and the trichloroacetic acid-precipitated material was processed for estimation of radioactivity as described previously [10].

**Identification of LPA-receptor mRNAs by reverse transcription (RT)-PCR**

Total RNA was extracted from clonally propagated PT cells (approx. 10^4 cells) using a commercially available reagent, TRIZOL®. After DNA digestion of the RNA sample with RNase-free DNase I, 2 μg of RNA was reverse-transcribed into cDNA using an oligo-dT primer and subjected to PCR amplification. The following sets of primers, the design of which was based on the published endothelial differentiation genes (Edg)-2, Edg-4 and Edg-7 cDNAs, were used in PCR: 5′-GAGAGGCAATTACGGTTTCC-3′ (459–480) and 5′-CATTTCTTTTGTCGCGTAGAG-3′ (980–959) for the Edg-2 cDNA [11], 5′-ACCCGCATTTTCTCTAGGTGC-3′ (709–730) and 5′-CCCTAGACGATGGCTGAAG-3′ (1393–1372) for the Edg-4 cDNA [12], and 5′-CTTTCTAGGATTCTACCATCCTACAAAAGCTGAAGG-3′ (1148–1127) for the Edg-7 cDNA [13]. Whole kidney total RNA (Clonetech, Palo Alto, CA, U.S.A.) was used as a positive control. Amplification was performed for 40 cycles at 94°C (1 min), 55°C (1 min) and 72°C (1 min). PCR products were separated by electrophoresis on a 3% agarose gel and revealed with SYBR Green I (Molecular Probes, Eugene, OR, U.S.A.) staining.

**Measurement of intracellular cAMP content**

Quiescent and subconfluent PT cells in 12-well culture dishes were stimulated with 30 μM LPA in the presence of 200 μM 3-isobutyl-1-methylxanthine (IBMX) for 3, 5, 10 or 20 min and the medium was removed by aspiration at the end of these times. Cyclic nucleotides were extracted from the sonicated cells with ethanol containing 20 mM HCl, as described previously [14]; the extract was freeze-dried and the cAMP content was determined by enzyme immunoassay (EIA) (Cyclic AMP Enzyme Immunoassay kit; Cayman Chemical Company, Ann Arbor, MI, U.S.A.). Protein determinations were performed using a Bio-Rad kit (Bio-Rad, Hercules, CA, U.S.A.) with BSA as a standard.

**Statistical analysis**

Data, presented as means ± S.E.M., were analysed by one-way analysis of variance followed by the unpaired Student’s t test. A value of P < 0.05 was considered statistically significant.

**RESULTS**

Quiescent PT cells, collected and cultivated from voided human urine, were stimulated with various concentrations of LPA (0, 10, 30 or 100 μM) for 2 days and the mitogenic action of LPA was assessed by counting the number of viable cells. As shown in Table 1, exposure of PT cells to LPA resulted in mitogenic activity, as demonstrated by an increase in cell number, with a maximal effect at 30 μM, where the cell number was 1.3-times greater than that of quiescent cells (P < 0.05, n = 5 wells). At 100 μM LPA, the mitogenic effect of LPA was not significantly different from that at 30 μM LPA.

To define the molecular basis of LPA-induced PT cell proliferation, we tested the sensitivity of PTX, an inactivator of G_i-proteins via ADP ribosylation, for the growth-stimulatory activity of LPA. As shown in Figure 1, pretreatment of PT cells with 100 ng/ml PTX for 4 h

**Table 1 Human PT cell proliferation stimulated by LPA**

<table>
<thead>
<tr>
<th>LPA (μM)</th>
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<tr>
<td>0</td>
<td>8.0 ± 0.4</td>
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<tr>
<td>10</td>
<td>9.0 ± 0.2*</td>
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<tr>
<td>30</td>
<td>10.3 ± 0.2*</td>
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<tr>
<td>100</td>
<td>10.0 ± 0.2*</td>
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* P < 0.05 compared with no added LPA.

**Figure 1 Inhibitory effect of PTX on LPA-induced human PT cell proliferation**

Serum-starved PT cells were preincubated with (100 ng/ml) or without PTX (P) for 4 h and then stimulated with 30 μM LPA (L) for 4 days. The number of viable cells (those excluding Trypan Blue) was counted. Values are the means ± S.E.M. (n = 4 wells). * P < 0.05 compared with unstimulated, quiescent cells (Q). ** P < 0.05 compared with LPA stimulation.
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Figure 2 Time course of the decrease in cAMP in response to LPA
Quiescent PT cells were stimulated with 30 µM LPA in the presence of 200 µM IBMX, and the cAMP content was measured using an EIA. Values are the means ± S.E.M. (n = 4 wells). *P < 0.05 compared with unstimulated, quiescent cells.

Figure 3 Inhibitory effect of PTX on LPA-induced cAMP decrease
Quiescent PT cells were preincubated with (100 ng/ml) or without PTX (P) for 4 h and then stimulated with 30 µM LPA (L) in the presence of 200 µM IBMX for 5 min. Intracellular cAMP was measured by EIA. Values are the means ± S.E.M. (n = 4 wells). *P < 0.05 compared with unstimulated quiescent cells (Q). **P < 0.05 compared with LPA stimulation.

Figure 4 Effect of wortmannin on LPA-induced DNA synthesis
Quiescent PT cells were stimulated with 30 µM LPA (L) in the presence or absence of 10 nM wortmannin (W) for 24 h, and labelled with 1.5 µCi/ml [3H]thymidine. Values are the means ± S.E.M. (n = 4 wells). *P < 0.05 compared with unstimulated, quiescent cells (Q). Not significant (NS) compared with LPA stimulation.

mediated by phosphodiesterase action. Figure 2 shows that LPA decreased intracellular cAMP content as early as 3 min after stimulation (186 ± 52.3 to 34.4 ± 4.3 pmol/mg of protein; n = 4 wells, P < 0.05). The decreased cAMP levels were observed for 3–5 min, at which time cAMP content was 13.4% of the initial value (24.9 ± 3.5 and 186 ± 52.3 pmol/mg of protein respectively; n = 4 wells, P < 0.05), and cAMP remained below the initial levels for the 20-min duration of the experiment. Furthermore, as shown in Figure 3, the decrease in cAMP content in PT cells 5 min after stimulation by LPA was significantly inhibited by pretreatment of the cells with PTX (24.9 ± 3.5 and 186 ± 52.3 pmol/mg of protein; n = 4 wells, P < 0.05). These results suggest that LPA induced PT cell proliferation while activating the PTX-sensitive G protein that inhibits adenylate cyclase.

To test whether phosphatidylinositol 3-kinase (PI3K) is essential for LPA-induced PT cell proliferation, we examined the effect of wortmannin, an inhibitor of PI3K (15), on the mitogenic effect of LPA. As shown in Figure 4, treatment of cells with 10 nM wortmannin did not significantly change LPA-induced DNA synthesis of PT cells, as estimated by [3H]thymidine incorporation [(39.2 ± 1.7) × 10³ and (39.2 ± 0.8) × 10³ d.p.m./well; n = 4 wells, P > 0.05]. The decrease in cAMP levels by LPA stimulation was not affected by the presence of 10 nM wortmannin (Figure 5). These data suggest that PI3K is not involved in the mitogenic action of LPA in PT cells.

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Figure 5 Effect of wortmannin on LPA-induced cAMP decrease
Quiescent PT cells were stimulated with 30 μM LPA (L) in the presence or absence of 10 nM wortmannin (W) for 5 min, and cAMP content was measured by EIA. Values are the means ± S.E.M. (n = 4 wells). *P < 0.05 compared with unstimulated, quiescent cells (Q). **P < 0.05 compared with wortmannin-treated cells.

Figure 6 Identification of LPA receptor mRNAs by RT-PCR
Total RNA (2 μg) from clonally cultured PT cells and human kidney tissue were digested with DNase I and assayed for Edg-2, Edg-4 and Edg-7 by RT-PCR. Expected sizes (in bp) were 522 (Edg-2), 685 (Edg-4) and 1135 (Edg-7).

RT-PCR studies were performed to confirm the expression of LPA-receptors in PT cells. Total RNA was isolated from clonally proliferated PT cells. Following digestion of 2 μg total RNA samples with DNase I, we reverse-transcribed total RNA and performed PCR. Figure 6 shows RT-PCR products for Edg-2, Edg-4, and Edg-7 separated on an agarose gel and stained with SYBR Green I. Cultured PT cells contained large amounts of Edg-2 mRNA and trace amounts of Edg-4 mRNA. In a parallel experiment, we examined the expression of LPA-receptors using total RNA samples which had been isolated from normal human kidney, and found that normal kidney tissue homogenates also expressed predominantly Edg-2 mRNA and a trace amount of Edg-4 mRNA. Under control RT-PCR conditions, including water alone or DNase-I-treated but not reverse-transcribed total RNAs, no bands were observed (results not shown). The exact predicted sequences were further confirmed by sequencing analysis (results not shown). Edg-7 mRNA was not detected in PT cells or in kidney tissue.

DISCUSSION

In renal tubular injury, loss of cell–cell contact and detachment of cells from the basement membrane leads to cell death, which must be followed by cell proliferation and a full differentiation sequence to restore the integrity of the epithelium. Based on the evidence that tubular cell multiplication occurs focally in the disordered internal milieu of the kidney, the mediator to promote tubular mitogenesis has been hypothesized to be produced and released locally [1]. In the present study, we focused on the properties of LPA, a lipid mediator produced and released from injured cells, and evaluated its mitogenic activity in PT cells obtained from voided human urine. In this study, we demonstrated that LPA stimulates DNA synthesis, as well as cell proliferation, in human PT cells, with maximal effects observed with 30 μM LPA, a concentration near to the physiological range in serum (2–20 μM) [16]. Although the local tissue concentrations of LPA in the vicinity of injured nephrons cannot be evaluated, our data suggest that LPA may act as a mitogen in promoting the regeneration of renal tubular cells at sites of injured tubules.

To date, it has been reported that both epidermal growth factor (EGF) and insulin-like growth factor (IGF)-1 have growth stimulatory activity in primary cultured human PT cells [17,18]. However, the involvement of these polypeptide growth factors in the rapid regenerative process of injured PT remains uncertain for several reasons: first, renal levels of EGF mRNA and the production of urinary EGF both decline rapidly during acute renal failure [19]; secondly, EGF is primarily localized in cells of the distal convoluted tubule/thick ascending limb of the loop of Henle [20]; thirdly, IGF-1 is also produced primarily in the medullary collecting duct [21]. Considering that these factors are produced in nephron segments more distal than PT, it seems unlikely that they will reach a damaged PT to achieve mitogenic activity in vivo. In chronic, progressive tubular damage, IGF-1, which can be produced in interstitial fibroblasts, may act paracriney on the basolateral membranes of PT...
cells, leading to the induction of the mitogenic response of PT cells [18].

Based on the following evidence, LPA is more likely to be directly involved in the proliferation of tubular cells in response to acute injury: (1) phospholipase A₂, a key enzyme that generates LPA by a breakdown of phospholipid, has been shown to be activated in the plasma membrane of anoxic and damaged proximal tubules [22,23]; (2) phospholipid depletion, accompanied by the accumulation of phospholipid breakdown products, has been observed during an ischaemic insult in rat kidneys [24]; (3) production and release of glycerophosphate mediators of the LPA family have been detected in epithelial cell injury, including in corneal keratocytes [8]. Although we have not as yet succeeded in identifying measurable amounts of LPA in urine samples of patients with acute renal failure by HPLC or by two-dimensional TLC methods, the studies mentioned above may be viewed as evidence that LPA can be produced and released from injured tubular cells and act autocrinely and/or paracrine to promote PT cell growth.

In the present report, the results show that LPA elicits growth stimulation of human PT cells via the LPA-receptor (Edg2 or Edg4 isotype) coupled to the PTX-sensitive G₁α-protein, which triggers inhibition of adenylate cyclase and promotes PT cell proliferation. In addition, we present evidence that the mitogenic reaction observed is independent of PI3K activity, since wortmannin did not block DNA synthesis or the cAMP decrease induced by LPA. The PI3K-independent LPA-induced mitogenic response in our experimental system is different from the results reported by Levine et al. [25], a study which showed that LPA-induced DNA synthesis was dependent on PI3K in mouse primary cultured PT cells isolated from kidney tissue. The exact reason for this discrepancy is not yet clear. As we have also observed that LPA stimulated DNA synthesis of rat primary cultured PT cells in a PI3K-insensitive manner (N. Kumagai and C. N. Inoue, unpublished work), and given that Roche et al. [26] have also demonstrated that, in rat cultured fibroblasts, microinjection of a neutralizing antibody against PI3K left the mitogenic action of LPA unchanged, it seems likely that the discrepancy is caused by the differences in animal species mouse, human and rat. Moreover, when the toxic, rather than growth-inhibitory effect, of wortmannin in primary cultured PT cells under serum-free conditions is considered, we suggest that PI3K may not be involved in DNA synthesis in human primary cultured PT cells. However, we can not rule out the possibility that the discrepancy might be due to the differences in experimental conditions, since Levine et al. [25] used serum-free medium for their growth studies, whereas 0.5% FBS and 0.1% BSA were added to the medium used in our studies to prevent cell deterioration and death by serum-deprivation. Thus it is possible that some growth factor(s) are derived from the small amounts of added serum, which constitutively activates PI3K, even in the quiescent phase, in our experimental system.

In summary, our data support the hypothesis that LPA, which is released in response to cell injury and during wound healing, is most likely a local growth factor which mediates PT cell growth after tubular injury.

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