Role of LPA and the Hippo pathway on apoptosis in salivary gland epithelial cells

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Lysophosphatidic acid (LPA) is a bioactive lysophospholipid involved in numerous physiological responses. However, the expression of LPA receptors and the role of the Hippo signaling pathway in epithelial cells have remained elusive. In this experiment, we studied the functional expression of LPA receptors and the associated signaling pathway using reverse transcriptase-PCR, microspectrofluorimetry, western blotting and immunocytochemistry in salivary gland epithelial cells. We found that LPA receptors are functionally expressed and involved in activating the Hippo pathway mediated by YAP/TAZ through Lats/Mob1 and RhoA/ROCK. Upregulation of YAP/TAZ-dependent target genes, including CTGF, ANKRDI and CYR61, has also been observed in LPA-treated cells. In addition, based on data suggesting that tumor necrosis factor (TNF)-α induces cell apoptosis, LPA upregulates TNF-induced caspase-3 and cleaved Poly(ADP-ribose)polymerase (PARP). However, small interfering RNA treatment to Yes-associated protein (YAP) or transcriptional co-activator with a PDZ-binding motif (TAZ) significantly decreased TNF-α- and LPA-induced apoptosis, suggesting that YAP and TAZ modulate the apoptotic pathway in salivary epithelial cells.

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the loss of physiological functions. In this study, we report the functional expression of LPA receptors and identify the Hippo signaling pathway as a regulator of apoptosis in salivary gland epithelial cells.

MATERIALS AND METHODS

Materials

LPA, Carbachol (CCh), BAPTA-AM, U73211, 2-APB and Y-27632 were purchased from Sigma (St Louis, MO, USA). Fura-2 AM was obtained from Molecular Probes (Eugene, OR, USA). A Hippo signaling antibody sampler kit, β-tubulin, lamin A/C caspase-3 and PARP were purchased from Cell Signaling Technology (Danvers, MA, USA). TAZ, p-TAZ and β-actin were obtained from Santa Cruz (Dallas, TX, USA).

Cell culture

The HSG cells were a generous gift from Professor Kazuo Hosoi at the Tokushima University in Japan. The cells were grown in suspension in 10-ml culture plates at 37 °C in 95% air–5% CO2 and were maintained in a minimum essential medium supplemented with 10% fetal bovine serum. Each plate was replated twice a week. The intracellular Ca2+ concentration ([Ca2+]i) in HSG cells loaded with 2 μM Fura-2 in 2 ml of culture medium for 30 min at 37 °C was measured. The experiments were conducted at an excitation wavelength of 340 nm to that measured at 380 nm (Ca2+ concentration)

Measurement of the cytoplasmic-free calcium concentration ([Ca2+]f)

The intracellular Ca2+ concentration ([Ca2+]f) in HSG cells loaded with 2 μM Fura-2 in 2 ml of culture medium for 30 min at 37 °C was measured. The cells were gently washed once by replacing the medium and then incubated in normal solution for 10 min prior to the experiment to allow for maximal de-esterification of the dye. The MetaFluor version 6.1 imaging system (Universal Imaging, West Chester, PA, USA) was used for recording and analysis. Fura-2 fluorescence was recorded at 37 °C at an excitation wavelength of 510 nm, and the results are presented as the ratio of fluorescence measured at 340 nm to that measured at 380 nm (Ca2+ fluorescence ratio, F340/F380).

Nuclear-cytoplasmic fractionation and western blot analysis

The cells were homogenized in lysis buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 100 mM NaCl, 10 mM tetrasodium pyrophosphate, 10 mM NaF, 1 mM EDTA, 1 mM NaV, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride and 1 μg ml−1 aprotinin, leupeptin and pepstatin) followed by gentle sonication on ice. For nuclear-cytoplasmic fractionation, intracellular proteins were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer’s protocol.

Following protein concentration determination, the proteins were separated on an SDS-polyacrylamide gel and transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane, which was then incubated with primary antibody.

Immunocytochemistry

The cells were grown in Dulbecco’s modified Eagle’s medium medium and allowed to adhere to coverslips for 2 h at 37 °C in 5% CO2. Treated (LPA) and untreated cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were washed in phosphate-buffered saline 3X for 5 min and then placed in blocking solution (2% bovine serum albumin, 5% fetal bovine serum (FBS) and 0.1% Triton X-100). The cells were then incubated overnight at 4 °C with primary antibody, and after being washed, the cells were incubated at room temperature for 1 h with secondary antibody.

Reverse transcriptase–PCR analysis

The amplification conditions included 60 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C for 35 cycles, and a final 5 min at 72 °C. The primers that were used (forward, reverse and product size (bp)) were as follows: LPA1, 5'ATCCGGGATACCATGATGAGT-3' and 5'-TCCGTTCACTTTCTC-3'; LPA2, 5'-GTGCCAAGTTTCGTTCTGA-3' and 5'-AACCTTTCTCCTGCTC-3'; LPA3, 5'-GTGTCACCGTGCTTCTC-3' and 5'-CATGTCATACCGTCTCACTGG-3'; LPA4, 5'-GTGCCGATTTTCACCCAT-3' and 5'-GAATTGCAAGGCAAAAGTG-3'; LPA5, 5'-GATTCCGGCCCTGACACCAACG-3' and 5'-AACCTTTCTCCTGCTC-3'; LPA6, 5'-TGTTGAGCTGTTACTG-3' and 5'-TTCGAGACTT-3'; and LPA7, 5'-ATCTGCTGAAAGGTCGAG-3', 362.

Real-time PCR

Total RNA was isolated from cells using TRIzol (Gibo-BRL, Invitrogen) and then used in reverse transcription reactions for cDNA synthesis (Invitrogen, Carlsbad, CA, USA). Real-time PCRs were performed with SYBR green premix buffer and an ABI Prism 7300 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), and real expression levels were determined after normalization to the threshold cycle (CT) values for β-actin. The gene-specific primers were as follows: for β-actin, 5'-CCGGAAGAGATGACAGCAGAGAGATCAGA-3' and 5'-AAAGGTTCGCTGCTTCTC-3'; for CTGF, 5'-CCATGACAGGCTCTGCTC-3' and 5'-TGGTGACGCCAAGAGTCTC-3'; for Cyr61, 5'-GCCGCCAGGCTGTGTCGCT-3' and 5'-GCGACAGGCTACCTC-3'; for ANKRD1, 5'-GACTCTTGAGCCACCTGC-3' and 5'-GCGACAGGCTACCTC-3'; and for EDN1, 5'-TGTTGTACCTCTGCTC-3' and 5'-GCGACAGGCTACCTC-3'.

small interfering RNA treatment

Cells were transfected with small interfering RNA (siRNA) against YAP or TAZ (Santa Cruz Biotechnology) using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions.

Statistical analysis

The band intensities were measured with ImageJ software (Bethesda, MD, USA), and statistical analysis was performed using Excel 2003. All data are expressed as the means ± s.e.m. Statistical significance was evaluated using Student’s unpaired t-test. A probability below 0.05 (P<0.05) was considered significant.

RESULTS

Functional expression of LPA receptors in HSG cells

We first identified the types of LPA receptors that are expressed in HSG cells using reverse transcriptase–PCR. The mRNA transcripts for all LPA receptor subtypes, from 1 to 6, were detected, and the expected size of the PCR products was 362 bp, 342 bp, 426 bp, 437 bp, 402 bp, 409 bp and 458 bp, respectively (Figure 1a). We then tested whether LPA could increase the intracellular free Ca2+ ([Ca2+]i) level, which has a vital role in salivary secretion. Application of 1 μM LPA
consistently induced an increase in the \([\text{Ca}^{2+}]\) (Figure 1b), and this increase was also observed in \([\text{Ca}^{2+}]\)-free medium (gray line in Figure 1b). However, preincubation of the cells with 10 \(\mu\text{M}\) U73211 or 50 \(\mu\text{M}\) 2-APB for 10 min significantly inhibited the LPA-induced \([\text{Ca}^{2+}]\) increase (gray lines in Figure 1c and d). After LPA stimulation, subsequent stimulation of cells with 100 \(\mu\text{M}\) CCh, a typical muscarinic agonist, or with LPA after CCh stimulation did not increase the \([\text{Ca}^{2+}]\) (Figures 1e and f).

### Serum and LPA-induced Hippo pathway activation

We next determined whether serum and LPA could activate the Hippo pathway in HSG cells. In serum-free conditions, most YAP proteins were phosphorylated in these cells (second lane in the upper panel in Figure 2a). However, addition of 2 or 5\% FBS into the extracellular medium gradually but significantly dephosphorylated YAP, and 10\% FBS completely dephosphorylated YAP (first lane in the upper panel in Figure 2a). The lower panel shows a summary of the results. The amount of phosphorylated YAP significantly decreased when 2, 5 and 10\% serum were added. Figure 2b shows the translocation of YAP from the cytoplasm to the nucleus. At the resting state, in serum-free media, YAP (red color) did not localize within the nucleus (blue color); however, addition of 10\% serum markedly caused the translocation of YAP from the cytosol to the nucleus (noted by the merged, purple color). After confirming the activation of the Hippo pathway by serum, we then examined whether LPA could activate the Hippo pathway. We determined that 0.1 \(\mu\text{M}\) of LPA was associated with minimal levels of dephosphorylated YAP, but 1 \(\mu\text{M}\) of LPA significantly dephosphorylated YAP (the top row in Figure 2c). Although the dephosphorylation effect was not prominent compared with that of YAP, 1 \(\mu\text{M}\) LPA also dephosphorylated TAZ proteins (the third row in Figure 2c). The lower histograms summarize the results and show the effects of various LPA concentrations on YAP and TAZ dephosphorylation. We next examined whether this LPA-induced dephosphorylation of YAP/TAZ was dependent on \(\text{Ca}^{2+}\). Preincubation of cells with 10 \(\mu\text{M}\) BAPTA-AM for 30 min did not inhibit the LPA-induced YAP/TAZ dephosphorylation.

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**Figure 1** Functional expression of lysophosphatidic acid (LPA) receptors in HSG cells. (a) Expression of mRNA transcripts for all LPA receptor subtypes, from 1 to 6, using reverse transcriptase-PCR. Lanes ‘M’ and ‘N’ indicate the kb marker and negative control, respectively. β-actin was used as the positive control. (b) LPA-induced a transient \([\text{Ca}^{2+}]\) increase in the presence or absence (gray line) of \([\text{Ca}^{2+}]\) in the medium. (c and d) LPA-induced the \([\text{Ca}^{2+}]\) response before and after (gray line) pretreatment of cells with 10 \(\mu\text{M}\) U-73211 and 10 \(\mu\text{M}\) 2-APB. (e) Subsequent stimulation of the cells with 100 \(\mu\text{M}\) carbachol (CCh) after LPA stimulation. (f) Subsequent stimulation of the cells with 1 \(\mu\text{M}\) LPA after CCh stimulation.
(the upper panel in Figure 2d). The lower histograms summarize the effects of various LPA concentrations on YAP and TAZ dephosphorylation after preincubation with BAPTA-AM. The LPA-induced translocation of YAP/TAZ from the cytosol to the nucleus is shown in Figure 2e. At the resting state, TAZ (green) and YAP (red) did not localize within the nucleus; however, 1 μM LPA markedly translocated YAP and TAZ from the cytosol to the nucleus (note the merged, purple and cobalt colors, respectively). Preincubation of cells with BAPTA-AM had little effect on the LPA-induced YAP/TAZ
translocation into the nucleus (the lower panels in Figure 2c), which confirms our western blot data in Figure 2d.

Hippo signaling pathway mediated by Lats and RhoA/ROCK

We next examined whether LPA could activate Lats, a signaling molecule in the pathway upstream of YAP/TAZ. LPA dephosphorylated Lats and Mob kinase activator 1 (Mob1), which interacts with Lats, in a concentration-dependent manner (first to third rows in Figure 3a). We finally tested the involvement of RhoA/ROCK in the LPA-induced Hippo pathway. Cells were preincubated with 10 μM Y27632, a Rho-kinase (ROCK) inhibitor, which inhibited both the LPA-induced YAP/TAZ dephosphorylation (Figure 3b) and the translocation of YAP/TAZ into the nucleus based on the western blot experiment (Figure 3b) and immunocytochemistry (Figure 3c). Our data demonstrate that LPA induces dephosphorylation of YAP/TAZ mediated by both the Lats and RhoA/ROCK pathways.

Localization of YAP/TAZ in the subcellular fractionation

We next determined the subcellular localization of YAP/TAZ using cell fractionation and western blotting. The cytoplasmic localization of YAP/TAZ in LPA-treated cells was decreased compared with that of untreated cells, whereas the nuclear localization of YAP/TAZ in LPA-treated cells was increased compared with that of nontreated cells (Figure 4). These results are consistent with Figure 2, showing that LPA induces the dephosphorylation of YAP/TAZ and induces YAP/TAZ to move into the nucleus.

Relative expression levels of genes by LPA stimulation

YAP/TAZ is a cofactor for TEA domain family transcription factors, and the activation/inhibition of YAP/TAZ may have an important role in the regulation of various genes. To identify whether LPA treatment can induce gene expression, we quantified the mRNA levels of connective tissue growth factor (CTGF), ankyrin repeat domain-containing protein 1 (ANKRD1), cysteine-rich angiogenic inducer 61 (CYR61), transgelin (TAGLN) and endothelin 1 (EDN1). As shown in Figure 5, the mRNA levels of CTGF, ANKRD1, and CYR61, but not of TAGLN or EDN1, increased in response to LPA treatment.

Involvement of YAP and TAZ in TNF-α and LPA-induced apoptosis

We finally investigated the roles of YAP and TAZ in cell apoptosis using siRNA. Figure 6a shows the decreased expression levels of YAP and TAZ after siRNA treatment. Treatment of the cells with 20 nM TNF-α increased the expression of caspase-3 and its substrate, cleaved PARP (the upper panels in Figures 6b and c). We then investigated the effect of LPA on TNF-α-induced caspase-3 activation. Simultaneous application of 1 μM LPA and TNF-α further increased the expression level of caspase-3 and cleaved PARP. Pretreatment of the cells with YAP or TAZ siRNA significantly decreased expression levels of these two proteins (the third columns in Figures 6b and c).

DISCUSSION

LPA receptors, which are G protein-coupled receptors, affect diverse physiological and pathophysiological functions, including cancer, fibrosis, bone metabolism and autoimmune disease. To date, six distinct G protein-coupled receptors (LPA1–6) have been identified and have been shown to be differentially expressed at varying levels in epithelial cells. In HSG cells, LPA, epidermal growth factor and basic fibroblast growth factor stimulate Rho proteins, which are essential for acinar formation. Recent reports have indicated that the Hippo signaling pathway affects salivary gland development and is associated with Sjogren’s syndrome. However, the functional expression of LPA receptors and the associated signaling pathway have not been rigorously studied in secretory epithelia, including salivary glands.

In our study, mRNA transcripts for all subtypes of the LPA receptors were detected in HSG cells. LPA receptors in HSG cells appeared to function because LPA treatment consistently increased the [Ca^{2+}]i. Such an LPA-induced [Ca^{2+}]i increase was also shown in Ca^{2+}-free medium, although it had a fast [Ca^{2+}]i decay without the [Ca^{2+}]i plateau that occurred in Ca^{2+}-containing medium (Figure 1b). The LPA-induced [Ca^{2+}]i increase was inhibited by U73211, a PLC inhibitor, and 2-APB, an IP_{3}-sensitive Ca^{2+} store blocker. Thus, our results suggest that LPA-induced [Ca^{2+}]i increases are due to both [Ca^{2+}]i influx and [Ca^{2+}]i release from the intracellular Ca^{2+}.
stores, particularly from IP₃-sensitive Ca²⁺ stores. Because no [Ca²⁺], response to the subsequent stimulation of cells with CCh after LPA stimulation was detected, this further result confirms that LPA uses the same intracellular Ca²⁺ stores as CCh, a typical muscarinic agonist.

Next, we examined whether the Hippo pathway exists in HSG cells. Serum is a complex component that consists of growth factors, nutrients, lipids and other factors which can regulate YAP/TAZ activation. Among these factors, serum lipid-containing S1P and LPA potently decreases YAP/TAZ

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phosphorylation. Therefore, we first examined whether serum could activate the Hippo pathway before LPA application. Incubation of cells with FBS dephosphorylated the YAP protein in a concentration-dependent manner, which resulted in the translocation of YAP from the cytoplasm to the nucleus (Figure 2b). After confirming the dephosphorylation effect of serum on YAP/TAZ, we tested the effect of LPA. As expected, LPA also activated the dephosphorylation of YAP/TAZ in a concentration-dependent manner. Preincubation of the cells with BAPTA-AM, a strong Ca\(^{2+}\) chelator, had little effect on LPA-induced YAP/TAZ activation (Figure 2d); however, extracellular Ca\(^{2+}\) depletion impaired cell tight junctions and triggered the shift of YAP/TAZ from the cytoplasm to the nucleus in Eph4 mouse mammary epithelial cells.

Reports have indicated that the main Hippo pathway is a serine/threonine kinase cascade that involves MST1/2 protein kinase and large tumor suppressor homologs (Lats1/2). MST1/2 phosphorylates Lats1/2 and scaffolding proteins such as Mob. After Mob1 is phosphorylated by MST1/2, Mob1 interacts with Lats1/2, which in turn leads to an increase in their kinase activity. We examined whether two signaling molecules, the Lats and Mob1 were involved in the upstream pathway involved in dephosphorylating YAP/TAZ and found that LPA dephosphorylated the Lats and Mob1 (Figure 3a). In our experiments, inhibition of ROCK decreased LPA-induced YAP/TAZ dephosphorylation. To date, many regulators that regulate the core of the Hippo pathway (YAP and TAZ) at different levels have been identified. In particular, LPA and S1P have been reported as the main G protein-coupled receptors that regulate the Hippo pathway. Generally, LPA and S1P regulate YAP and TAZ via the RhoA/ROCK pathway and/or the MST/Lats pathway, causing the dephosphorylation and nuclear accumulation of YAP and TAZ. However, the upstream core Hippo signaling pathway can differ according to the LPA receptor subtypes that are expressed in each cell. For example, LPA acts through LPA2 and LPA3 to induce YAP activity mediated by RhoA/ROCK/PP1A, but not by Lats kinase, in OVCA433 EOC cells, whereas LPA acts through LPA1 and an LPA3 receptor to stimulate YAP activity mediated by Lats pathways in HEK293 cells. In our

**Figure 5** The lysophosphatidic acid (LPA)-stimulated mRNA levels of CTGF, ANKRDK, CYR61, TAGLN and EDN1. HSG cells were treated with 1 \(\mu\)M LPA for 2 h, followed by quantification of the mRNA levels of CTGF, ANKRDK, CYR61, TAGLN and EDN1 by real-time PCR and normalization to the level of \(\beta\)-actin. \(P<0.05\) and \(**P<0.01\) when compared with the control. The bar represents the means \(\pm\) s.e.m. \((n=3)\).

**Figure 6** Involvement of YAP/TAZ in TNF-\(\alpha\) and LPA-induced apoptosis. (a) Reduced levels of YAP and TAZ proteins after YAP and TAZ siRNA treatment. (b and c) Cells were transfected with YAP and TAZ siRNA. After 24 h, the cells were treated with 20 nM TNF-\(\alpha\) in the presence or absence of 1 \(\mu\)M LPA for 48 h. LPA, lysophosphatidic acid; TAZ, transcriptional co-activator with a PDZ-binding motif; TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); YAP, Yes-associated protein.
experiments, we found that mRNA transcripts for all LPA subtype receptors were expressed in HSG cells (Figure 1a) and that LPA dephosphorylated YAP/TAZ through both the Lats and RhoA/ROCK pathways (Figures 2 and 3), suggesting that more than two types of LPA receptors are functionally expressed in HSG cells.

To the best of our knowledge, this is the first study reporting the expression of LPA receptors and Hippo signaling pathway activation by LPA in salivary gland epithelial cells. LPA also enhanced TNF-α-induced caspase-3 and cleaved PARP, which was prevented by YAP and TAZ siRNA. This result demonstrates that the Hippo signaling pathway affects the apoptotic pathway, TNF-induced caspase-3 and cleaved PARP, which significantly decreased the downregulation of YAP and TAZ (Figures 6b and c). It has been reported that the Hippo pathway promotes cell survival and differentiation. However, our data show that stimulation of YAP/TAZ by LPA is required for TNF-α-induced LPA synergistic apoptosis (Figure 6) and that the mRNA of YAP/TAZ-dependent target genes, including CTGF, ANKRDI and CYR61, was upregulated in LPA-treated HSG cells (Figure 5). CTGF is an inflammatory mediator that increases interleukin-6 production. CYR61 promotes the apoptotic function of inflammation cytokines such as TNF-α, whereas ANKRDI1 is induced during wound repair to enhance healing. Therefore, it is possible that YAP/TAZ activated by LPA enhances the production of more CTGF and CYR61 mRNA than of survival factors such as ANKRDI. This result implies that upregulation of genes markedly increases the susceptibility of HSG cells to apoptosis. In conclusion, our results strongly suggest that LPA has an important role in apoptotic cell death through YAP and TAZ in the Hippo pathway.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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