Role of acylglycerol kinase in LPA-induced IL-8 secretion and transactivation of epidermal growth factor-receptor in human bronchial epithelial cells

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Kalari S, Zhao Y, Spannhake EW, Berdyshev EV, Natarajan V. Role of acylglycerol kinase in LPA-induced IL-8 secretion and transactivation of epidermal growth factor-receptor in human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 296: L328–L336, 2009. First published December 26, 2008; doi:10.1152/ajplung.90431.2008.—LPA (lysophosphatidic acid) is a potent bioactive phospholipid, which regulates a number of diverse cellular responses through G protein-coupled LPA receptors. Intracellular LPA is generated by the phospholipase D-mediated hydrolysis of membrane phospholipids. In this study, we show that overexpression of AGK wild type increased intracellular LPA production (~1.8-fold), enhanced LPA-mediated IL-8 secretion, and stimulated tyrosine phosphorylation of p38 MAPK, JNK, and NF-κB in human bronchial epithelial cells (HBEpCs). Expression of AGK in HBEpCs was detected by real-time PCR, and overexpressed AGK was mainly localized in mitochondria as determined by immunofluorescence and confocal microscopy. Overexpression of lentiviral AGK wild type increased intracellular LPA production (~1.8-fold), enhanced LPA-mediated IL-8 secretion, and stimulated tyrosine phosphorylation of p38 MAPK, JNK, and NF-κB activation. Furthermore, downregulation of native AGK by AGK small interfering RNA decreased intracellular LPA (~2-fold) and attenuated LPA-induced p38 MAPK, JNK, and NF-κB activation, tyrosine phosphorylation of p38 MAPK, JNK, and NF-κB activation, and IL-8 secretion. These results suggest that native AGK regulates LPA-mediated IL-8 secretion involving MAPKs, NF-κB, and transactivation of EGF-R. Thus AGK may play an important role in innate immunity and airway remodeling during inflammation.

LPA-Rs plays an important role in cellular responses such as proliferation, differentiation, motility, and survival (27, 28, 32, 34).

We have earlier demonstrated that LPA is a potent stimulator of IL-8 gene expression and secretion via NF-κB and AP-1 transcription (6, 26, 40), and IL-8 secretion by LPA was dependent on ligation to LPA1,3 expressed on the cell surface of human bronchial epithelial cells (HBEpCs) (26). In addition to signaling via its cognate LPA-Rs, ligation of LPA to its receptors resulted in transactivation of epidermal growth factor receptor (EGF-R), PDGF-Rβ, and C-Met (14, 36, 39, 40), which partly regulated IL-8 secretion (39) in HBEpCs. Furthermore, in HBEpCs, lipid phosphate phosphatase-1 (LPP1) regulated the LPA-induced IL-8 gene expression and secretion via calcium release as well as NF-κB activation (41), suggesting that extracellularly added LPA was dephosphorylated to monoacylglycerol (MAG) (2, 41). MAG derived by the action of LPPs can be subsequently rephosphorylated to intracellular LPA by lipid kinase(s). Acylglycerol kinase (AGK) has been recently identified as a potential lipid kinase that generates intracellular LPA from MAG in prostate cancer cells, and increased expression of AGK resulted in sustained activation of ERK1/2 enhanced cell proliferation and migration (2). The role of extracellular LPA in regulating cellular functions such as cytokine secretion has been well studied; however, very little is known about the role of AGK and intracellular LPA in signaling and cytokine production in airway epithelium.

As HBEpCs express higher AGK mRNA compared with human lung endothelial cells, we investigated the potential role of AGK in intracellular generation of LPA and IL-8 secretion. In this study, we show that overexpression of AGK wild type increased intracellular LPA production, enhanced intracellular LPA-mediated activation of p38 MAPK and NF-κB, IL-8 secretion, and EGF-R transactivation in HBEpCs. Furthermore, downregulation of native AGK expression by AGK small interfering RNA decreased intracellular LPA levels and attenuated LPA-induced p38 MAPK and NF-κB activation, tyrosine phosphorylation of EGF-R, and IL-8 secretion. These results demonstrate a novel role for AGK in LPA-mediated IL-8 secretion via NF-κB and EGF-R transactivation in HBEpCs.

MATERIALS AND METHODS

Materials. 1-Oleoyl (18:1) LPA and standards of LPA with different fatty acid chains (17:0 as internal standard, 18:0, 18:1, 18:2, and 20:4, where the first number indicates the number of carbon atoms and the second the number of double bonds) were purchased from Avanti Polar Lipids (Alabaster, AL). 

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Cells were incubated with 50 μCi/ml 32Porthophosphate in complete medium for 3 h, and lipids were extracted from the cells with 1 ml of cold methanol:HCl (100:1 vol/vol) followed by addition of 1 ml of chloroform. The mixture was incubated on ice for 30 min followed by the addition of 1 ml of chloroform and 1.8 ml of 0.1 N HCl. The extraction of proteins by Western blotting was obtained from Amersham Biosciences. The ELISA kit for IL-8 measurement was purchased from BIOSOURCE International (Camarillo, CA). siRNA for AGK was from Dharmacon (Lafayette, CO). Phosphorus-32 as H332PO4 in HCl-free water (specific activity 285.6 Ci/mg as phosphorus) was purchased from Perkin Elmer Life and Analytical Science (Boston, MA). Basal serum-free essential medium (BEBM) plus growth factor bullet kit was obtained from Lonza (Rockland, ME). All other reagents were of analytical grade.

**Cell culture.** Primary human bronchial epithelial cells were purchased from Lonza (Walkersville, MD). The isolated P1 (passage 1) HBEpCs were seeded at a density of ~1.5 × 10^5 cells/cm² onto Vitrogen-coated (1:75 in sterile water; Cohesion, Palo Alto, CA) 100-mm dishes in BEBM supplemented with growth factors. Cells were incubated at 37°C in 5% CO2 and 95% air to ~80% confluence and subsequently propagated in 35-mm or six-well collagen-coated dishes. All experiments were carried out between passages 1 and 4.

**RNA isolation, reverse transcription, and quantitative PCR.** Total RNA was isolated from cultured HBEpCs using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and RNA was quantified by spectrophotometry. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed to assess expression of AGK and 18S using primers designed based on human mRNA sequences. AGK primers: forward 5′AGG ATG CTG GCG TCA AAG TT 3′ and reverse 5′TGA GTC TGA GCC CAC TCC TT 3′ and 18S primers: forward 5′GTA ACC CGT TGA ACC CCA TT 3′ and reverse 5′CCA TCC AAT CGG TAG TAG CG 3′. Real-time PCR was performed using iQ SYBR Green Supermix and the iCycler real-time PCR detection system (Bio-Rad). Amplicon expression in each sample was normalized to its 18S RNA content. The relative abundance of target mRNA in each sample was calculated as 2 raised to the negative of its threshold cycle value times 10^−6 after being normalized to the abundance of its corresponding 18S, e.g., [(2−(IL-8 threshold cycle))/2−(18S threshold cycle)] × 10^6.

**Cloning of AGK.** PCR amplification of AGK for cloning was achieved by using 5′CACC ATG CTG GTG TCC TTT AAA ACG 3′ as left primer and 5′CTG GTT GGG GCT TGT GAG CAT 3′ as right primer from cDNA synthesized from HBEpCs as mentioned above. This amplified AGK gene was cloned into the pLent6/V5-DTOPO vector in such a way that AGK present the V5 tag in the COOH-terminal position. The control vector and the viral producing kit were purchased from Invitrogen, and the company protocols for TOPO cloning and lentiviral particle generation were followed. Vector alone was used to generate control lentiviral particles.

**Transfection of lentiviral constructs.** Infection of HBEpCs (~60% confluence) with the purified empty lentiviral vector and lentiviral vectors of AGK was carried out in six-well plates. Different volumes of viral particles were directly placed into 1 ml of basal essential growth medium for 48 h; the virus-containing medium was then replaced with complete BEBM, and experiments were performed.

**Measurement of AGK activity in intact cells.** HBEpCs seeded in six-well dishes were infected with lentiviral LacZ (vector) or V5-tagged lentiviral AGK wild type for 48 h in complete BEGM medium.
extracts were subjected to vigorous vortexing, and phase separation was achieved by centrifuging at 1,500 g for 15 min. The organic chloroform phase was transferred into 4-ml glass tubes, dried under N₂, and redissolved in 100 μl of chloroform. The lipid extracts were subjected to two-dimensional thin-layer chromatography (TLC) on silica gel 60 plates with chloroform/methanol/ammonia (65:35:5 vol/vol/vol) as the solvent system for the first dimension and chloroform/methanol/acetic acid/acetone/water (10:6:2:4:1 vol/vol/vol/vol/vol) as the solvent for the second dimension. Cold LPA was added as a carrier during TLC separation. Dried plates were subjected to autoradiography, the area corresponding to labeled LPA was excised, and radioactivity was determined by liquid scintillation spectrometry (23). The data were normalized to total radioactivity in the lipid extract.

Analysis of LPA by liquid chromatography tandem mass spectrometry. HBEpCs were seeded in six-well dishes, infected with vector, lentiviral LacZ (vector), or V5-tagged lentiviral AGK. Viral infection was allowed to proceed in complete BEGM for a further 48 h, and cells were incubated for 1 h with MAG. Both cells (for extracellular LPA) and cells (for intracellular LPA) were collected separately for LPA analysis. Lipids were extracted from media and cells (10). Briefly, media (0.5 ml) was added to 2 ml of methanol and 1 ml of chloroform with a subsequent addition of the internal standard (C17:0-LPA). Extraction was allowed for an hour on ice, and then 1 ml of chloroform and 1.3 ml of 0.1 N hydrogen chloride were added to achieve phase separation after vortexing and centrifugation at 2,000 g for 10 min. Cells were scraped into 2 ml of methanol (1 ml, twice), 1 ml of chloroform was added, followed by addition of the internal standard (C17:0-LPA) and 0.5 ml of 0.1 N hydrogen chloride. Lipids were extracted at 4°C overnight with subsequent addition of 1 ml of chloroform and 1.3 ml of 0.1 N hydrogen chloride to achieve phase separation after vortexing and centrifugation at 2,000 g for 10 min. The organic phase was transferred into 4-ml glass tubes, dried under N₂, and redissolved in 0.1 ml of methanol. LPA content and individual molecular species composition were measured by electrospray ionization MS/MS analysis after lipid separation by HPLC. An API-4000 Q-trap hybrid triple quadrupole-ion trap mass spectrometer coupled with Agilent 1100 LC system was used (3, 4). Lipids were separated on Zorbax Eclipse XDB-C8, 4.6 × 150-mm, 5-μm particle size column using methanol/water/formic acid, 75/25/0.5 vol/vol/vol, with 5 mM ammonium formate as solvent A and methanol/water/formic acid, 100/50/0.5 vol/vol/vol, with 5 mM ammonium formate as solvent B. LPA was analyzed in the negative ion mode with declustering potential and collision energy optimized for 17:0, 18:0, 18:1, 18:2, and 20:4 LPA. Multiple reaction monitoring parameters for nine other LPA molecular species were selected with the closest possible approximation to available LPA standards. The following transitions

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**Fig. 2.** Effect of AGK overexpression on lysophosphatidic acid (LPA) synthesis and IL-8 secretion. HBEpCs were seeded in 6-well dishes and infected with vector (lenti LacZ) or V5-tagged lentiviral AGK wild type for 48 h. Media were aspirated, and 1 μM monoacylglycerol (MAG), in basal media without any growth factors, was added to cells and incubated for an additional 1 h. Lipids were extracted from both cells and media for LPA measurements as described in MATERIALS AND METHODS. **A:** intracellular (cells) analysis of LPA accumulation compared between vector and V5-tagged lentiviral AGK wild type infected cells, either in presence or absence of MAG, by LC-MS/MS. **B:** extracellular media were analyzed for LPA accumulation in vector and V5-tagged lentiviral AGK wild type transfected cells, either in presence or absence of MAG, by LC-MS/MS. **C:** HBEpCs were seeded in 6-well dishes and infected with vector control or V5-tagged AGK wild type for 48 h and incubated for 3 h with 50 μCi/ml [³²P]orthophosphate. Labeled LPA generated in cells was quantified after separation of the lipid extracts by thin-layer chromatography and scintillation counting as described in MATERIALS AND METHODS. Values are means ± SD from 3 independent experiments. *Significantly different from vector control cells at P < 0.05. **Significantly different from vector control cells at P < 0.001.
were monitored: 407.0/153.0 (16:1 LPA); 409.0/153.1 (16:0 LPA); 423.0/153.1 (17:0 LPA internal standard); 431.0/153.0 (18:3 LPA); 433.0/153.0 (18:2 LPA); 435.1/152.9 (18:1 LPA); 437.0/153.0 (18:0 LPA); 455.1/153.0 (20:5 LPA); 457.0/153.0 (20:4 LPA); 459.1/153.0 (20:3 LPA); 461.1/153.0 (20:2 LPA); 481.1/153.0 (22:6 LPA); 483.1/153.0 (22:5 LPA); 485.1/153.0 (22:4 LPA).

Transfection of siRNA for AGK. Smartpool RNA duplexes corresponding to AGK were purchased from Santa Cruz Biotechnology. Scrambled control #2 siRNA was from Dharmacon Research (Lafayette, CO). HBEpCs (P1 or P2) were cultured onto six-well plates. At 40–50% confluence, transient transfection of siRNA was carried out using TransMessenger transfection reagent (Qiagen, Valencia, CA). Briefly, siRNA (10 nM) was condensed with Enhancer R and formulated with TransMessenger reagent according to the manufacturer’s instructions. The transfection complex was diluted into 900 μl of BEBM medium and added directly to the cells. The medium was replaced with complete basal essential growth medium after 3 h. Cells were analyzed by Western blotting at 72 h after transfection.

Measurements of IL-8 secretion. HBEpCs were cultured in six-well plates. Cells were challenged in BEBM containing 0.1% bovine serum albumin with or without LPA at the indicated concentrations for 3 h. At the end of the experiment, cell supernatants were collected, centrifuged at 1,000 g for 5 min at 4°C, and frozen at −80°C for later analysis of IL-8 by ELISA, which was performed according to the manufacturer’s instructions.

Preparation of cell lysates and Western blotting. After the indicated treatments, HBEpCs were rinsed twice with ice-cold phosphate-buffered saline and lysed in 200 μl of buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM MgCl2, 1% Triton X-100, 1 mM sodium orthovanadate, 10 μg/ml protease inhibitors, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Cell lysates were incubated at 4°C for 10 min, sonicated on ice for 10 s, and centrifuged at 5,000 g for 5 min at 4°C in a microcentrifuge. Protein concentrations were determined with a BCA protein assay kit (Pierce) using bovine serum albumin as the standard. Equal amounts of protein (10 μg) were subjected to 10% or 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, blocked with 5% (wt/vol) nonfat dry milk in TBST [25 mM Tris–HCl (pH 7.4), 0.137 mM NaCl, and 0.1% Tween 20] for 1 h, and incubated with primary antibodies in TBST containing 5% (wt/vol) bovine serum albumin for 1–2 h at room temperature. The membranes were washed at least three times with TBST at 15-min intervals and then incubated with either mouse or rabbit horseradish peroxidase-conjugated secondary antibody (1:3,000 dilution) for 1 h at room temperature. The membranes were developed with enhanced chemiluminescence detection system according to the manufacturer’s instructions.

Immunocytochemistry. HBEpCs grown on coverslips to ~60% confluence were transfected with lentiviral V5-tagged AGK wild type for 48 h. Coverslips were rinsed with PBS and treated with 3.7% formaldehyde in phosphate-buffered saline at room temperature for 20 min. After being washed with PBS, coverslips were incubated in blocking buffer (1% bovine serum albumin in TBST) for 1 h, and cells were subjected to immunostaining with V5 antibody (1:200 dilution) for 1 h and washed three times with TBST followed by staining with Alexa Fluor 488 (1:200 dilution in blocking buffer) for 1 h. After being washed at least three times with TBST, the coverslips were mounted using commercial mounting medium for fluorescent microscopy (Kirkgegaard and Perry laboratories, Gaithersburg, MD) and were examined by an immunofluorescent microscope with a Hamamatsu digital camera using a ×60 oil-immersion objective and MetaVue software.

Statistical analyses. All results were subjected to statistical analysis using one-way analysis of variance, and, whenever appropriate, analyzed by Student-Newman-Keuls test. Data are expressed as means ± SD of triplicate samples from at least three independent experiments, and statistical significance was accepted at a level of P < 0.05.
PCR, which matched the expected size (1,269 bp) on agarose gel. Furthermore, the PCR product was cloned into TOPO lentiviral vector and confirmed by nucleotide sequencing. Comparison of the expression of AGK mRNA by real-time RT-PCR showed an ~14-fold higher expression in HBEpCs compared with human lung endothelial cells (Fig. 1B). Additionally, transfection of HBEpCs with V5-tagged lentiviral AGK construct confirmed the expression of the overexpressed protein to run at ~47 kDa on Western blot (Fig. 1C). Confocal microscopy images of overexpressed AGK in HBEpCs with anti-V5 antibody showed a predominant mitochondrial localization of the expressed protein as confirmed by coinmunostaining with MitoTracker Red CMXRos; however, some distribution in the cytoplasm was also observed (Fig. 1D). These results indicate that AGK mRNA is highly expressed in HBEpCs and that overexpressed AGK has a predominant mitochondrial localization.

AGK regulates intracellular LPA levels and IL-8 secretion. To determine the functional significance of AGK, we analyzed the effect of AGK expression on intracellular LPA synthesis and IL-8 secretion. As determined by LC-MS/MS, overexpression of V5-tagged lentiviral AGK wild type for 48 h increased the intracellular levels of LPA (~1.8-fold compared with vector control cells), which was further enhanced (~2.5-fold) on addition of exogenous MAG to cells for 1 h (Fig. 2A). In contrast to accumulation of LPA in cells, LPA was not detected in the medium either in the presence or absence of MAG (Fig. 2B). In parallel experiments, HBEpCs overexpressing AGK were labeled with [32P]orthophosphate, and accumulation of labeled LPA was determined by thin-layer chromatography of the total lipid extract. As shown in Fig. 2C, a similar increase in intracellular generation of [32P]LPA was observed in AGK overexpressing cells. Furthermore, overexpression of AGK enhanced basal- and LPA-induced IL-8 secretion (vector con-

![Fig. 4. Role of MAPK in AGK-mediated IL-8 secretion. HBEpCs grown in 6-well dishes to ~95% confluence were infected with either vector control or V5-tagged lentiviral AGK wild type for 48 h or transfected with scrambled siRNA or AGK siRNA (10 nM) for 48 h before LPA (1 μM) challenge. In A and B, cells were exposed to LPA for 10 min, and cell lysates (20 μg proteins) were subjected to SDS-PAGE and Western blotted with phospho-p38 and total p38 (A) and phospho-JNK and total JNK antibodies (B). Blots were scanned and analyzed by image analyzer, and increase in phosphorylation was normalized with total p38 MAPK or total JNK. Shown are representative blots, and values are means ± SD from 3 independent experiments. *Significantly different from controls at P < 0.01. **Significantly different from LPA-treated cells at P < 0.005. In C and D, HBEpCs grown in 6-well dishes to ~60% confluence were transfected with scrambled siRNA or AGK siRNA for 48 h and then challenged with LPA (1 μM) for 10 min. Cell lysates (20 μg proteins) were subjected to SDS-PAGE and Western blotted with phospho-p38 and total p38 (C) and phospho-JNK and total JNK antibodies (D). Shown are representative blots, and values are means ± SD from 3 independent experiments. *Significantly different from controls at P < 0.01. **Significantly different from LPA-treated cells at P < 0.005.
AGK is a lipid kinase that phosphorylates MAG and DAG to LPA, 12.6 ± 0.2 pg/µg protein; Fig. 3C). Interestingly, AGK siRNA did not alter the mRNA levels of LPP1 and sphingosine kinase 1 in HBEpCs. These results show that overexpression of AGK upregulates intracellular LPA production and IL-8 secretion, whereas knocking down of native AGK downregulates intracellular LPA production and IL-8 secretion in HBEpCs.

Role of AGK on LPA-induced MAPK and NF-κB activation. We have previously demonstrated a role for p38 MAPK and JNK in LPA-induced IL-8 secretion via NF-κB and AP-1 transcriptional factors, respectively, in HBEpCs (26). As AGK regulates LPA-induced IL-8 secretion (Fig. 2D), we next investigated the effect of AGK on LPA mRNA expression on p38 MAPK/JNK signaling and NF-κB activation by LPA. HBEpCs were infected with vector control or V5-tagged lentiviral AGK wild type for 48 h and then challenged with medium alone or vehicle plus LPA (1 µM) for 15 min. As shown in Fig. 4, A and B, overexpression of AGK wild type enhanced LPA-induced phosphorylation of p38 MAPK and JNK without altering the basal phosphorylation; furthermore, downregulation of AGK with AGK siRNA attenuated LPA-induced p38 MAPK and JNK phosphorylation (Fig. 4, C and D). Transfection of cells with scrambled siRNA had no effect on the protein expression of p38, MAPK, and JNK, indicating specificity of the siRNA (Fig. 4, C and D). As activation of p38 MAPK and JNK pathways by LPA regulates NF-κB/AP-1 transcriptional factors (27), we next investigated the effect of AGK on NF-κB activation. Overexpression of V5-tagged lentiviral AGK (5 MOI) for 48 h significantly enhanced LPA-induced IkB phosphorylation compared with either vector control or LPA-treated cells, whereas AGK siRNA blocked LPA-induced IkB phosphorylation (Fig. 5, A and B). These results suggest that LPA-induced activation of p38 MAPK/JNK and NF-κB signaling is partly regulated by native AGK in HBEpCs.

Role of AGK on LPA-induced transactivation of EGF-R. We have previously demonstrated earlier that LPA transactivates PDGF-Rβ (37), EGF-R (14, 39), and C-Met (40); however, transactivation of EGF-R (39), but not PDGF-R (36), by LPA enhanced IL-8 secretion in HBEpCs. To further determine the role of AGK on LPA-induced EGF-R transactivation, HBEpCs were infected with V5-tagged lentiviral AGK wild type or transfected with AGK siRNA (10 nM) for 48 h before stimulation of LPA (1 µM) or EGF (20 ng/ml). As shown in Fig. 6, A and B, overexpression of AGK wild type enhanced LPA-, but not EGF-, induced tyrosine phosphorylation of EGF-R. Down-regulation of AGK with AGK siRNA attenuated LPA-mediated tyrosine phosphorylation of EGF-R; however, it had no effect on EGF-induced tyrosine phosphorylation of EGF-R (Fig. 6, C and D). These results show that native AGK modulates LPA-, but not EGF-, induced tyrosine phosphorylation of EGF-R in HBEpCs.

DISCUSSION

We have previously demonstrated that LPA is a potent stimulator of IL-8 expression and secretion in HBEpCs, which was dependent on p38 MAPK-NF-κB, JNK-AP-1, and EGF-R transactivation (26, 41). The present study demonstrates that native AGK plays an important role in regulating LPA-induced IL-8 secretion mediated by p38 MAPK-JNK-NF-κB and EGF-R transactivation signaling in HBEpCs.

AGK is a lipid kinase that phosphorylates MAG and DAG to LPA and PA, respectively (2). Our studies with V5-tagged lentiviral AGK show colocalization of AGK with MitoTracker red, and a similar mitochondrial localization of AGK has been
shown in NIH/3T3 fibroblasts, HEK-293, and PC-3 cells (2). Cell lysates of overexpressing V5-tagged lentiviral AGK phosphorylated MAG and DAG, but not sphingosine or ceramide (data not shown); however, AGK expressed in bacteria phosphorylated MAG and DAG as well as the sphingoid bases (30, 35). AGK expression was upregulated in prostate cancers compared with normal prostate tissues, and expression of AGK in PC-3 prostate cancer cells increased the formation and secretion of LPA (2). In normal primary HBEpCs, overexpression of AGK increased intracellular production of LPA; however, the intracellularly generated LPA was not secreted, suggesting differences between the prostate cancer cell line and the primary HBEpCs in the mechanism of action of intracellularly generated LPA signaling and IL-8 secretion.

Recently, a novel role of AGK in producing intracellular LPA/PA and modulation of EGF-R transactivation and sustained ERK1/2 stimulation, culminating in enhanced cell proliferation of PC-3 prostate cancer cells, has been demonstrated (2, 30). Our earlier studies have identified at least two separate mechanisms involved in LPA-induced IL-8 secretion in HBEpCs. The first pathway is dependent on the activation of p38 MAPK/NF-κB and JNK/AP-1 by LPA, whereas the second pathway involves LPA-dependent transactivation of EGF-R (14, 39). In the present study, we have shown a role for native AGK in LPA-induced activation of signaling cascades regulating IL-8 secretion in HBEpCs. Downregulation of native AGK with AGK siRNA attenuated intracellular production of LPA from C18:1 MAG, LPA-induced activation of p38 MAPK, JNK, IkB phosphorylation, and EGF-R tyrosine phosphorylation, and IL-8 secretion while overexpression of AGK enhanced the above signaling targets. Both in PC-3 prostate cancer cells (2) and HBEpCs, AGK overexpression markedly increased MAPK activation, EGF-R transactivation, and IL-8 secretion to LPA challenge. Downregulation of AGK in PC-3

Fig. 6. Role of AGK in EGF-R transactivation by LPA. In A and B, HBEpCs grown in 6-well dishes to ~95% confluence were infected with either vector control or V5-tagged lentiviral AGK wild type for 48 h and then challenged with either LPA (1 μM) or EGF (20 ng/ml) for 15 min. Cell lysates (20 μg proteins) were subjected to SDS-PAGE and Western blotted with phospho-EGF-R, total EGF-R, or total ERK1/2 antibodies. Shown are representative blots, and values are means ± SD from 3 independent experiments normalized to total ERK. *Significantly different from controls at P < 0.005. **Significantly different from either LPA- or EGF-treated cells at P < 0.02. In C and D, HBEpCs grown in 6-well dishes to ~60% confluence were transfected with scrambled siRNA or AGK siRNA (10 nM) for 48 h and then challenged with either LPA (1 μM) or EGF (20 ng/ml) for 15 min. Cell lysates (20 μg proteins) were subjected to SDS-PAGE and Western blotted with phospho-EGF-R, total EGF-R, or total ERK1/2 antibodies. Shown are representative blots, and values are means ± SD from 3 independent experiments normalized to total ERK. *Significantly different from controls at P < 0.01. **Significantly different from LPA-treated cells at P < 0.001. ***Significantly different from EGF-treated cells at P < 0.05.
prostate cancer cells reduced EGF-, but not LPA-, induced wound closure and IL-8 secretion (2); however, in HBEpCs, knockdown of AGK with AGK siRNA attenuated LPA-induced IL-8 production. Furthermore, this modulation by AGK siRNA involved both the canonical and transactivation of EGF-R pathways meditated by LPA. Our results on the involvement of native AGK in IL-8 secretion by LPA are different with PC-3 cells (2, 30). In PC-3 cells, AGK siRNA reduced EGF (from 2.6-fold to basal level) or LPA (from 4- to 2.5-fold)-induced IL-8 secretion, which was smaller compared with the effect in HBEpCs. Furthermore, in PC-3 cells, down-regulation of AGK reduced EGF-, but not LPA-, induced ERK1/2 activation (2); however, no such change in ERK activation by EGF (data not shown) or EGF-induced tyrosine phosphorylation of EGF-R was observed after knockdown of AGK in HBEpCs (Fig. 6D). Thus, our results suggest that endogenous AGK plays an important role in cross talk between LPA-Rs and transactivation of EGF-R in HBEpCs.

LPA-induced IL-8 secretion in epithelial cells and ovarian cancer cells is regulated at the transcriptional level by NF-kB and AP-1 (26, 41). Previously, we have shown that LPA induces COX-2 expression and PGE2 production through EGF-R transactivation and C/EBPβ activity in HBEpCs; the present results provide evidence for the involvement of native AGK in EGF-R transactivation by LPA. Additionally, AGK siRNA experiments indicate that LPA-induced COX-2 expression was dependent on C/EBPβ, which was downstream to EGF-R activation (data not shown). Earlier studies have demonstrated activation of COX-2 by LPA in renal mesangial (11), human colon cancer LoVo (29), and ovarian cancer cell lines (31). The mechanism of native AGK regulation of EGF-R transactivation, C/EBPβ activation, and COX-2 expression is unclear and requires further investigation. While results from PC-3 cells show production and secretion of LPA by AGK induces G1-dependent cell proliferation via LPA-Rs, our results show that LPA produced by native AGK is not secreted by HBEpCs (Fig. 2, A and B), suggesting that the observed effects of AGK are not due to secreted LPA acting via its receptors. We have previously shown intracellular localization of LPA1 in HBEpCs by immunocytochemistry (37), which suggests that intracellularly generated LPA via AGK may act via the intracellularly localized LPA-Rs. Further studies are necessary to characterize the mechanism(s) of intracellularly generated LPA by AGK in signaling pathways regulating IL-8 secretion.

The physiological significance of AGK localization and production of LPA in the mitochondria is yet to be defined. AGK can phosphorylate DAG to produce PA in the mitochondria, and PA is also generated via phosphorylation of DAG by DAG kinase (42) and agonist-induced activation of phospholipase D1/D2 (5, 15) in mammalian cells. PA, thus generated, is a second messenger and regulates numerous cellular responses such as mTOR activation (7, 8), PDGF-Rβ tyrosine phosphorylation (36), and COX-2 expression (19). Interestingly, an LPA phosphatase with homology to human prostatic acid phosphatase is also localized in the mitochondria (16), suggesting potential regulation of LPA biosynthesis and catabolism in the mitochondria. Furthermore, LPA generated in the mitochondria is also exported to other organelles, in part, is dependent on AGK.

In summary, our results indicate an important role for native AGK in LPA-induced IL-8 secretion in HBEpCs. Evidence is provided for AGK-dependent regulation of IL-8 release by LPA via p38 MAPK/JNK/NF-κB signaling and EGF-R transactivation. Our data suggest that AGK may modulate LPA-induced pro- and anti-inflammatory signals that regulate innate immunity in the airway epithelium.

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REFERENCES

ACYLGLYCEROL KINASE IN LPA SIGNALING


