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Report

Different Effects of Endoplasmic Reticulum Stress Inducers on Lysophosphatidic Acid-induced A431 Cell Dispersal

Ryo Saito,^{*,a,b} Yoshikatsu Eto,^a and Norihisa Fujita^b

^aAdvanced Clinical Research Center, Southern Tohoku Research Institute for Neuroscience, 255 Furusawa, Asao-ku, Kawasaki 215-0026, Japan; ^bLaboratory of Pharmacoinformatics, College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Noji-Higashi, Kusatsu 525-8577, Japan

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Lysophosphatidic acid (LPA), a small ubiquitous lipid found in vertebrate and non-vertebrate organisms, mediates diverse biological actions. LPA activates mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase, and low-molecular-weight G-proteins by binding to multiple LPA receptors (LPA₁₋₆, and GPR87). We previously demonstrated that colonies of A431 cells, a human epidermoid carcinoma cell line, were dispersed by LPA₁ and GPR87 activation. This LPA-induced A431 cell dispersal is accompanied by epithelial-mesenchymal transition (EMT) and is believed to contribute to tumor progression. Endoplasmic reticulum (ER) stress has been implicated in tumor progression and growth. A recent study found that activation of the inositol-requiring enzyme 1 α /X-Box binding protein 1 pathway promotes tumor progression and EMT in colorectal carcinoma. In addition, another report indicated that ER stress preconditioning using stress inducers promotes transforming growth factor β 1-induced EMT and apoptosis in human peritoneal mesothelial cells. To investigate the effect of ER stress preconditioning on LPA-induced cell dispersal, we analyze the crosstalk between LPA-induced and ER stress-induced cellular responses using A431 cells. Interestingly, preconditioning via tunicamycin, an ER stress inducer, inhibited LPA-induced A431 cell dispersal, whereas thapsigargin, another inducer, promoted cell dispersal. Furthermore, western blot analysis illustrated that LPA-induced p38 MAPK phosphorylation was enhanced by thapsigargin pretreatment but not by tunicamycin. These results indicate that ER stress inducers differentially alter LPA-induced A431 cell dispersal by modifying LPA-related signals.

Key words lysophosphatidic acid, endoplasmic reticulum stress, cell colony dispersal, epithelial-mesenchymal transition, tumor progression

INTRODUCTION

Lysophosphatidic acid (LPA) is a lysophospholipid produced via autotoxin enzymatic activity that acts as a lipid mediator and induces several physiological effects.¹⁻³ Several reports demonstrated that LPA activates mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase, and low-molecular-weight G-proteins by binding to at least seven specific G-protein coupled receptors known as LPA receptors.¹⁻⁷ LPA receptors can be classified into two major subfamilies. Specifically, LPA₁, LPA₂, and LPA₃, which share > 50% homology, are known as endothelial differentiation gene members, whereas LPA₄, LPA₅, LPA₆, and a putative LPA receptor, GPR87 are members of the P2Y purinergic family.⁵⁻⁷ These LPA receptors are expressed in various mammalian cells to transduce LPA-induced biological signals.² However, unwanted ectopic activation was known to contribute to the regulation of cell viability, proliferation, migration, and invasion in several human tumors.⁶ Similarly to these reports, our previous study also demonstrated that the colony dispersal of human squamous carcinoma-derived A431 cells was induced by LPA through LPA₁ and GPR87 activation.⁸ In addition, we revealed that epidermal growth factor (EGF) receptor transactivation

and p38 MAPK, Akt, and JNK signaling activation are strongly associated with this cell dispersal.⁸ Furthermore, we also found that LPA stimulation of A431 cells causes a change in subcellular localization of E-cadherin from the plasma membrane to the cytoplasm. This colony dispersal and changes in the subcellular localization of E-cadherin comprise epithelial-mesenchymal transition (EMT), which occurs during tumor migration and transition.^{9,10} In addition to A431 cells, the malignant transformation of tumors by LPA has been proven in other cell types. Our recent study demonstrated that the migration of human cervical carcinoma-derived ME180 cells was also induced by LPA through LPA₁ and GPR87 activation.¹¹ However, unlike A431 cells, ME180 cell migration was revealed to involve transactivation of EGF receptors and activation of Erk, p38 MAPK, and JNK but not Akt signaling.¹¹ These reports support the hypothesis that ectopic activation of LPA receptor strongly contributes to the malignant progression of tumor.

Endoplasmic reticulum (ER) plays a crucial role in the synthesis of membrane and secretory proteins, lipid biosynthesis, and homeostasis of intracellular Ca²⁺ levels. Various stresses, including hypoxia, glucose starvation, and viral infection, affect ER function and lead to ER stress, which is characterized by accumulation of unfolded proteins in the ER. As pro-

*To whom correspondence should be addressed. e-mail: sai.1986.s61@gmail.com

protective response to mitigate ER stress, mammalian cells have a specific signaling pathway called unfolded protein response (UPR). UPR is primarily mediated by three initiator/sensor transducers, namely inositol-requiring enzyme 1 α (IRE1 α), protein kinase R-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which are normally associated with 78-kDa glucose-regulated protein.^{12,13} Recent reports indicated that hypoxia, a major hallmark of tumor microenvironment, activates the UPR pathway and thus regulates cell viability via PERK/eukaryotic initiation factor 2 α /ATF4/CHOP signaling pathway.¹⁴ In other cases, IRE1 α /X-Box binding protein 1 (XBP1) pathway is reported to contribute to colorectal carcinoma cell proliferation through regulating cyclin D1 expression, and this pathway may also play an important role in EMT.¹⁵ Furthermore, it has been reported that ER stress inducers tunicamycin and thapsigargin can trigger EMT via SMAD2/3 phosphorylation and increase nuclear translocation of β -catenin and expression of Snail. In addition, taurine-conjugated ursodeoxycholic acid, an ER stress blocker, has been demonstrated to prevent transforming growth factor β 1-induced EMT and apoptosis in human peritoneal mesothelial cells.¹⁶

As previously mentioned, the effects of LPA on the malignancy of tumors and the significance of UPR to the microenvironmental adaptation of tumors have been individually reported. However, it remains unclear whether LPA signaling and UPR interact during tumor malignant transformation. In this study, we investigated the effect of ER stress inducers on LPA-induced A431 cell colony dispersal.

MATERIALS AND METHODS

Colony Dispersal Assay A431 cells (JCRB0004; JCRB Cell Bank, Osaka, Japan) were seeded at a density of 2×10^4 cells/cm² and maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Fujifilm-Wako, Osaka, Japan) in a humidified atmosphere of 5% CO₂ at 37°C. For dispersal assays, cells were pre-cultured for 24 h in serum-free DMEM containing 0.5 ng/mL tunicamycin or 1.0 nM thapsigargin and then treated with 1.0 μ M 1- α -LPA (oleoyl sodium salt, LPA 18:1; Avanti Polar Lipid Inc., Alabaster, AL, USA). Cells were visualized using a BZ-X700 microscope (KEYENCE, Osaka, Japan), and pictures of several fields of view per well (200-400 cells/well) were randomly acquired. The peripheral cells of a colony that appeared to be moving away from the colony or isolated cells that appeared to spread out were designated as scattered cells. The total number of scattered cells was divided by total number of cells, and the ratio was expressed as % scattered cells under the indicated condition.

WST-8-based Cytotoxicity Assay A431 cells were seeded into a 24-well flat-bottomed tissue culture plate in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37°C for 48 h. The culture medium was then replaced with serum-free DMEM containing tunicamycin (50 pg/mL to 0.5 μ g/mL) or thapsigargin (50 pM to 0.5 μ M) and incubated at 37°C for 24 h. Cell viability was evaluated using Cell Count Reagent SF (Nacalai Tesque) according to the manufacturer's instructions. Briefly, Cell Count Reagent SF was added to each well, fol-

lowed by incubation for 2 h at 37°C. Then, viable cells were assessed by measuring the optical density at 450 nm.

p38 MAPK and Akt Activities To investigate the effects of ER stress inducers on p38 MAPK- and Akt-activation, A431 cells were stimulated with 0.5 ng/mL tunicamycin or 1.0 nM thapsigargin for various time intervals. On the other hand, to investigate the combined effects of ER stress inducers and LPA, A431 cells were precultured for 24 h in serum-free DMEM containing 0.5 ng/mL tunicamycin or 1.0 nM thapsigargin and then treated with 1.0 μ M LPA for 15 min. Western blotting was performed using the extracts of these cells to determine the levels of phosphorylation of p38 MAPK and Akt. Total proteins were extracted using Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and quantified using a Pierce 660 nm Protein Assay Kit and Ionic Detergent Compatibility Reagent (Thermo Fisher Scientific, Waltham, MA, USA). An aliquot of the lysates was resolved via 10% SDS-PAGE and then transferred to Immobilon membranes (Merk Millipore, Burlington, MA, USA). After blocking procedure, the membranes were reacted with primary antibodies, all of which were purchased from Cell Signaling Technology (Danvers, MA, USA), according to the manufacturer's protocols. The immunocomplexes were visualized using anti-rabbit IgG conjugated with horseradish peroxidase and ImmunoStar Zeta or ImmunoStar LD (Fujifilm-Wako). The bands were detected using a LAS-4000 mini luminescent image analyzer (GE Healthcare, Chicago, IL, USA). Quantitative analysis was performed using Multi Gauge software (GE Healthcare).

Statistical Analysis Results were compared using two-tailed Student's *t*-test or two-tailed multiple *t*-test with ANOVA followed by Bonferroni's correction. All data are expressed as mean $\pm$ standard error of mean. A *p* value less than 0.05 was considered statistically significant.

RESULTS

Effects of ER Stress Inducers on A431 Cell Viability, Colony Dispersal and Intracellular Signaling Prolonged ER stress induces apoptosis via the PERK/ATF4/CHOP signaling pathway. However, because the efficiencies of inducers to causing ER stress varies by cell line, we first investigated the effects of tunicamycin and thapsigargin on A431 cell viability. Each reagent induced concentration-dependent cytotoxicity, and cell viability was significantly reduced by high concentrations of tunicamycin (> 5 ng/mL) and thapsigargin (> 50 nM) (Fig. 1A, B). We next assessed the effects of ER stress inducers on A431 cell colony dispersal at a range of concentrations that did not significantly affect cell viability. Interestingly, low concentrations of tunicamycin suppressed the effect on colony dispersal by about 10% (Fig. 2A), whereas thapsigargin significantly promoted colony dispersal in a concentration-dependent manner (0.5 nM and 1.0 nM thapsigargin showed about 35% and 50% colony dispersal, respectively; Fig. 2B). As the inducers appeared to have inhibitory or activating effects on colony dispersal (Fig. 2A, B), we analyzed the activation of p38 MAPK and Akt associated with EMT in A431 cells employing western blot analyses. Although transient activations of Akt were observed as early as 15 min at low concentration of both ER stress inducers, there was no sustained Akt activation by either inducer. No significant activation of p38 MAPK was observed at all meas-

Fig. 1. Effects of Endoplasmic Reticulum Stress Inducers on A431 Cell Viability

(A, B) A431 cells were treated with tunicamycin (Tm, 50 pg/mL to 0.5 μ g/mL: A) or thapsigargin (Tg, 50 pM to 0.5 μ M: B) for 24 h, and the cell viability was assessed using WST-8-based cytotoxicity assay. Data are normalized to the internal DMSO control. Statistical analysis were performed using ANOVA followed by Bonferroni's correction ($N = 3$; $*P < 0.05$ vs. control).

urement points in the reaction of tunicamycin nor thapsigargin (Fig. 2C). As shown in Fig. 2C, we were unable to identify the pharmacological evidence to prove the opposite effects of these inducers on colony dispersal. Then, we decided to investigate the combined effects of these inducers with LPA, which induces colony dispersal via activation of p38 MAPK and Akt.

Combined Effects of ER Stress Inducers and LPA on A431 Cell Colony Dispersal and Intracellular Signaling Our previous study illustrated that the colony dispersal of A431 cells was induced by LPA through LPA₁ and GPR87 activation.⁸⁾ Consistent with our previous report, 1.0 μ M LPA increased A431 cell colony dispersal more than 2.0-fold, whereas this colony dispersal remained at non-treated control levels under pretreatment with 0.5 ng/mL tunicamycin (Fig. 3A). This result suggested that tunicamycin has opposing effects on LPA-induced colony dispersal. Contrarily, pretreatment with 1.0 nM thapsigargin promoted LPA-induced colony dispersal by about 64%, suggesting that colony dispersal increased about 2.3-fold in LPA alone, whereas it increased about 3.0-fold in thapsigargin pretreatment and LPA stimulation relative to non-treated control (Fig. 3B). Therefore, these results indicate that thapsigargin possesses additive effect on LPA to induce colony dispersal.

To verify the crosstalk of intracellular signaling caused by LPA and ER stress inducers, we performed western blotting. Because our previous report revealed that the activation of p38 MAPK and Akt signaling is strongly associated with A431 cell colony dispersal,⁸⁾ we re-evaluated the activation of these signaling pathways under pretreatment with tunicamycin or thapsigargin. Consistent with our previous report, the phosphorylation levels of p38 MAPK and Akt were increased by 1.0 μ M LPA as shown in Fig. 3C. Notably, pretreatment with thapsigargin significantly enhanced LPA-stimulated p38 MAPK activation by about 1.9-fold, but not Akt-activation. On the other hand, pretreatment with tunicamycin did not affect LPA-stimulated activation of p38 MAPK nor Akt (Fig. 3C). These results strongly suggest that tunicamycin and thapsigargin have different effects on LPA-induced A431 cell colony dispersal. Furthermore, the enhanced activity of p38 MAPK should be one of the reasons thapsigargin increased the LPA-induced colony dispersal.

DISCUSSION

Aberrant activation of LPA receptor-mediated signaling is known to contribute to malignant tumor progression by promoting tumor growth, survival, migration, and metastasis. On the other hand, it is also known that tumor cells adapt to microenvironmental changes, such as hypoxia and nutrient starvation, by skillfully exploiting UPR. However, it remains unclear whether LPA signaling and UPR interact during tumor malignant transformation. In this study, we investigated the effects of ER stress on LPA-induced A431 cell colony dispersal using the chemical ER stress inducers tunicamycin and thapsigargin.

Tunicamycin and thapsigargin non-specifically activate UPR by disturbing *N*-glycan synthesis and calcium homeostasis, respectively. A comparative analysis of the expression patterns of UPR-related genes by several ER stress inducers also showed that tunicamycin and thapsigargin activate all stress sensors (IRE1, PERK and ATF6), unlike the other ER stress inducers, such as eeyarestin I, monensin and brefeldin A.¹⁷⁾ However, it is possible that tunicamycin and thapsigargin have different effects on other intracellular signals because of different mechanisms of action; that is, tunicamycin have a specific effect on *N*-glycosylation-dependent signals, whereas thapsigargin have a specific effect on Ca²⁺-dependent signals. The induction of EMT after UPR activation was first observed in rat alveolar epithelia cell lines upon treatment with tunicamycin or thapsigargin. In this case, activation of a number of pathways, including MAPK, SMAD, β -catenin, and Src kinase signaling, has been observed following treatment with 0.5 μ g/mL tunicamycin. In particular, activation of IRE1 α /XBP1 signaling and subsequent activation of SMAD2/3 and Src signaling are known to play important roles in UPR-induced EMT.^{18,19)} Several UPR-induced EMT cascades has been reported, but it is important to note that the intracellular responses involved in EMT differ by cell type.²⁰⁾ In this study, we newly discovered that human squamous carcinoma-derived A431 cells also exhibit thapsigargin-induced EMT. However, the precise molecular mechanism in thapsigargin-induced EMT are unknown. Our previous study raised the possibility that the activation of MAPK and Akt signaling contribute to thapsigargin-induced A431 cell dispersal.⁸⁾ In addition, other research groups found that LPA activates SMAD2 during colony dispersal.²¹⁾ Furthermore, the important role of intracellular Ca²⁺ for cytoskeletal reorganization, cell migration, and cancer metastasis have also been reported.²²⁾ Therefore, thapsigargin-induced A431 cell colony dispersal may be mediated by activation of MAPK, Akt, SMAD2/3, and Ca²⁺-related signal cascades. In support of these hypotheses, thapsigargin was revealed to significantly enhance LPA-stimulated p38 MAPK activation in the present study. Although the possibility that thapsigargin also contributes to SMAD and/or Ca²⁺-related signal cascades remains unknown, the enhancement of p38 MAPK signaling is one of the reasons that thapsigargin potentiated the LPA-induced colony dispersal. However, thapsigargin alone did not activate p38 MAPK, suggesting that thapsigargin indirectly contributes to the LPA-stimulated p38 MAPK activation. It is known that thapsigargin irreversibly inhibits Ca²⁺-ATPase on the ER membrane, inhibiting Ca²⁺ uptake into the ER and simultaneously causing Ca²⁺ to leak from the ER into the cytoplasm. Therefore, the cytosolic Ca²⁺ is increased by thapsigargin. Interestingly, both LPA₁ and

Fig. 2. Effects of Endoplasmic Reticulum Stress Inducers on A431 Cell Colony Dispersal and Intracellular Signaling

(A) A431 cells were cultured in serum-free medium with 0.05 or 0.5 ng/mL tunicamycin (Tm) for 48 h. Phase contrast micrographs showing A431 scattering at 48 h after Tm treatment. The quantitative values of scattered cells are normalized to the control, and the results are expressed relative to the untreated control. Statistical analysis was performed using ANOVA followed by Bonferroni's correction ($N = 3$; $*P < 0.05$ vs. control). (B) A431 cells were cultured in serum-free medium with 0.5 or 1.0 nM thapsigargin (Tg) for 48 h. Phase contrast micrographs showing A431 scattering at 48 h after Tg treatment. The quantitative values of scattered cells are normalized to the control, and the results are expressed relative to the untreated control. Statistical analysis was performed using ANOVA followed by Bonferroni's correction ($N = 3$; $*P < 0.05$ vs. control). (C) A431 cells were treated with 0.5 ng/mL Tm or 1.0 nM Tg for various time intervals. The total cell lysates were analyzed by Western blotting using the indicated antibodies. The experiment was independently repeated three times with similar results.

GPR87 have been reported to conjugate with G_{aq} protein and, on activation by LPA, increase cytosolic Ca^{2+} via release from the ER.^{2,5)} Therefore, pretreatment with thapsigargin may have enhanced colony dispersal by increasing the sensitivity of the Ca^{2+} response to LPA stimulation. Thus, experiments focusing on the Ca^{2+} responses are required to clarify the effects of thapsigargin on LPA signaling.

Conversely, A431 colony dispersal was strongly inhibited by tunicamycin treatment, in contrast to previous reports.^{16,18,19)} Recent reports demonstrated that tunicamycin suppresses tumor growth and metastasis by inhibiting the Akt signaling pathway.^{23–26)} However, we observed the transient activation of Akt in the presence of tunicamycin, as well as in the presence of thapsigargin (Fig. 2C). Therefore, we assume that the activation of Akt is not directly related to the ER stress inducers-induced colony dispersal. Because *N*-glycosylation contributes significantly to protein maturation and trafficking, we specu-

late that tunicamycin may have inhibited expression and intracellular trafficking of LPA receptors and signaling molecules related to colony dispersal and thus attenuated LPA activity. In fact, LPA_1 and GPR87 are predicted to have *N*-glycosylation sites within their molecules (UniProt ID: Q92633 and Q9BY21). In addition, validation using other inhibitors, such as 2-deoxy-D-glucose, is necessary to confirm the inhibition of A431 cells colony dispersal via inhibition of *N*-glycosylation and to identify relevant signals. As the inhibitory effect of tunicamycin on colony dispersal is so strong that the inhibitory mechanism would provide crucial information for a novel target for drug design against cancer metastasis.

In summary, our present study revealed that tunicamycin and thapsigargin, both of them are ER stress inducers, showed opposite effects on A431 cell colony dispersal in the presence or absence of LPA. The potentiation effect of thapsigargin was demonstrated to be attributable to enhanced activation

Fig. 3. Combined Effects of Lysophosphatidic Acid (LPA) and Endoplasmic Reticulum Stress Inducers on A431 Cell Colony Dispersal and Intracellular Signaling

(A) A431 cells were precultured in serum-free medium with 0.5 ng/ml tunicamycin (Tm) for 24 h. Phase contrast micrographs showing A431 scattering at 24 h after 1.0 μ M LPA treatment. The quantitative values of scattered cells are normalized to the control, and the results are expressed relative to the untreated control. Statistical analysis was performed using ANOVA followed by Bonferroni's correction ($N = 3$; $*P < 0.05$). (B) A431 cells were precultured in serum-free medium with 1.0 nM thapsigargin (Tg) for 24 h. Phase contrast micrographs showing A431 scattering at 24 h after 1.0 μ M LPA treatment. The quantitative values of scattered cells are normalized to the control, and the results are expressed relative to the untreated control. Statistical analysis was performed using ANOVA followed by Bonferroni's correction ($N = 3$; $*P < 0.05$). (C) A431 cells were pretreated with 0.5 ng/mL Tm or 1.0 nM Tg for 24 h prior to 1.0 μ M LPA stimulation. The total cell lysates were analyzed by Western blotting using the indicated antibodies at 15 min after LPA stimulation. Data are normalized to the total protein levels, and the results are expressed as the fold increase versus the protein levels in cells treated with LPA alone. Statistical analysis was performed using ANOVA followed by Student's t -test ($N = 4$; $*P < 0.05$ vs. without LPA) or Bonferroni's correction ($N = 4$; $\dagger P < 0.05$ vs. LPA alone).

of p38 MAPK, while the signaling mechanism of the attenuation effect of tunicamycin remains unclear. Since the ER stress inducers have different effects on tumor cells, such as promot-

ing or inhibiting malignancy and inducing apoptosis, depending on their pharmacological effect and concentrations,¹⁶⁾ it is necessary to accurately determine the alteration of intracellular

signaling specific to the cell type, inducer, and concentration. Based on the present results, the pharmacological mechanisms of these ER stress inducers should be intensively investigated in order to develop a novel drug design against malignant tumor progression.

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Conflict of interest The authors declare no conflict of interest.

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