Regular Article

 β -Estradiol Enhanced Secretion of Lipoprotein Lipase from Mouse Mammary Tumor FM3A CellsTomoyasu Fujii,*^a Mizuho Ogasawara,^{a,b} Jun Kamishikiryo,^a and Tetsuo Morita*^a^aDepartment of Biochemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University; 985 Higashimura, Fukuyama, Hiroshima 729–0292, Japan: and ^bDepartment of Pharmacy, Kochi Health Sciences Center; 2125–1 Ike, Kochi 781–8555, Japan.

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The role of β -estradiol (E_2) in lipoprotein metabolism in mammary tumors is unclear, therefore, we investigated the effect of E_2 on the secretion of lipoprotein lipase (LPL) from mouse mammary tumor FM3A cells. E_2 -treated cells increased the secretion of active LPL from FM3A cells in a time- and dose-dependent manner. Activity of mitogen-activated protein kinase (MAPK) was increased in the tumor cells treated with E_2 , and enhanced secretion of LPL was suppressed by MAPK kinase 1/2 inhibitor, PD98059, extracellular signal-regulated kinase (ERK) 1/2 inhibitor, FR180204, p38 MAPK inhibitor, SB202190, and phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002. In addition, the effect of E_2 on LPL secretion was markedly suppressed by an inhibitor of mammalian target of rapamycin complex (mTORC) 1 and 2, KU0063794, but were not by a mTORC1 inhibitor, rapamycin. Furthermore, a small interfering RNA (siRNA)-mediated decrease in the expression of rapamycin-insensitive companion of mTOR (Rictor), a pivotal component of mTORC2, suppressed secretion of LPL by E_2 . These results suggest that the stimulatory secretion of LPL by E_2 from the tumor cells is closely associated with an activation of mTORC2 rather than mTORC1 possibly via the MAPK cascade.

Key words lipoprotein lipase; β -estradiol; mammary tumor; mitogen activated-protein kinase; mammalian target of rapamycin

INTRODUCTION

Breast cancer is one of the most serious cancers in women.¹⁾ Epidemiological evidence indicates that elevated endogenous estrogen levels are associated with an increase in breast cancer in postmenopausal women.^{2–4)} In addition, the risk of breast cancer is linked to increases in estrogen level due to obesity.^{5–7)} Estrogen is known to affect nutrient supply by regulating the expression and activities of various enzymes involved in metabolic pathways, and the signal transduction and transcriptional actions of estrogen are also believed to contribute to the growth of cancer cells.⁸⁾ Furthermore, the role of lipids on the supply of the energy and cell components are particularly important in tumor growth and proliferation^{9,10)}; however, the function of β -estradiol (E_2) on lipid metabolism in cancer cells is not fully understood.

Lipoprotein lipase (LPL) is responsible for supplying energy sources and cell components in lipid metabolism¹¹⁾; LPL is synthesized in extrahepatic tissues^{12,13)} and adheres to cell surfaces and to the luminal endothelium of blood vessels.¹⁴⁾ Triacylglycerol components in chylomicron and very low-density lipoproteins in plasma are hydrolyzed by LPL, and the intracellular absorption of free fatty acids and the chylomicron remnants and intermediate-density lipoproteins is markedly dependent on LPL activities.¹¹⁾

The effects of E_2 on lipoprotein metabolism are not well understood with regards to regulation of LPL from mammary tumor cells. In this study, we investigated the enhanced secretion of LPL from mouse mammary tumor FM3A cells by E_2 , and the association on the stimulation of LPL with the mammalian target of rapamycin (mTOR) pathway.

MATERIALS AND METHODS

Materials Chemicals were sourced as follows: Mouse mammary tumor FM3A cells (RIKEN Bio Resource Center, Tsukuba, Japan); β -estradiol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan); PD98059, FR180204, SB202190, SP600125, LY294002, rapamycin, and KU0063794 (Funakoshi Co., Ltd., Tokyo, Japan); RPMI 1640 medium (Nippon Suisan Kaisha, Ltd., Tokyo, Japan); triolein [$\text{carboxyl-}^{14}\text{C}$] (3.7 MBq/mL) and adenosine 5'-triphosphate [$\gamma\text{-}^{32}\text{P}$] (370 MBq/mL) (PerkinElmer, Inc., MA, U.S.A.); anti-LPL antibody (LabFrontier, Seodaemun-gu, Seoul, Korea); anti-Rictor (53A2) antibody (Cell Signaling Technology, Inc., Beverly, MA, U.S.A.); anti- β -actin antibody (Proteintech, Tokyo, Japan); anti-rabbit secondary antibodies conjugated with horseradish (Bio-Rad, Hercules, CA, U.S.A.); goat anti-mouse immunoglobulin G (IgG)-heavy and light chain antibody horseradish peroxidase (HRP) conjugated (Bethyl Laboratories, Inc., Montgomery, AL, U.S.A.). All other chemicals used were of analytical grade.

Cell Culture Mouse mammary tumor FM3A cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 0.2 mM alanine, 0.15 mM kanamycin, 2 mM glutamine, and 1 mM sodium pyruvate under a humidified atmosphere at 37°C and 5% CO_2 . Medium was removed by aspiration following centrifugation ($150 \times g$, 3 min at 4°C) and cells were seeded at a density of 1×10^7 cells/mL in HBSS(+) without phenol red (FUJIFILM Wako Pure Chemical Corporation). Tumor cells were incubated with E_2 in the presence of various inhibitors. After incubation, suspension cells were centrifuged ($860 \times g$, 3 min at 4°C). The supernatant was used as sample

*To whom correspondence should be addressed. e-mail: t-fujii@fukuyama-u.ac.jp; morita@fukuyama-u.ac.jp

Fig. 1. Stimulatory Effects of β -Estradiol on Secretion of LPL Activity from Mouse Mammary Tumor FM3A Cells

FM3A cells were incubated for 60 min with (●) 10 nM E₂ or without (○) for indicated time (a, b). Changes on secretion of LPL the cells incubated for 60 min with various concentrations of E₂ (c, d). The activity (a, c) and protein (b, d) of LPL secreted into the medium was measured as described in Materials and Methods. * p < 0.05 and ** p < 0.01, compared with groups without E₂.

containing secreted LPL, and cells were used as sample for intracellular mitogen-activated protein kinase (MAPK) and LPL. Cells were sonicated with homogenate buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) [pH 7.2], 210 mM mannitol, 70 mM sucrose, 1 mM ethylene glycol bis(2-aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA)), 5 μ g/mL leupeptin, 1 mM β -glycerophosphate and 1 mM sodium pyrophosphate. Cellular debris was removed by centrifugation (16000 $\times g$ 10 min at 4°C) and processed for further experiments.

Determination of LPL Activity LPL activity was determined by a radioisotopic method using triolein [carboxyl-¹⁴C]-substrate.¹⁵⁾ The liberated ¹⁴C-fatty acids were separated from the reaction mixture.¹⁶⁾ LPL activity was determined by subtracting the non-LPL-dependent activity from the total lipolytic activity and expressed as nmol of free fatty acids (FFA) produced/h/10⁶ cells.

Determination of MAPK Activity MAPK activity was determined by modification of the published method.¹⁷⁾ Enzyme solutions were incubated in 80 mM Tris buffer (pH 7.5), 0.5 mg/mL myelin-basic protein (Sigma, St. Louis, MO, U.S.A.), 10 mM MgCl₂, 1 mM MnCl₂, 0.2 mM ATP, adenosine 5'-triphosphate [γ -³²P]- at 25°C for 20 min. The reaction was stopped by adding 0.1 N HCl containing 1 mg/mL bovine serum albumin (BSA). The phosphorylated substrate was applied to Whatman cellulose chromatography paper P81 and

washed with 0.5% (v/v) phosphoric acid. The paper strips were dried and analyzed for their radioactivity. The MAPK activity was expressed pmol of [³²P] phosphorylated myelin/min/10⁶ cells.

Suppression of Rictor Expression FM3A cells, which were seeded at a density of 8 $\times$ 10⁴ cells/mL, were transfected with 10 nM small interfering RNA (siRNA) targeting mouse Rictor (SASI_Mm01_00137729, Sigma) or siRNA control (MISSION siRNA Universal Negative Control #1, Sigma) using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Waltham, MA, U.S.A.) and Opti-MEM (Gibco, Waltham, MA, U.S.A.). After the incubation, knock-down efficiency of transfection was confirmed by Western blotting.

Western Blotting Cell culture supernatant was used for protein quantification directly, and protein concentrations were determined by bicinchoninic acid (BCA) protein assay method. Five to twenty micrograms of total proteins were loaded in 10% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. After transfer, the membrane was blocked in 1% skim milk in TBS-T (20 mM Tris-buffer containing 137 mM NaCl, 1% (v/v) Tween 20) for 1 h at room temperature and incubated with various primary antibodies diluted in TBS-T containing skim milk overnight at 4°C. The membrane was washed in TBS-T and then incubated with the horseradish peroxidase-conjugated secondary antibody

for 1 h at room temperature. The blots were visualized by using Western blot chemiluminescence reagent (ImmunoStar, FUJIFILM Wako Pure Chemical Corporation).

Statistical Analysis All results are presented as mean $\pm$ standard error (S.E.) of six observations. Similar results were obtained with at least two separate experiments. The data were analyzed by Student's *t*-test.

RESULTS

Effect of E_2 on Secretion of LPL Figure 1(a) shows the time-dependent secretion of LPL activity from FM3A cells

Fig. 2. Effects of E_2 on MAPK Activity in FM3A Cells

FM3A cells were incubated with (●) 10 nM E_2 or without (○) over 90-min period. MAPK activity was measured as described in Materials and Methods. ***p* < 0.01, compared with groups without E_2 .

incubated with 10 nM E_2 over a 90-min period. Secreted LPL activity was 1.5- to 2-fold higher than that of vehicle at 60 min. However, the amount of LPL protein secreted into the medium was not found to change compared to vehicle (Fig. 1b). Cells were then incubated with E_2 (0–10 nM) for 60 min, which significantly enhanced the secretion of LPL activity in a dose-dependent manner up to 10 nM (*p* < 0.01) (Fig. 1c). The amount of LPL protein secreted into the medium was also not dose-dependent (Fig. 1d). These results suggested increases in specific activity of secreted LPL by E_2 . When E_2 enhanced the secretion of LPL from the cells into the medium, the enzyme activity in the cells decreased (data not shown).

Effect of E_2 on Intracellular MAPK Activity E_2 is known to phosphorylate MAPK.^{18,19)} Therefore, to determine whether the stimulatory secretion of LPL activity by E_2 is involved in MAPK phosphorylation. Intracellular MAPK activity significantly increased with E_2 supplementation in a time-dependent manner (*p* < 0.01 at 90 min) (Fig. 2).

Effects of Various Inhibitor on Stimulatory Secretion of LPL by E_2 Cells were incubated with E_2 in the presence of a various inhibitors of the MAPK cascade pathway. The stimulatory secretion of LPL by E_2 was suppressed by MAPK kinase 1/2 inhibitor, PD98059, extracellular signal-regulated kinase (ERK) 1/2 inhibitor, FR180204, p38 MAPK inhibitor, SB202190 (Figs. 3a–c), but not by a c-Jun N-terminal kinase (JNK) inhibitor, SP600125 (Fig. 3d). To examine the association of phosphatidylinositol 3-kinase (PI3K) on E_2 -enhanced secretion of LPL, cells were incubated with E_2 in the presence of a PI3K inhibitor, LY294002, which significantly reduced the E_2 -enhanced secretion at 10 μ M (*p* < 0.01) (Fig. 4).

In addition, we investigated the effects of mammalian target

Fig. 3. Effects of MAPK Inhibitor on Stimulatory Secretion of LPL by E_2

FM3A cells were incubated for 60 min with 10 nM E_2 or without in presence of PD98059 (a), FR180204 (b), SB202190 (c), SP600125 (d). The activity of LPL secreted into the medium was measured as described in Materials and Methods (**p* < 0.05, ***p* < 0.01).

of rapamycin (mTOR) inhibitors on the secretion of LPL by E_2 . The E_2 -enhanced secretion of LPL was markedly reduced by an mTORC1/2 inhibitor, KU0063794 (Fig. 5a) ($p < 0.01$, 100 nM), but was not suppressed by an mTORC1 inhibitor,

Fig. 4. Effects of PI3K Inhibitors on Stimulatory Secretion of LPL by E_2

FM3A cells were incubated for 60 min with 10 nM E_2 or without in presence of LY294002. The activity of LPL secreted into the medium was measured as described in Materials and Methods (** $p < 0.01$).

Fig. 5. Effects of mTOR Inhibitors on Stimulatory Secretion of LPL by E_2

FM3A cells were incubated for 60 min with 10 nM E_2 or without in presence of rapamycin (a) and KU0063794 (b). The activity of LPL secreted into the medium was measured as described in Materials and Methods (* $p < 0.05$, ** $p < 0.01$).

Fig. 6. Effects of Rictor Knock-Down on Stimulatory Secretion of LPL by E_2

FM3A cells were transfected with Rictor siRNA and control siRNA (a). Transfected cells were incubated for 60 min with E_2 (b). The activity of LPL secreted into the medium was measured as described in Materials and Methods (* $p < 0.05$, ** $p < 0.01$).

rapamycin (Fig. 5b).

Effects of Rictor Knock-Down on E_2 -Enhanced Secretion of LPL To further investigate whether stimulatory secretion of LPL by E_2 is mainly *via* mTORC2. Rictor is a novel binding partner, and is essential for activation of mTORC2.^{20,21} Therefore, we decreased expression of Rictor by siRNA knockdown of *Rictor* mRNA in FM3A cells (Fig. 6a). Subsequently, when knock-down cells were incubated with E_2 , the stimulatory secretion of LPL by E_2 was markedly suppressed ($p < 0.01$, 10 nM E_2) (Fig. 6b).

DISCUSSION

Estrogen has important physiological functions in both females and males, and in particular, the growth and proliferation of breast cancer cells are dependent on estrogen concentration. However, the role of estrogen in breast cancer cells is not fully understood. Lipoprotein lipase is a secreted protein and a key enzyme in lipid metabolism and essential in cell growth.^{22,23} Few studies have investigated the effects of estrogen on lipoprotein metabolism, particularly in the secretion and activity of LPL from breast cancer cells.

In this study, E_2 enhanced the secretion of active LPL from mouse mammary tumor FM3A cells in a dose- and time-dependent manner (Figs. 1a, c). However, the amount of LPL as protein secreted into the medium was not markedly

increased (Figs. 1b, d). LPL is synthesized in rough endoplasmic reticulum (ER), it is modified in ER and golgi, and is then secreted as an active form.²⁴⁾ It was recognized that LPL is catalytically active only as a homodimer,^{25–27)} and both active and inactive forms of LPL have been found.²⁸⁾ A recent report showed that some monomers of LPL may be enzymatically active,²⁹⁾ suggesting that the increase of secreted active LPL by E_2 is not due to an increase in amount of LPL protein, but is due to an increase in LPL specific activity by the promotion of post-translational modification to increase abundance of the active form. In addition, the decrease in intracellular LPL activity and invariant protein content by E_2 may be due to the preferential secretion of active LPL.

There are several reports that E_2 activates the MAPK signal transduction pathway.^{18,19)} In our data, E_2 also elevated MAPK activity in the tumor cells (Fig. 2). The stimulatory secretion of LPL activity by E_2 was suppressed by various inhibitors, except SP600125 of the MAPK subfamily (Figs. 3a, b, c, d). Therefore, we investigated changes to PI3K activity, which is also reported to be activated by E_2 .³⁰⁾ The E_2 -enhanced secretion of LPL activity was reduced by a PI3K inhibitor, LY294002 (Fig. 4). In bladder carcinoma, an mTOR is suggested to affect the downstream regulation of PI3K transduction pathway.³¹⁾ E_2 activation of mTOR *via* the estrogen receptor has been observed in various cells.^{32–34)} An inhibitor of mTORC1 and C2, KU0063794 decreased the stimulatory secretion of LPL by E_2 , but an mTORC1 inhibitor, rapamycin did not (Figs. 5a, b). Finally, we determined that the stimulatory secretion of LPL by E_2 was suppressed with the siRNA knock-down of Rictor in mTOR (Fig. 6b).

These results suggest that the stimulatory secretion of the more active LPL by E_2 from mouse mammary tumor FM3A cells is due to the activation of mTORC2 with an association of MAPK–PI3K transduction pathway through the estrogen receptor.

Conflict of Interest The authors declare no conflict of interest.

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