β-Estradiol Enhanced Secretion of Lipoprotein Lipase from Mouse Mammary Tumor FM3A Cells

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The role of β-estradiol (E2) in lipoprotein metabolism in mammary tumors is unclear, therefore, we investigated the effect of E2 on the secretion of lipoprotein lipase (LPL) from mouse mammary tumor FM3A cells. E2-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 E2, 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 E2 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 E2. These results suggest that the stimulatory secretion of LPL by E2 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.2–7) In addition, the risk of breast cancer is linked to increases in estrogen level due to obesity.2–7) 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.8–10) Furthermore, the role of lipids on the supply of the energy and cell components are particularly important in tumor growth and proliferation;10,11) however, the function of β-estradiol (E2) on lipid metabolism in cancer cells is not fully understood.

Lipoprotein lipase (LPL) is responsible for supplying energy sources and cell components in lipid metabolism.13) LPL is synthesized in extrahepatic tissues12,13) 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 E2 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 E2, 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, LY294002, rapamycin, and KU0063794 (Funakoshi Co., Ltd., Tokyo, Japan); RPMI 1640 medium (Nippon Suisan Kaisha, Ltd., Tokyo, Japan); triolein [carboxyl-14C]- (3.7MBq/mL) and adenosine 5′-triphosphate [γ-32P]- (370MBq/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, 0.2mM alanine, 0.15mM kanamycin, 2mM glucoseamine, and 1mM sodium pyruvate under a humidified atmosphere at 37°C and 5% CO2. Medium was removed by aspiration following centrifugation (150×g, 3 min at 4°C) and cells were seeded at a density of 1×105 cells/mL in HBSS(+) without phenol red (FUJIFILM Wako Pure Chemical Corporation). Tumor cells were incubated with E2 in the presence of various inhibitors. After incubation, suspension cells were centrifuged (860×g, 3 min at 4°C). The supernatant was used as sample

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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 µg/mL leupeptin, 1 mM β-glycerophosphate and 1 mM sodium pyrophosphate. Cellular debris was removed by centrifugation (16000 × 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. 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/10^6 cells.

**Determination of MAPK Activity** MAPK activity was determined by modification of the published method. 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/10^6 cells.

**Suppression of Rictor Expression** FM3A cells, which were seeded at a density of 8 × 10^5 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.

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.
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 ± 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 E₂ on Secretion of LPL** Figure 1(a) shows the time-dependent secretion of LPL activity from FM3A cells incubated with 10 nM E₂ 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 E₂ (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 E₂. When E₂ enhanced the secretion of LPL from the cells into the medium, the enzyme activity in the cells decreased (data not shown).

**Effect of E₂ on Intracellular MAPK Activity** E₂ is known to phosphorylate MAPK. Therefore, to determine whether the stimulatory secretion of LPL activity by E₂ is involved in MAPK phosphorylation. Intracellular MAPK activity significantly increased with E₂ supplementation in a time-dependent manner ($p < 0.01$ at 90 min) (Fig. 2).

**Effects of Various Inhibitor on Stimulatory Secretion of LPL by E₂** Cells were incubated with E₂ in the presence of a variety of inhibitors of the MAPK cascade pathway. The stimulatory secretion of LPL by E₂ 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 phosphatidyl inositol 3-kinase (PI3K) on E₂-enhanced secretion of LPL, cells were incubated with E₂ in the presence of a PI3K inhibitor, LY294002, which significantly reduced the E₂-enhanced secretion at 10 µM ($p < 0.01$) (Fig. 4).

In addition, we investigated the effects of mammalian target...
of rapamycin (mTOR) inhibitors on the secretion of LPL by E2. The E2-enhanced secretion of LPL was markedly reduced by an mTORC1/2 inhibitor, KU0063794 (Fig. 5a) \((p<0.01, 100 \text{nM})\), but was not suppressed by an mTORC1 inhibitor, rapamycin (Fig. 5b).

**Effects of Rictor Knock-Down on E2-Enhanced Secretion of LPL.** To further investigate whether stimulatory secretion of LPL by E2 is mainly via mTORC2, Rictor is a novel binding partner, and is essential for activation of mTORC2.\(^\text{20,21}\) 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 E2, the stimulatory secretion of LPL by E2 was markedly suppressed \((p<0.01, 10 \text{nM} \text{E2})\) (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.\(^\text{22,23}\) 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, E2 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. It was recognized that LPL is catalytically active only as a homodimer, and both active and inactive forms of LPL have been found. A recent report showed that some monomers of LPL may be enzymatically active, suggesting that the increase of secreted active LPL by E2 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 E2 may be due to the preferential secretion of active LPL.

There are several reports that E2 activates the MAPK signal transduction pathway. In our data, E2 also elevated MAPK activity, which is also reported to be activated by E2. An E2-enhanced secretion of LPL activity was reduced by a PI3K inhibitor, (Figs. 4). In bladder carcinoma, an mTOR is suggested to affect the downstream regulation of PI3K transduction pathway. E2 activation of mTOR via the estrogen receptor has been observed in various cells. An inhibitor of mTORC1 and C2, dramatically decreased the stimulatory secretion of LPL by E2, but an mTORC1 inhibitor, rapamycin did not (Figs. 5a, b). Finally, we determined that the stimulatory secretion of LPL by E2 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 E2 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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