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The Effect of Medroxyprogesterone Acetate on the Secretion of Lipoprotein Lipase in Mouse Mammary Tumor Cells

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Progesterone (P4) is a corpus luteum hormone associated with the development of the mammary gland and uterus. The actions of P4 on lipid metabolism in breast cancer are unclear. In this study, we investigated medroxyprogesterone acetate (MPA) on the secretion of lipoprotein lipase (LPL) from mouse mammary tumor FM3A cells. The tumor cells were incubated with MPA and other agents. The treated cells were used in the mitogen-activated protein kinase (MAPK) and LPL activity assay. The supernatant was used in the LPL activity assay and western blotting. The increased secretion of LPL in the tumor cells treated with MPA was observed. The MPA-stimulated secretion of LPL was suppressed by a protein kinase A (PKA) inhibitor. The activity of MAPK increased in the tumor cells treated with MPA, and various MAPK inhibitors suppressed the stimulatory secretion of LPL. The effect of MPA on LPL secretion was markedly suppressed by an inhibitor of the mechanistic target of rapamycin complex (mTORC) 1 and 2, KU0063794, but not the mTORC1 inhibitor, rapamycin. Furthermore, a small interfering RNA-mediated decrease in Rictor's expression, a pivotal component of mTORC2, suppressed the stimulatory secretion of LPL. These results suggest that the stimulatory secretion of LPL in the tumor cells treated with MPA is closely associated with activation of mTORC2, possibly via the MAPK signaling associated with PKA activation.

Key words lipoprotein lipase, progesterone, mechanistic target of rapamycin, cAMP-dependent protein kinase, mitogen-activated protein kinase

INTRODUCTION

Lipid metabolism plays a vital role in tumor progression.[1,2] Lipoprotein metabolism is significantly associated with cancer growth, metastasis, and invasion.[3,4] Therefore, the investigation of lipid metabolism in tumor cells is essential for discovering new therapeutic strategies.

Lipoprotein lipase (LPL; EC 3.1.1.34) is an important enzyme responsible for lipoprotein metabolism and is synthesized in extrahepatic tissues or adheres to cell surfaces and the luminal endothelium of blood vessels.[5] It is also recognized that LPL hydrolyzes triacylglycerol components in chylomicrons and very-low-density lipoproteins in plasma. Moreover, LPL expression has been confirmed in various kinds of cancer cells.[6–8] Sakayama *et al*. indicated high levels of LPL activity in some human sarcomas and carcinomas that were proliferating actively.[9] And Trost *et al*. also reported that high LPL activity in non-small cell lung cancer tissue predicted shorter patient survival.[10]

Progesterone (P4) is involved in the development of the mammary gland and uterus and pregnancy continuation. The expression of the P4 receptor (PR) is a well-known risk factor for breast cancer.[11] High doses of medroxyprogesterone acetate (MPA), which is used as a P4 medicament, are applied to PR-positive breast cancer patients.[12] Furthermore, it is considered to have an effect of suppressing cancer cachexias.[13] On the other hand, an increase in the growth of breast cancer caused by P4 has been reported.[14] As described above, several studies have been reported the relationship between the growth of breast cancer and P4, however, effects of P4 on lipoprotein metabolism, especially LPL, in breast cancer cells are not fully elucidated in detail.

In this study, we show that MPA causes the stimulatory secretion of LPL in mouse mammary tumor FM3A cells. FM3A cells have been often used in the investigation for breast cancer, to possess the high proliferative potential and hormone receptor positivity.[15,16] Moreover, the action of MPA is partly associated with a pathway of the mechanistic target of rapamycin (mTOR).

MATERIALS AND METHODS

Materials FM3A cells were obtained from RIKEN Bio Resource Center (Tsukuba, Japan). MPA was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Triolein [carboxyl-^{14}C]- (3.7 MBq/mL) and ATP [γ-^{32}P]- (370 MBq/mL) were purchased from Perkin Elmer (Waltham, MA, USA). cAMP EIA kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). Small interfering RNA (siRNA) Rictor (SASI_Mm01_00137729) and siRNA control (MISSION siRNA Universal Negative Control #1) were purchased from Sigma (Louis, MO, USA). All other chemicals used were of

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analytical grade.

Cell Preparation FM3A cells were cultured in the medium containing RPMI1640 supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. After removing the medium, the cells were incubated in Hanks' balanced salt solution (+) with MPA in the presence of various inhibitors. After incubation, the supernatant and the cells were separate by centrifugation, and served as the preparation for studying the activities of LPL and mitogen-activated protein kinase (MAPK), for analyses of western blotting, and for measurements of cAMP contents as described.[17] Cell growth was counted with an Olympus BX50 microscope (Tokyo, Japan).

LPL Assay LPL activity was determined by a radioisotopic method using triolein [carboxyl-^{14}C]-labeled substrate.[17–19] LPL activity was expressed as nmol of free fatty acids produced/h/10^6 cells.

Measurement of cAMP Contents According to the manufacturer's protocol, cAMP contents in cells were lysed in a 0.1 M HCl solution for 20 min were determined using enzyme immunoassay (EIA) using a cAMP EIA kit. cAMP content was expressed pmol of cAMP/protein mg.

MAPK Assay Measurement of MAPK activity was performed by a radioisotopic method using ATP [γ-^{32}P]- labeled substrate.[17,20] The MAPK activity was expressed pmol of [^{32}P] phosphorylated myelin/min/10^6 cells.

Rictor Knockdown FM3A cells, which were seeded at a density of 8×10^4 cells/mL in 150 cm^2 suspension cell culture flasks from Sumitomo Bakelite (Tokyo, Japan), were transfected to 10nM siRNA Rictor or siRNA control using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Waltham, MA, USA) and Opti-MEM (Gibco, Waltham, MA, USA) following the manufacturer's instructions. The knockdown efficiency of transfection was confirmed using western blotting.

Western Blotting Antibodies of LPL and Rictor (53A2) were purchased from LabFrontier (Seodaemun-gu, Seoul, Korea) and Cell Signaling Technology (Beverly, MA, USA), respectively. Western blotting was performed as described previously.[17]

Statistical Analysis Results are shown as mean ± standard error (SE) of four to six observations for two separate experiments. The data were analyzed by Student's t-test or one-way ANOVA followed by Tukey multiple comparisons test.

RESULTS

Effects of MPA on FM3A Cells We investigated the effects of MPA on the growth of FM3A cells. The cell growth increased up to 10 nM (Fig. 1a). Figure 1b shows the secreted LPL activity from FM3A cells incubated in the presence of 1 nM MPA over a 90-min period. However, the amount of LPL protein secreted into the medium was not found to change with time (Fig. 1c). Then, the cells were incubated with MPA (0–50 nM) for 60 min; the secreted LPL activity significantly increased as the dosage increased up to 10 nM ($P < 0.05$) (Fig. 1d). The amount of LPL protein secreted into the medium was not influenced by dosage (Fig. 1e).

Effect of MPA on cAMP Content in FM3A Cells To examine whether the stimulation of the LPL secretion by MPA was associated with the activation of Gs protein-coupled receptor and protein kinase A (PKA), the cells were incubated with MPA in the presence of H-89, a PKA inhibitor.[21] The MPA-stimulated secretion of LPL was resuced by H-89 (Fig. 2a). Furthermore, the cells were incubated with 1 nM MPA over a 5-min period. A time-dependent increase in cAMP content in the tumor cells was observed up to 1 min (Fig. 2b).

Effect of MPA on MAPK and MAPK Inhibitors There was a marked increase in intracellular MAPK activity after treatment with MPA up to 60 min ($P < 0.01$) (Fig. 3a). The tumor cells were incubated with MPA in the presence of various inhibitors of the MAPK signaling pathway, such as U0126,[22] FR180204,[23] SB202190,[24] SP600125.[25] The stimulation of LPL secretion by MPA was suppressed by U0126[22] (a MAPK kinase 1/2 inhibitor), FR180204[23] (an extracellular signal-regulated kinase (ERK) 1/2 inhibitor), and SB202190[24] (a p38 MAPK inhibitor) (Fig. 3b–d), but not by SP600125[25] (a JNK inhibitor) (Fig. 3e). The concentration of the MAPK inhibitors used is based on the references cited.[22–25]

Effects of Rictor Knockdown on MPA-Stimulated Secretion of LPL The MPA-stimulated secretion of LPL was markedly reduced by an inhibitor of mTORC1 and 2, KU0063794 (Fig. 4a) ($P < 0.01$, 100 nM) but was not suppressed by the mTORC1 (mTORC1 inhibitor, rapamycin (Fig. 4b)). To further investigate whether the stimulation of LPL secretion by MPA is essential via mTORC2, we knocked down Rictor expression by siRNA in the tumor cells that is specific components of mTORC2 (Fig. 4c).[26] Subsequently, when the knockdown cells were incubated with MPA, the stimulation of LPL secretion by MPA was strongly inhibited (Fig. 4d) ($P < 0.01$, 1 nM MPA).

DISCUSSION

In our studies, MPA enhanced the proliferation of mouse mammary tumor FM3A cells at a concentration of approximately 10 nM. According to the studies,[14,27] MPA was proven to enhance the mammary tumor growth by P4. Moreover, a high dose of MPA is used to treat endometrial and breast cancers.[12,13] These findings suggest that the growth of the tumor cells is changed by any concentration of MPA. The results showed that MPA-stimulated active LPL in mouse mammary tumor FM3A cells up to 10 nM (Fig. 1d). However, the amount of LPL protein secreted into the medium was not markedly increased (Fig. 1e). LPL is synthesized in the rough endoplasmic reticulum (rER), modified in the rER and Golgi body, and then secreted as an active form.[5] Therefore, these results suggest that the increase in the secreted active LPL due to MPA is not caused by the increase in the amount of LPL protein but by the post-translational modification promotion.[5]

The P4 receptor is localized in various cell organelles such as the nucleus, cytoplasm, and cell membrane.[28,29] Among them, the membrane progesterone receptor (mPR) is identified, but mPR which is either G protein-coupled receptor, is still under unknown.[30,31] In this study, MPA increased cAMP contents within 1 min (Fig. 2b), and this suggests that the mPR might be a Gs protein-coupled receptor. Figure 2a show that H-89 (a PKA inhibitor) suppresses the secretion of active LPL by MPA. Therefore, it is considered that the secretory process of LPL by MPA is involved in an activation of PKA with an increase in cAMP contents.

According to the reports, P4 activates the MAPK signaling.[27,32] In this report, MPA also stimulated MAPK activity in the tumor cells (Fig. 3a). The secreted LPL activity by MPA was reduced by the inhibitors on the MAPK pathway, except

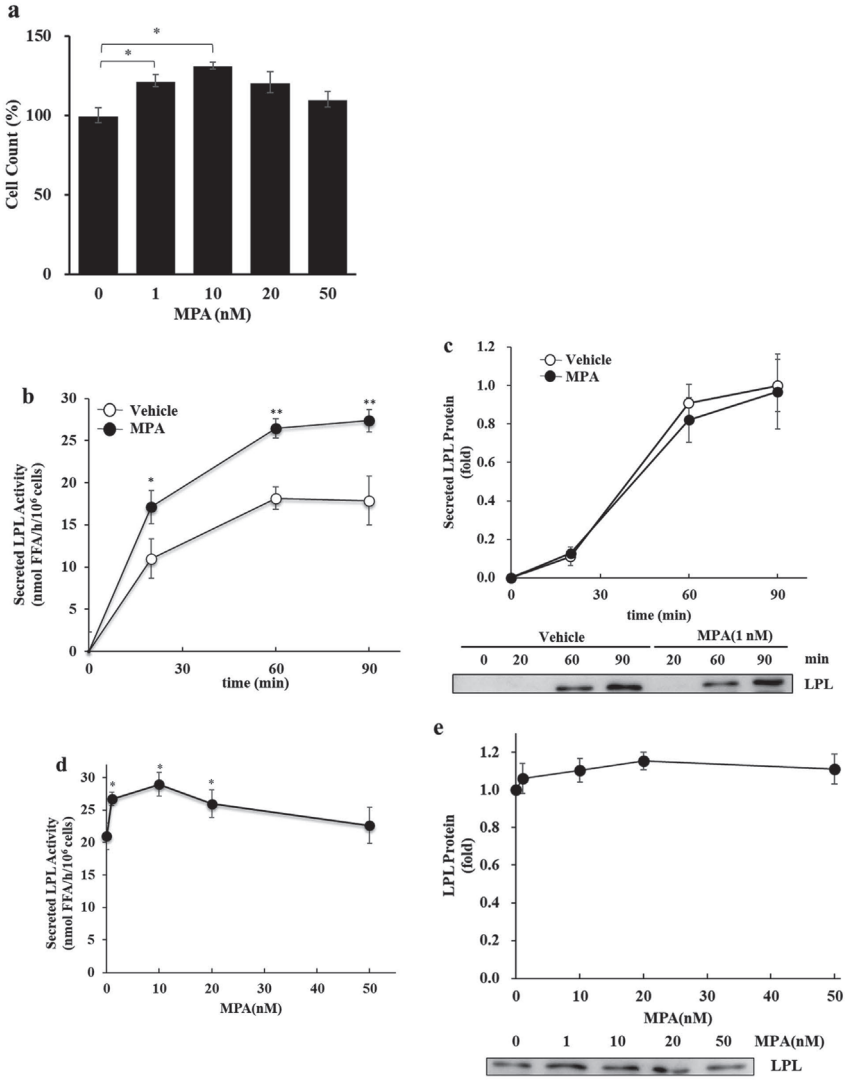

Fig. 1. Effects of Medroxyprogesterone Acetate (MPA) on Mouse Mammary Tumor FM3A Cells

(a) FM3A cell growth after treatment with various MPA concentrations for 72 h. (b, c) Secreted lipoprotein lipase (LPL) and protein activity in FM3A cells incubated with (●) or without (○) 1 nM MPA for the indicated time. (d, e) Changes in the secretion of LPL from the cells incubated for 60 min with various concentrations of MPA were examined. (b, d) The activity of LPL and (c, e) LPL protein secreted into the medium. Data were shown as mean ± SE. Significantly different from no MPA (n = 6) by Student's t-test (b, c, e) or one-way ANOVA followed by Tukey multiple comparisons test (a, d). *P < 0.05, **P < 0.01

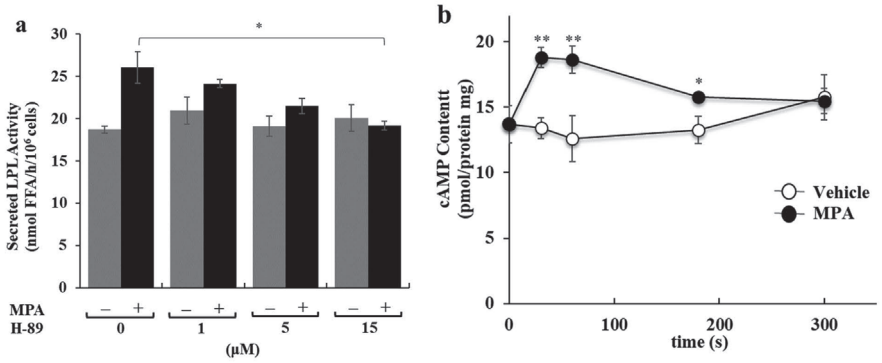

Fig. 2. Effect of MPA on cAMP Contents in FM3A Cells

(a) Secreted LPL activity in FM3A cells incubated for 60 min with or without 1 nM MPA in the presence of H-89. Significantly different from no H-89 by one-way ANOVA followed by Tukey multiple comparisons test. (n = 4) *P < 0.05 (b) cAMP content in FM3A cells incubated with (●) or without (○) 1 nM MPA over 300 s. Data were shown as mean ± SE. Significantly different from no MPA by Student's t-test. (n = 6) *P < 0.05, **P < 0.01

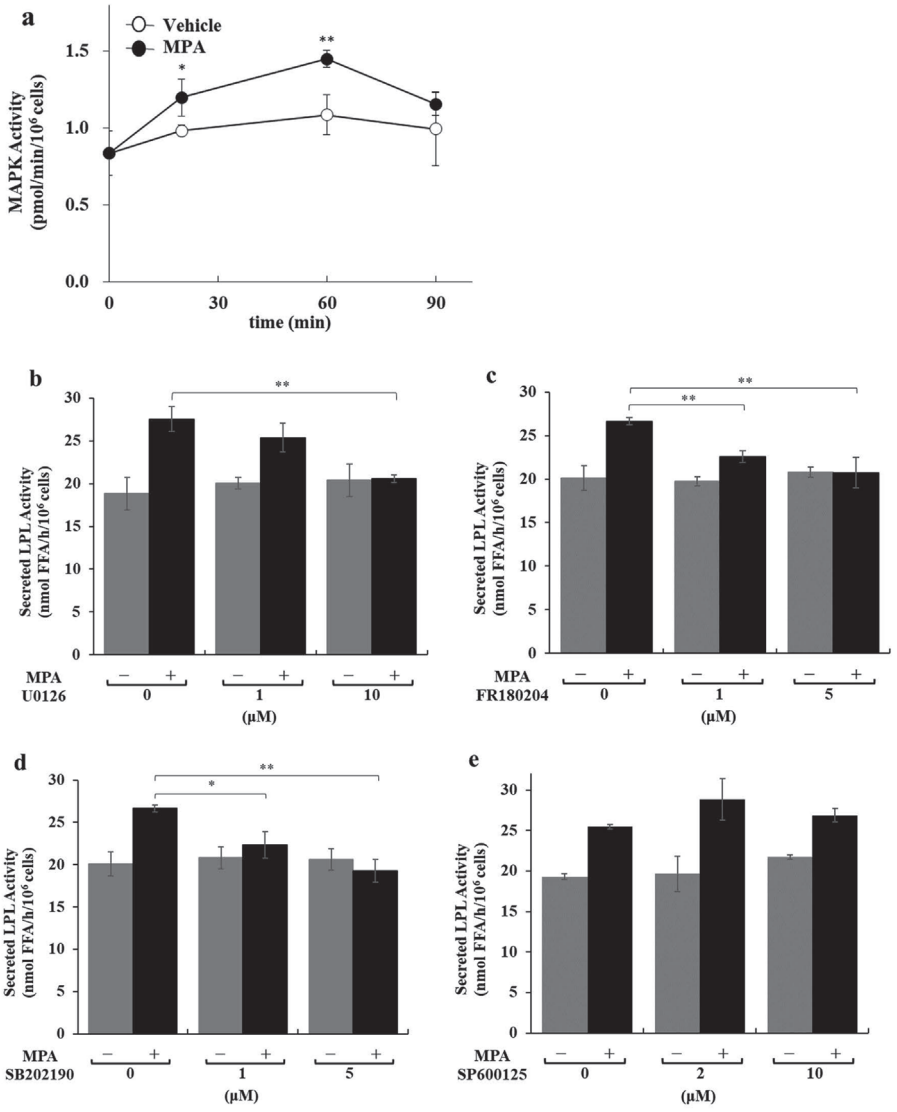

Fig. 3. Effect of MPA on Mitogen-Activated Protein Kinase (MAPK) and MAPK Inhibitors

(a) MAPK activity in FM3A cells incubated with (●) or without (○) 1 nM MPA over a 90-min period. Secreted LPL activity in FM3A cells incubated for 60 min with or without 1 nM MPA in the presence of U0126 (b), FR180204 (c), SB202190 (d), and SP600125 (e). Data were shown as mean ± SE. Significantly different from treated with (b-e, n = 6) or without (a, n = 6) MPA by Student's t-test. *P < 0.05, **P < 0.01

SP600125 (an inhibitor of JNK in the MAPK subfamily) (Fig. 3b-e). From our results, the stimulatory secretion of LPL by MPA was mediated through ERK 1/2 and the p38 MAPK subfamily pathways. These MAPK pathways have been shown to activate the mTOR pathway.[33,34] The mTORC1 and mTORC2 inhibitor, KU0063794, decreased the stimulation of LPL secretion by MPA (Fig. 4a). However, the mTORC1 inhibitor, rapamycin, did not suppress the stimulatory secretion of LPL (Fig. 4b). Finally, we determined that the siRNA Rictor knockdown suppressed MPA stimulated secretion of LPL in the mTOR (Fig. 4d).

In conclusion, our study suggests that MPA increased in the secretion of the active LPL from FM3A cells due to the stimulation of mTORC2 with an activation of the PKA–MAPK signaling pathway through the mPR. Moreover, these results suggest that the low concentration of P4 may increase the progression of breast cancer by promoting lipoprotein metabolism.

Conflict of interest The authors declare no conflict of interest.

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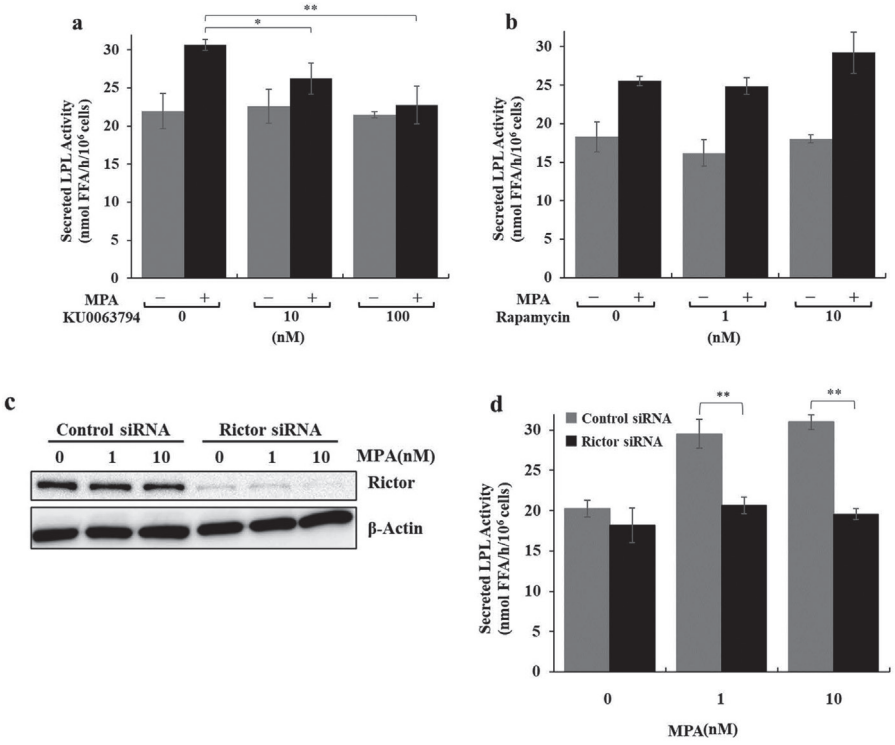

Fig. 4. Effects of Rictor Knockdown on MPA-Stimulated Secretion of LPL

Secreted LPL activity in FM3A cells incubated for 60 min with or without 1 nM MPA in the presence of KU0063794 (a) and rapamycin (b). (c) FM3A cells were transfected with Rictor siRNA and control siRNA. (d) Secreted LPL activity in transfected FM3A cells incubated for 60 min with MPA. Data were shown as mean ± SE. Significantly different from treated with MPA (a, b, n = 6) or without rictor knockdown (d, n = 4) by Student's t-test. *P < 0.05, **P < 0.01

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