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Evodiamine inhibits adipogenesis via the EGFR–PKCα–ERK signaling pathway

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ABSTRACT

The molecular mechanism of the anti-adipogenic effect of evodiamine (which has several capsaicin-like pharmacological actions) was investigated. The evodiamine effect was not blocked by the specific TRPV1 antagonist capsazepine in 3T3-L1 preadipocytes, whereas its effect was greatly curtailed by inhibitors of protein kinase C (PKC) and epidermal growth factor receptor (EGFR). Signal analyses showed that evodiamine stimulated the phosphorylation of EGFR, PKCα, and ERK, all of which were reduced by an EGFR inhibitor. Silencing experiments of EGFR mRNA supported the involvement of these signaling molecules in the inhibitory effect of evodiamine. An unidentified mechanism whereby evodiamine inhibits adipogenesis via the EGFR–PKCα–ERK signaling pathway was revealed.
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1. Introduction

Adipocytes are the major cellular component in adipose tissues and have important roles in obesity, insulin resistance, and diabetes mellitus. Because the progress of these diseases is intimately related to adipocyte hypertrophy and the recruitment of new adipocytes from preadipocytes, manipulation of adipogenesis may lead to a promising strategy to reduce the incidence of obesity and diabetes mellitus [1]. The discovery of anti-adipogenic compounds and understanding of their mode of action would contribute to the development of new dietary interventions and/or clinical therapies against obesity and diabetes mellitus.

Evodiamine is a major alkaloidal compound extracted from the fruit of *Evodia fructus* (*Evodia* rutaecarpa Benth., Rutaceae), which has been used for many years as a traditional Chinese herbal medicine for the treatment of pain, vomiting, and pyresis. Studies demonstrated that evodiamine exhibited anti-tumor and anti-inflammatory bioactivities [2,3]. These activities are similar to those of capsaicin and are blocked competitively by capsazepine, a specific antagonist of the capsaicin receptor/transient receptor potential (TRP) V1 channel [4–6]. Evodiamine is therefore thought to act as an agonist for TRPV1. TRP channels are non-selective cation channels found in many animals and are implicated in diverse cellular functions, including pain sensation, temperature, osmolality and taste sensation [7]. Kobayashi et al. reported a capsaicin-like anti-obese activity of evodiamine, which could be due to serial stimulation of the sympathetic nervous system and uncoupling protein-1 thermogenesis in brown adipose tissue (BAT) [8]. Uncoupling protein-1 allows fuel energy to be burned as heat.

We recently demonstrated that evodiamine improves diet-induced obesity in an uncoupling protein-1-independent manner [9]. In that report, we revealed that evodiamine (but not capsaicin) increased phosphorylation of extracellular signal-regulated kinase/mitogen-activated protein kinases (ERK/MAPK), reduced the expression of transcription factors such as peroxisome proliferator-activated receptor-γ, and strongly inhibited adipocyte differentiation. The identity of the target of evodiamine and how the signal is transduced to ERK activation, leading to inhibition of adipogenesis, is not known.

In the present study, we explored the anti-adipogenic mechanism of evodiamine, in which evodiamine stimulates protein kinase C alpha (PKCα) via phosphorylation of the epidermal growth factor receptor (EGFR), leading to ERK activation. This is the first report that clearly shows a novel inhibitory mechanism of evodiamine distinct from that of capsaicin on adipogenesis.

Abbreviations: EGFR, epidermal growth factor receptor; ERK/MAPK, extracellularly regulated kinase/mitogen-activated protein kinase; MEK, MAPK kinase; PKC, protein kinase C; PLC, phospholipase C; TRP, transient receptor potential

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2. Materials and methods

2.1. Cell culture

3T3-L1 preadipocytes, which are mouse embryonic fibroblasts capable of differentiating into adipocytes, were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) containing 10% calf serum (CS; ICN Biomedicals, Aurora, OH, USA). Adipocyte differentiation was carried out as described [10]. Briefly, 2 days after confluence, the medium was changed to DMEM containing 10% fetal bovine serum (ICN), 10 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. Dexamethasone and 3-isobutyl-1-methylxanthine were withdrawn after 2 days of exposure, and insulin withdrawn after 4 days. We induced 2-day post-confluent preadipocytes to differentiate in the presence of evodiamine (Kishida Chemicals, Osaka, Japan) or capsaicin (Wako Pure Chemicals, Osaka, Japan) for 4 days and then in its absence for 6 days. Capsazepine (Tocris Bioscience, Bristol, UK), ruthenium red (Sigma–Aldrich, St. Louis, MO, USA), PD98059 (Cell Signaling Technology, Danvers, MA, USA), AG1478 and chelerythrine chloride (Calbiochem, La Jolla, CA, USA) were added to the medium together with evodiamine as required. After 10 days of differentiation, cells were stained with Oil Red O (Muto Pure Chemicals, Tokyo, Japan).

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from tissues and cultured cells with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After DNase treatment of the total RNA fraction, 1 μg of the RNA sample was reverse transcribed to single-stranded cDNA using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA). Detection of TRPV genes was done using cDNA templates and PCR. The primer sequences used for gene amplification were: TRPV1, ATGGAACAACGGGCTAGCTT and TTGTGCAGATTGAGCATGGC; TRPV2, CTACACCTTGGCTTCAGCCT and GTGGAGCCTTCTGTGTATGC; TRPV3, GTCTCCTCAGGATGATGTGA and TCAGGGTGATGTTGTAGAAG; TRPV4, AGTGGTGGTACCCCTGGATA and AATGTAGGTGGGAGCGAAGG; β-actin, AAGTACCCCATTGAACACGG and CTCCGGAGTCCATCACAATG (forward and reverse primer, respectively).

2.3. Western blot analysis

Total cell lysates were prepared and analyzed as described [9]. Briefly, the proteins of cell lysates were separated by 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred onto a polyvinylidene difluoride membrane. Immunoblotting and immunoprecipitation were done using cell lysates (30 μg and 500 μg, respectively) and specific antibodies against phospho-tyrosine 4G10 and EGFR (Upstate, Charlottesville, VA, USA), β-tubulin (Santa Cruz Biotechnology, Santa Cruz, USA), p44/42 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), MAPK kinase (MEK)1/2, Phospho-MEK1/2 (Ser217/221), PKCα, Phospho-PKCα/β (Thr638/641), and phospholipase Cγ1 (PLCγ1, Cell Signaling Technology, Beverly, MA, USA). The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences, Amersham, UK).

2.4. siRNA transfection

siRNA oligos and control for knockdown of endogenous EGFR protein were prepared by using the siRNA SMARTpool™ EGFR (EGFR siRNA/siAB™ assay kit, Dharmacon, Incorporated, Lafayette, CO, USA). siRNAs were transfected into cells using LipofectAMINE 2000 (Invitrogen) according to manufacturer's instructions.

3. Results and discussion

To study the upstream effectors of the ERK signal stimulated by evodiamine, we initially examined if PKC is involved in the inhibitory effect of evodiamine on adipocyte differentiation because PKC has been implicated in the proliferation, differentiation and transformation of cells [11]. With respect to adipogenesis regulation, PKCγ and PKCε are required to promote adipogenic commitment [12,13], whereas PKCα is highly expressed in preadipocytes but this expression decreases upon adipocyte differentiation [14]. ERK phosphorylation by evodiamine stimulation was blocked completely when chelerythrine chloride, an effective inhibitor of PKC [15], was added to the culture of 3T3-L1 preadipocytes (Fig. 1A). Treatment of the PKC inhibitor during preadipocyte differentiation inhibited the evodiamine effect, and stimulated adipogenesis similar to that observed in the control culture (Fig. 1B). These results suggested that evodiamine stimulated ERK phosphorylation and inhibited adipocyte differentiation via PKC activation.

We next investigated the target molecules of evodiamine, which leads to activation of PKC and ERK in 3T3-L1 preadipocytes. The predicted candidate was TRPV1 because evodiamine has cap-

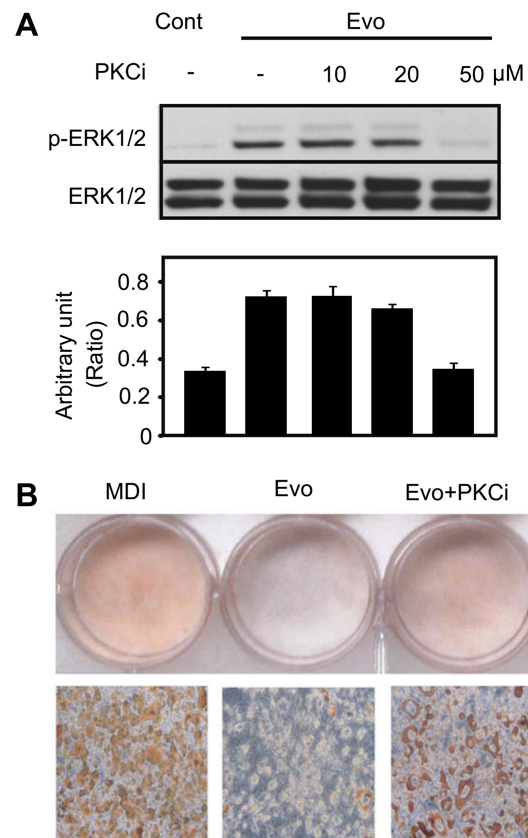

Fig. 1. PKC is involved in the inhibitory effect of evodimine on adipogenesis and regulation of ERK activation. (A) Effect of chelerythrine chloride (PKC inhibitor (PKCi)) on ERK phosphorylation. Two-day post-confluent 3T3-L1 preadipocytes were serum-deprived for 4 h and treated with 20 μM evodiamine (Evo) or an equal volume of dimethylsulfoxide control (Cont) for 10 min. The PKCi was added at different concentrations 1 h before evodiamine addition. Lysates were analyzed for ERK phosphorylation. Data are expressed as the mean ± S.E. ($n = 3$). (B) Effect of PKCi on adipogenesis. Cells were cultured in the differentiation medium with 1 μM Evo in the absence or presence of 10 μM PKCi. Lipid accumulation in cells was evaluated by Oil Red O staining. Data are representative of three independent experiments.

saicin-like bioactivities [4–6]. Zhang et al. recently reported that TRPV1 was abundantly expressed in 3T3-L1 preadipocytes and that capsaicin inhibited its adipogenesis [16]. We failed to detect the transcript of TRPV1 mRNA as well as TRPV3 mRNA in 3T3-L1 preadipocytes, although very faint signals of these genes were observed in differentiated adipocytes (Fig. 2A). This observation is consistent with a report by Motter and Ahern stating that capsaicin failed to evoke Ca^{2+} responses in 3T3-L1 preadipocytes [17]. Gene expression of TRPV2 and TRPV4 mRNAs was observed in preadipocytes and adipocytes, and expression of TRPV4 mRNA was much higher than that of TRPV2 mRNA, particularly in preadipocytes. Transcripts of the four TRPV genes were detected in adipose tissues, which contain various cell types. Likewise, we could not detect any effects of capsaicin on ERK phosphorylation nor adipogenesis in 3T3-L1 cells (Fig. 2B and C and in our previous study [9]). We do not know the reason for the differences in TRPV1 expression and the effects of capsaicin in 3T3-L1 preadipocytes observed in our study and in Zhang's study. Nevertheless, our results strongly suggested that evodiamine stimulates adipogenesis through an unknown target that is not TRPV1.

We detected a high mRNA level of TRPV4 (which shares about 40% amino-acid homology with TRPV1), so we examined the possible involvement of TRPV4 in adipocyte differentiation as the target of evodiamine. Ruthenium red (an inhibitor of TRP channels including TRPV4) and capsazepine (TRPV1 antagonist) did not prevent the evodiamine effects on ERK phosphorylation and adipogenesis in 3T3-L1 preadipocytes (Fig. 2B and C). Primary preadipocytes derived from the white adipose tissues of TRPV4-deficient mice differentiated normally into mature adipocytes and evodiamine increased ERK phosphorylation in the cells and blocked their differentiation (Wang, T., Unpublished observation). These results strongly suggested that TRPV4 is not a target of evodiamine to induce activation of the ERK signaling pathway.

We investigated the receptor tyrosine kinase EGFR as another candidate of the evodiamine receptor because EGFR signaling has been reported to have a role in adipogenesis regulation [18]. Inhibition of adipocyte differentiation by evodiamine was recovered by co-treatment with an EGFR inhibitor, suggesting involvement of the EGFR in the evodiamine effect (Fig. 3A). The signal analysis revealed that evodiamine stimulated the tyrosine phosphorylation of EGFR in 3T3-L1 preadipocytes, and that its phosphorylation was blocked completely by addition of an EGFR inhibitor (Fig. 3B). In parallel with EGFR phosphorylation, PKCα/β was significantly phosphorylated: it was reduced by the EGFR inhibitor. PKCα is the most abundant classical PKC isoform and PKCβ is not expressed at the preadipocyte stage [13], so the PKC signal appeared to be that of PKCα. There were no changes in the phosphorylation levels of the PKC isoforms PKCδ, PKCθ, and PKC$_1$/λ, in evodiamine-stimulated 3T3-L1 preadipocytes (data not shown); however, the involvement of PKCγ and PKCε in the evodiamine effect remains to be addressed. Increased phosphorylation of MEK and ERK was observed after evodiamine stimulation in 3T3-L1 preadipocytes, whereas their phosphorylation levels were reduced by an EGFR inhibitor. These results suggested that an EGFR–PKCα–ERK signal cascade was stimulated by evodiamine in 3T3-L1 preadipocytes.

We conducted a gene-knockdown experiment using siRNA for EGFR to confirm the involvement of the EGFR–PKCα–ERK signal pathway in the inhibitory effect of evodiamine on adipogenesis. The siRNA silenced about 50% of EGFR protein in 3T3-L1 preadipocytes (which differentiated into addipocytes even under evodiamine stimulation) whereas non-coding RNA did not prevent the evodiamine effects (Fig. 4A and B). PLC has a significant role in

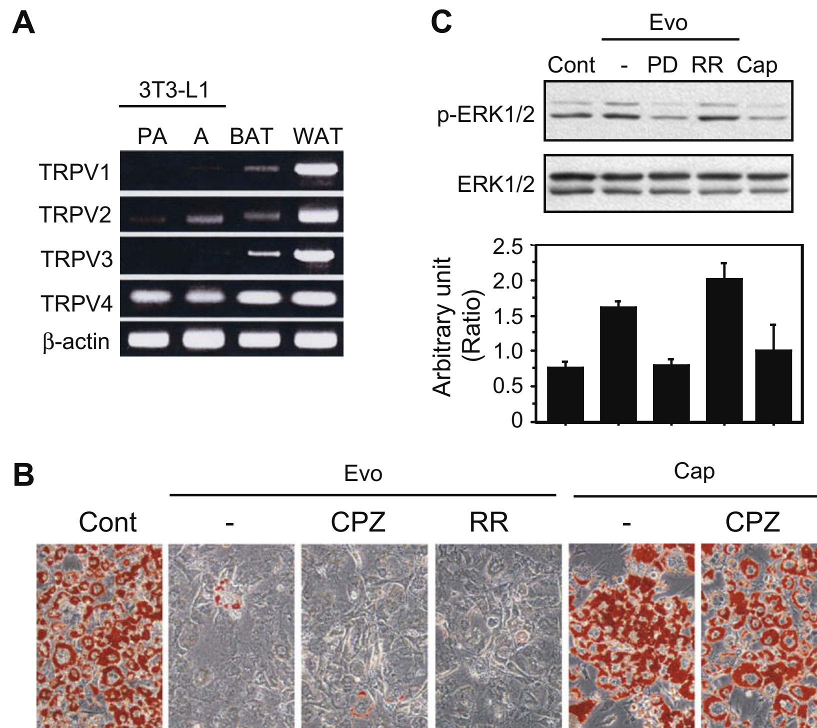

Fig. 2. TRPV1 and TRPV4 are not involved in the evodiamine effects on ERK phosphorylation and adipogenesis. (A) Expression of TRPV channels in 3T3-L1 cells and adipose tissues. RT-PCR using 3T3-L1 preadipocytes (PA) and mature adipocytes (A), brown adipose tissues (BAT), and white adipose tissues (WAT) was done as described in Section 2. (B) Effects of capsaicin (Cap), capsazepine (CPZ) and/or ruthenium red (RR) on adipogenesis. Two-day post-confluent 3T3-L1 preadipocytes were cultured in the differentiation medium with 1 μM evodiamine (Evo) or Cap in the absence or presence of 1 μM CPZ or RR. Lipid accumulation in cells was evaluated by Oil Red O staining. Data are representative of three independent experiments. (C) Effect of PD98059 (PD) or RR on Evo-stimulated ERK phosphorylation. Cells were treated with 10 μM Evo, Cap, or an equal volume of dimethylsulfoxide control (Cont) for 1 h using the same protocol as in Fig. 1A. PD (10 μM) or RR (10 μM) was also added before Evo stimulation. Data are expressed as the mean ± S.E. (n = 3).

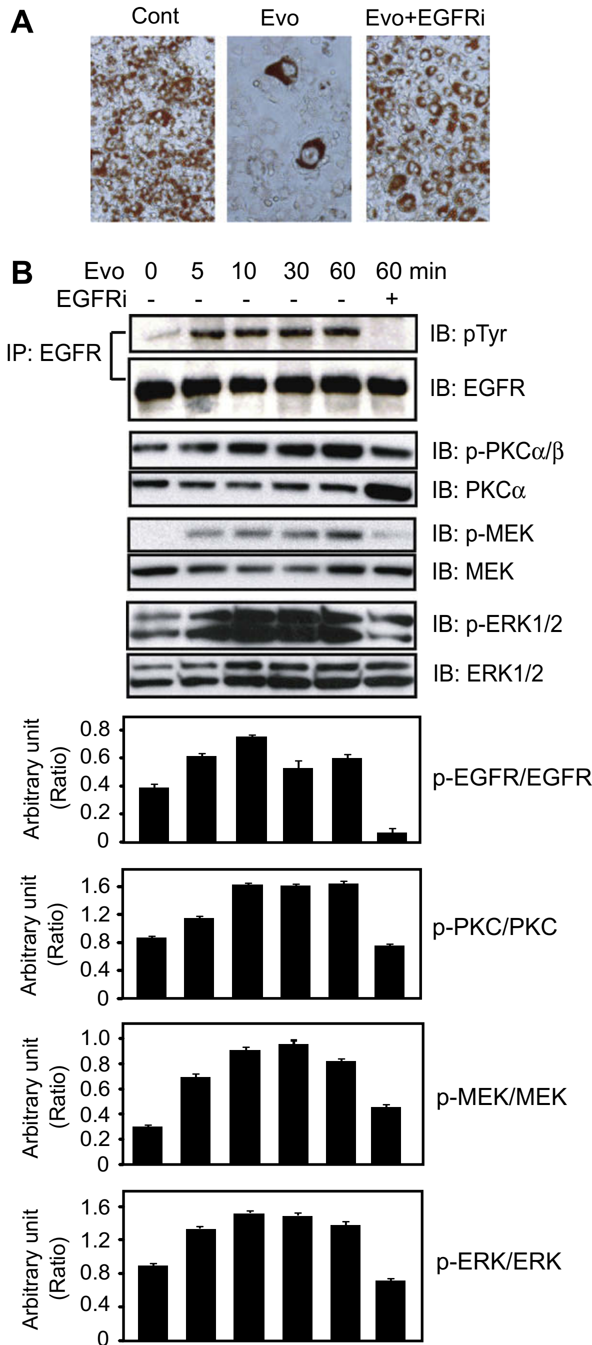

Fig. 3. Evodiamine inhibits adipogenesis through the EGFR signaling passway. (A) Effect of AG1478 (EGFR inhibitor (EGFRi)) on adipogenesis. Two-day post-confluent 3T3-L1 preadipocytes were cultured in the differentiation medium with 1 μM evodiamine (Evo) in the absence or presence of 5 μM EGFRi. Lipid accumulation in cells was evaluated by Oil Red O staining. Data are representative of three independent experiments. (B) Time-course of phosphorylation of EGFR, PKCα/β, MEK, and ERK by evodiamine. Cells were serum-deprived for 4 h and then treated with 50 μM evodiamine for the indicated time together with or without 1-h pretreatment with 5 μM EGFRi. Immunoprecipitation (IP) and immunoblot (IB) analyses were done using cell lysates and antibodies specific for each molecule as described in Section 2. Data are expressed as the mean ± S.E. ($n = 3$).

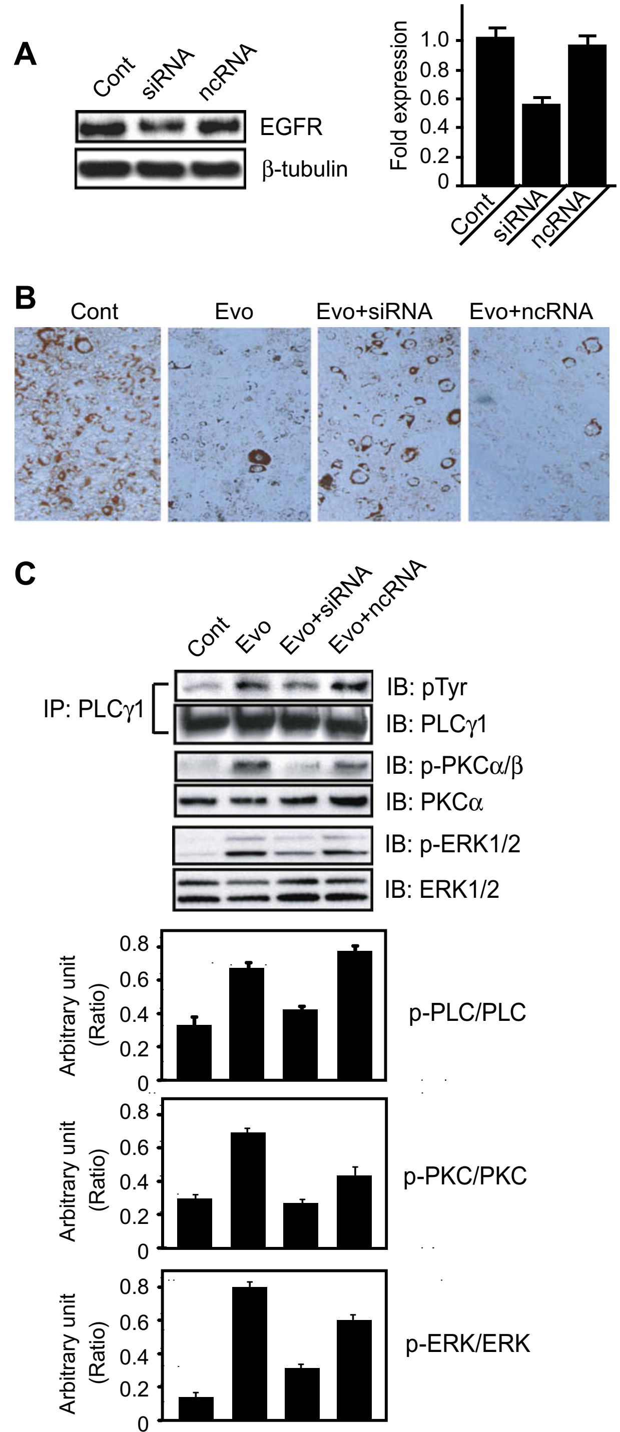

Fig. 4. Downregulation of EGFR induced by siRNA reduces evodiamine-induced ERK activation and its anti-adipogenic effect. (A) Effect of EGFR siRNA on the expression of EGFR protein. 3T3-L1 preadipocytes were transfected with EGFR siRNA or non-coding RNA (ncRNA). Three days after transfection, cells were harvested and analyzed for EGFR expression by western blotting. EGFR level was normalized by β-tubulin level. Data are expressed as the mean ± S.E. ($n = 3$). (B) Effect of EGFR siRNA on the anti-adipogenic effect of evodiamine (Evo). Transfected or non-transfected cells were cultured in the differentiation medium with 1 μM Evo and lipid accumulation evaluated by Oil Red O staining. Data are representatives of three independent experiments. (C) Phosphorylation of PLCγ1, PKCα/β, and ERK in Evo-stimulated cells with or without siRNA transfection. Cells were serum-deprived for 4 h and then treated with 50 μM Evo or an equal volume of dimethylsulfoxide control (Cont) for 1 h. Immunoprecipitation (IP) and immunoblot (IB) analyses were done using cell lysates and antibodies specific for each molecule as described in Section 2. Data are expressed as the mean ± S.E. ($n = 3$).

transmembrane signaling in response to extracellular stimuli such as hormones and growth factors [19]. Among PLC family members, PLCγ forms a complex with EGFR and is phosphorylated on tyrosine residues. The activated PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-triphosphate and diacylglycerol, which leads to PKC phosphorylation [20]. Downregula-

tion of EGFR reduced PLCγ1 phosphorylation stimulated by evodiamine in 3T3-L1 preadipocytes (Fig. 4C). We found reduced levels of PKCα/β and ERK phosphorylation in cells transfected with EGFR siRNA, which were similar to the data obtained from experiments using an EGFR inhibitor. As expected, non-coding RNA had no effects on the phosphprylation of PLCγ1, PKCα/β, and ERK in evodiamine-stimulated cells. These results support the idea that evodiamine stimulates EGFR phosphorylation and transduces a signal to PLCγ1, which activates the PKCα and MEK/ERK signaling pathways in the early step of preadipocyte differentiation. Sustained ERK activation would then inhibit adipogenesis as reported [9,21].

We revealed that EGFR (but not TRPV1 nor TRPV4) is a novel target of evodiamine in preadipocytes, although evodiamine is known to be an agonist for TRPV1 in the isolated atria and bronchus of guinea pigs [4,5]. We also clarified the mechanism that evodiamine inhibits adipogenesis via activating the EGFR–PKCα–ERK signaling pathway. Evodiamine may offer a useful strategy for obesity treatment because adipogenesis regulation appears to be important for the expansion of adipose tissues.

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