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Evodiamine Improves Diet-Induced Obesity in a Uncoupling Protein-1-Independent Manner: Involvement of Antiadipogenic Mechanism and Extracellularly Regulated Kinase/Mitogen-Activated Protein Kinase Signaling

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Evodiamine is an alkaloidal compound with antiobesity effects that have been thought to be due to uncoupling protein-1 (UCP1) thermogenesis similar to the effects of capsaicin, but the underlying mechanisms are poorly understood. To clarify the mechanisms, we first examined whether the antiobesity effect of evodiamine could be attributed to the involvement of UCP1. When UCP1-knockout mice were fed a high-fat diet with 0.03% evodiamine (wt/wt) for 2 months, the increases in body weight, adiposity, and the serum levels of leptin and insulin were reduced in a manner indistinguishable from control mice fed a high-fat diet with evodiamine, suggesting that evodiamine triggered a UCP1-independent mechanism to prevent diet-induced obesity. By using preadipocyte cultures, we found that evodiamine, but not capsaicin, increased phosphorylation of ERK/MAPK, reduced the expression of transcription factors such as peroxisome proliferator-activated receptor-γ, and strongly inhibited adipocyte differentiation. Evodiamine treatment also reduced insulin-stimulated phosphorylation of Akt, a crucial regulator of adipocyte differentiation; and the reduction of phosphorylated-Akt and augmentation of phosphorylated ERK were reversed by blockade of the MAPK kinase/MAPK signaling pathway, restoring adipogenesis in the cultures. The changes in ERK and Akt phosphorylation levels were also observed in white adipose tissues of UCP1-knockout mice fed the evodiamine diet. These findings suggest that evodiamine has a potential to prevent the development of diet-induced obesity in part by inhibiting adipocyte differentiation through ERK activation and its negative cross talk with the insulin signaling pathway. (Endocrinology 149: 358–366, 2008)
Evodiamine, a major alkaloidal compound in the fruit of *Evodia fructus* (*Evodia rutaecarpa* Bentham, Rutaceae) was previously reported to exhibit capsaicin-like antiobesity effects (14). The major mechanism eliciting the effect was postulated to be enhancement of energy dissipation by UCP1 thermogenesis, probably through β3-adrenergic stimulation in brown adipose tissue (BAT). Capsaicin, the pungent main principle of red pepper, has also been reported to decrease body weight by reducing food intake in rats (15), although the molecular basis on this antiobesity effect of capsaicin is still obscure. If evodiamine has a high potential for preventing obesity, this compound may be suitable for dietary supplementation because it has no perceptible taste, unlike capsaicin. However, the mechanisms underlying the antiobesity effects of evodiamine are still not clear. In this report, we demonstrate that evodiamine inhibited adipocyte differentiation through stimulation of an ERK/MAPK pathway and that dietary supplementation with this nonpungent compound could ameliorate diet-induced obesity in animals lacking UCP1 thermogenesis. This work may lead to the development of drugs and therapeutic strategies for treatment of obesity in adult humans who are virtually UCP1 deficient.

**Materials and Methods**

**Experimental animals**

Ucp1tm1 knockout (KO) mice on a C57BL/6 background (16) were kindly provided by Dr. Leslie Kozak (Pennington Biomedical Research Center, Baton Rouge, LA), and N13-N15 generations were used in the experiments. The mice were maintained according to our institutional guidelines for animal care under artificial lighting for 12 h/d and provided a standard chow (11.6% kcal from fat; Diet CE-2; CLEA Japan, Inc., Tokyo, Japan) and tap water ad libitum in our animal facility at 23 ± 1°C. In the experiments on the effects of evodiamine, UCP1-KO and the control (wild-type and hetero-type) littermates mice were fed the standard chow until they were 4 months old and then were fed a high-fat diet (HF: 41.9% kcal from fat; Diet B15040; CLEA Japan, Tokyo, Japan) and tap water ad libitum in our animal facility at 23 ± 1°C. In the experiments on the effects of evodiamine, UCP1-KO and the control (wild-type and hetero-type) littermates mice were fed the standard chow until they were 4 months old and then were fed a high-fat diet (HF: 41.9% kcal from fat; Diet B15040; CLEA Japan, Tokyo, Japan) and tap water ad libitum in our animal facility at 23 ± 1°C. In the experiments on the effects of evodiamine, UCP1-KO and the control (wild-type and hetero-type) littermates mice were fed the standard chow until they were 4 months old and then were fed a high-fat diet (HF: 41.9% kcal from fat; Diet B15040; CLEA Japan, Tokyo, Japan) and tap water ad libitum in our animal facility at 23 ± 1°C. In the experiments on the effects of evodiamine, UCP1-KO and the control (wild-type and hetero-type) littermates mice were fed the standard chow until they were 4 months old and then were fed a high-fat diet (HF: 41.9% kcal from fat; Diet B15040; CLEA Japan, Tokyo, Japan) and tap water ad libitum in our animal facility at 23 ± 1°C. In the experiments on the effects of evodiamine, UCP1-KO and the control (wild-type and hetero-type) littermates mice were fed the standard chow until they were 4 months old and then were fed a high-fat diet (HF: 41.9% kcal from fat; Diet B15040; CLEA Japan, Tokyo, Japan) and tap water ad libitum in our animal facility at 23 ± 1°C. In the experiments on the effects of evodiamine, UCP1-KO and the control (wild-type and hetero-type) littermates mice were fed the standard chow until they were 4 months old and then were fed a high-fat diet (HF: 41.9% kcal from fat; Diet B15040; CLEA Japan, Tokyo, Japan) and tap water ad libitum in our animal facility at 23 ± 1°C.

**Cell culture**

3T3-L1 cells, which were provided kindly by Dr. Masayoshi Imagawa (Nagoya City University, Nagoya, Japan), were grown in DMEM (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Northern blot analysis was performed by using total RNA (WAT: 20 µg, 3T3-L1 cells: 10 µg), as described earlier (17). Blots were hybridized successively with probes (labeled with [32P]dCTP) for the mRNAs of UCP2, β3-adrenergic receptor (AR), PPARy, leptin, adipocyte fatty acid-binding protein (aP2), resistin, and 18S RNA. In the analysis of β3-AR, three transcripts of 2.1, 2.8, and 3.6 kb were detected in WAT, as reported (21). Like probes for UCP2, β3AR, aP2, and resistin mRNAs and 18S rRNA (16, 17), probes for PPARy and resistin mRNAs were produced by the RT-PCR technique. The sequences used were the following: PPARy, positions 464-1945 of the mouse sequence (GenBank accession no. U01841), and resistin, positions 38-558 of the mouse sequence (GenBank accession no. AF320380). The PCR products were sequenced after subcloning into the pCRII vector (Invitrogen). Hybridization signals were quantified with Bioimage (FUJIFILM; Fuji, Tokyo, Japan).

**Histological analysis**

Tissues were fixed immediately in 10% formaldehyde in neutral buffer solution (Kishida Chemical) and embedded in paraffin. Tissue sections of 3 µm were cut and then stained with hematoxylin and eosin.

**Immunoblotting and immunoprecipitation**

Total cell lysates were prepared and analyzed as described previously (22). Briefly, cells in 100-mm dishes were washed with ice-cold PBS
containing 1 mM Na$_3$VO$_4$ and lysed with a lysis buffer (pH 7.2) consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EGTA, 25 mM NaF, 1 mM Na$_3$VO$_4$, and 0.25% protease inhibitor mixture solution (Sigma). The proteins of cell lysates were separated by 4–20% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane. Immunoblotting and immunoprecipitation were performed by using cell lysates (30 and 500 μg, respectively) and specific antibodies against CCAAT/enhancer-binding protein (C/EBP)-β, PPARγ, insulin receptor (IR)-β, IGF-I receptor (IGF-IRβ), phosphatidylinositol 3-kinase (PI3K) p85 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-tyrosine (4G10; Upstate, Charlottesville, VA), insulin receptor substrate (IRS)-1, p85 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-tyrosine (4G10; Upstate, Charlottesville, VA), insulin receptor substrate (IRS)-1, and phospho-Akt (Cell Signaling Technology, Danvers, MA). The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis

Data were expressed as the mean ± se. Significant differences between groups were assessed by ANOVA or Student’s $t$ test.

Results

Effects of evodiamine on the development of diet-induced obesity in UCP1-KO mice

We provided UCP1-KO and the control mice with a HF diet with or without evodiamine for 2 months. Body weight gain and adiposity index were significantly lower in the mice with evodiamine (+Evo) than in the mice without it (-Evo) in both groups (Fig. 1, A and B), even though there was no significant difference in food intake between the +Evo and -Evo groups (0.43 ± 0.02 and 0.43 ± 0.02 kcal/d/g body weight in the control mice, 0.45 ± 0.01 and 0.48 ± 0.01 kcal/d/g body weight in the KO mice, respectively). The serum leptin levels in the +Evo group were reduced to 27 and 43% of the -Evo group in the control and KO mice, respectively (Fig. 1C). Although the nonfasting glucose level in the mice was not changed by the evodiamine diet (Fig. 1D), the insulin levels in mice treated with evodiamine were decreased to about one third of those without it in both groups (Fig. 1E). Moreover, the evodiamine diet improved the impaired glucose tolerance in UCP1-KO mice fed the HF diet, bringing it close to that in the mice fed the standard chow diet (Fig. 1F).

In addition to the data on adiposity (Fig. 1B), histological analysis supported the effectiveness of evodiamine in reducing fat accumulation in WAT and BAT, as well as fatty liver, in the mice fed the HF diet in both groups (Fig. 2). Liver mass in the -Evo group and +Evo group was 1.535 ± 0.113 and 1.286 ± 0.056 g in the control mice and 1.540 ± 0.119 and 1.391 ± 0.100 g in the KO mice, respectively. When the accumulation of hepatic lipids was evaluated, the triglyceride content tended to be reduced in the +Evo group, compared with that in the -Evo group (16.4 ± 2.9 and 57.5 ± 22.2 mg/g liver, in the mice fed the HF diet in both groups (Fig. 2). Liver mass in the -Evo group and +Evo group was 1.535 ± 0.113 and 1.286 ± 0.056 g in the control mice and 1.540 ± 0.119 and 1.391 ± 0.100 g in the KO mice, respectively. When the accumulation of hepatic lipids was evaluated, the triglyceride content tended to be reduced in the +Evo group, compared with that in the -Evo group (16.4 ± 2.9 and 57.5 ± 22.2 mg/g liver, in the mice fed the HF diet in both groups (Fig. 2).
mg/g liver in the control mice, 25.1 ± 9.3 and 51.3 ± 10.8 mg/g liver in the KO mice, respectively). There was no difference in the total cholesterol content in the liver between the +Evo and −Evo groups (3.98 ± 0.37 and 3.42 ± 0.38 mg/g liver in the control mice, 3.75 ± 0.58 and 4.05 ± 0.47 mg/g liver in the KO mice, respectively).

In the analysis of gene expression in WAT, similar effects of evodiamine in the control and KO mice were observed (Fig. 3). Namely, reductions in the mRNA levels of leptin (control, 53%; KO, 26%), UCP 2 (control, 29%; KO, 22%), and PPARγ (control, 17%; KO, 26%) were found in the +Evo group, compared with those levels in the −Evo group, whereas the mRNA level of β3-AR increased in the +Evo group (WT: 201%; KO: 135%; Fig. 3), consistent with the effects of increased adiposity on the expression of these genes (17). There was no difference in the mRNA level of UCP1 in the BAT between the +Evo and −Evo groups in the control mice (data not shown). We also could not detect a significant difference in the mRNA levels of UCP2 and UCP3 in the BAT and muscles between the mice with or without evodiamine in either group (data not shown).

**Evodiamine inhibits adipocyte differentiation in 3T3-L1 cells**

The phenotypes of evodiamine in vivo could be mediated by changes in energy expenditure and lipolysis or through its effects on adipogenesis. Accordingly, we examined the effects of evodiamine on adipocyte differentiation in vitro. When the differentiation of 3T3-L1 preadipocytes was assessed quantitatively in terms of triglyceride contents, evodiamine dose-dependently inhibited the differentiation of the preadipocytes incubated in differentiation medium containing insulin, dexamethasone, and 1-isobutyl-3-methylxanthine (Fig. 4A). The addition of 1 μM evodiamine to the differentiation medium almost completely inhibited the adipocyte differentiation as evidenced by oil Red O staining of lipids. This inhibitory effect of evodiamine was reduced when it was added 2 d after the start of stimulation of differentiation, and the alkaloid did not suppress differentiation at all when it was added after d 4 (data not shown). Not only did evodiamine inhibit fat accumulation in adipocytes, but also the greatly reduced expression of aP2, leptin, and resistin indicated that adipogenesis in 3T3-L1 cells was strongly suppressed (Fig. 4B).

We then analyzed the induction of transcription factors regulating adipogenesis in the cells (Fig. 4C). In the absence of evodiamine, C/EBPβ, a crucial regulator in an early step of the transcriptional cascade in adipogenesis, and PPARγ were expressed in a time-dependent manner as the 3T3-L1 cells proceeded toward adipocyte differentiation, as previously described (23, 24). Treatment of postconfluent cultures with evodiamine severely suppressed the early induction of C/EBPβ and then blocked the induction of PPARγ (Fig. 4C), indicating that evodiamine acted to inhibit the early transcriptional steps of adipocyte differentiation. When the intracellular signaling molecules involved in adipocyte differentiation were examined, an increased level of phosphorylated ERK1/2 was found in
the cells treated with evodiamine, compared with that in the control cells (Fig. 4C). In the control cells, the very high level of ERK phosphorylation in growth medium before differentiation stimulation (d 0) was reduced greatly within the first 2 d, and it disappeared thereafter. On the other hand, a steady level of ERK phosphorylation was detected in Evo-treated cells in the first 2 d, and phosphorylated ERK was detectable by d 8. Evodiamine did not stimulate ERK phosphorylation in the 3T3-L1 mature adipocytes (data not shown).

**Evodiamine inhibits adipocyte differentiation by the sustained activation of ERK in 3T3-L1 cells**

To further assess the involvement of ERK signaling in the effects of evodiamine on adipocyte differentiation, we examined the acute effects of insulin and evodiamine on the stimulation of ERK phosphorylation. As previously reported (25), a transient increase in ERK phosphorylation that peaked after 5 min was observed in 3T3-L1 cells after insulin stimulation (Fig. 5A). Evodiamine addition led to a modest but considerable stimulation of ERK phosphorylation that lasted over a 1-h period (Fig. 5B), indicating the differences in mode of action between insulin and evodiamine. When the evodiamine effect was checked in a longer time course, ERK phosphorylation lasted 18 h after evodiamine stimulation (data not shown). Cotreatment of evodiamine with PD98059, a specific inhibitor of MAPK kinase (an upstream kinase for ERK), reduced ERK phosphorylation (Fig. 5B) and restored adipocyte differentiation (Figs. 5C). Interestingly, capsaicin had no effect on ERK phosphorylation (Fig. 5D) or adipocyte differentiation (Fig. 5E). In addition, the effect of evodiamine on ERK phosphorylation was detected in a nonadipogenic cell line, human hepatoma HepG2 cells. Similar to the results in 3T3-L1 preadipocytes, ERK phosphorylation greatly increased 1 h after evodiamine stimulation, and its increased level was detectable even after 24 h (Fig. 5F). As expected, the increase in ERK phosphorylation was blocked by cotreatment of evodiamine with PD98059.

**Effects of evodiamine on insulin signaling pathway**

We examined whether evodiamine would affect insulin signaling through the PI3K/Akt pathway during adipocyte differentiation because this pathway is important in transducing the proadipogenic effects of insulin through IR and/or IGF-IR (26, 27). As shown in Fig. 6A, evodiamine did not affect the tyrosine phosphorylation of either IR or IGF-IR in 3T3-L1 preadipocytes. Likewise, the tyrosine phosphorylation of IRS-1 and its binding with the PI3K p85 subunit in the preadipocytes were not changed by the evodiamine treatment (Fig. 6B). Evodiamine also did not affect PI3K activity (data not shown). However, we found that evodiamine strongly reduced the insulin-induced phosphorylation of Akt Ser473, a regulatory site of Akt activity, in the 3T3-L1 preadipocytes, which contrasted with the steady level of ERK phosphorylation (Fig. 7A). Reduced phosphorylation of Akt Ser308 was also detected in the evodiamine-treated cells (data not shown). When the cells were pretreated with PD98059, this inhibitory effect of evodiamine was strongly blocked, and Akt phosphorylation was restored by about 70% of level in the insulin treatment. The stimulation of ERK phosphorylation and inhibition of insulin-stimulated Akt phosphorylation were also observed in primary cultures of human adipose tissue.
adipocyte precursor cells (Fig. 7B). Moreover, the effects of evodiamine on ERK and Akt signaling were determined in the WAT derived from the mice in the diet study (Fig. 8A). Compared with those for the −Evo group, the phosphorylation levels of ERK and Akt were significantly higher (2-fold) and lower (about half), respectively, in the WAT of the −Evo group in KO mice. In the control mice, the phosphorylation level of Akt in the +Evo group was reduced to 28% of the −Evo group, whereas the effect on ERK phosphorylation was not clear. The effects of evodiamine on ERK and Akt signaling were also confirmed in the WAT of mice administered with the compound. An injection of evodiamine significantly up-regulated ERK phosphorylation (1.7-fold) and down-regulated Akt phosphorylation (about a fourth) in the WAT of mice, compared with those for the control mice (Fig. 8B).

**Discussion**

It was previously reported that evodiamine showed an antiobesity effect, which was thought to depend on the enhancement of UCP1 thermogenesis through β3-adrenergic stimulation in BAT (14). To clarify whether the antiobesity effect of evodiamine depended on energy dissipation mediated by UCP1 thermogenesis, we first examined the effects of evodiamine on the development of diet-induced obesity in UCP1-KO mice in the present study. Interestingly, we found that evodiamine showed a potent effect of preventing the increases in body weight and adiposity even in the UCP1-KO mice fed the HF diet, which was indistinguishable from the effects of this compound in the control mice. The data on blood parameters suggested the improvement of leptin resistance and insulin sensitivity in the mice fed the evodiamine diet. In addition, IPGT data supported the improvement of glucose metabolism in the mice treated with the evodiamine diet, in which the phenotypes in fed glucose and insulin levels were similar to those in PPARγ−/− mice (24). These results were unexpected because the diet-induced obe-
Fig. 7. Evodiamine negatively regulates insulin-stimulated Akt activation through an ERK pathway in preadipocytes. A, 3T3-L1 preadipocytes were serum deprived for 4 h and then treated with 20 μM evodiamine (Evo) for 1 h and with 20 nM insulin (Ins) for the last 10 min. PD98059 (PD; 20 μM) was added 1 h before the evodiamine treatment. Western blot analyses for ERK and Akt were performed by using tissue lysates (50 μg). The cells were serum deprived for 4 h and then treated with 20 μM evodiamine for 1 h and with 20 nM insulin for the last 10 min. Representative images of three independent experiments are shown.

Fig. 8. Effects of evodiamine on the phosphorylation of ERK and Akt in vivo. Phosphorylation levels of ERK and Akt in the WAT of mice fed the HF diet with or without evodiamine (Evo) in the diet study (A) and in the WAT of wild-type mice treated with evodiamine (3 mg/kg, ip) or vehicle for 24 h (B). Western blot analyses for ERK and Akt were performed by using tissue lysates (50 μg protein) of epididymal WAT from the mice. Data are expressed as the mean ± SE (n = 4). *, P < 0.05 and **, P < 0.01 vs. –Evo group (Student’s t test).
adipogenesis via regulation of C/EBPβ and PPARγ transcriptional activities (30). Of note, unlike evodiamine, capsaicin neither stimulated ERK phosphorylation nor inhibited adipogenesis, even though these compounds show similar actions in vivo on vasorelaxation and hypothermia (14, 31, 32). Considering the results on the signal analysis of mouse WAT, it is likely that evodiamine contributed to the suppression of diet-induced obesity in mice by inhibiting adipogenesis, although other mechanisms could be involved in the antiobesity effects of evodiamine.

We also found an effect of evodiamine on ERK phosphorylation in HepG2 cells. Similar to 3T3-L1 preadipocytes, evodiamine significantly stimulated ERK phosphorylation in the nonadipogenic cells. Kosone et al. recently suggested an involvement of ERK in a reducing effect of hepatocyte growth factor on lipid accumulation in HepG2 cells through induction of several genes related to lipid metabolism (33). Because the fatty liver observed in HF diet-induced obesity was improved considerably in the mice fed the evodiamine diet, it would be of interests to know the effect of evodiamine on lipid metabolism in hepatocytes.

In addition to ERK/MAPK signaling, PI3K/Akt is an important intracellular signal cascade in the regulation of many cellular activities including growth, glucose metabolism, and adipogenesis (34–36). Insulin stimulates tyrosine phosphorylation of the IR and/or IGF-IR, which promotes the activation of Akt via phosphorylation of PI3K. Differentiation of 3T3-L1 preadipocytes is stimulated strongly by the expression of a constitutively active form of Akt (37). On the other hand, adipogenesis is blocked in cultured cells or mice lacking Akt (26, 36). In the present study, the upstream signals within the insulin/IGF-I pathway were not affected by evodiamine in 3T3-L1 cells. However, we found that insulin-stimulated Akt phosphorylation was inhibited strongly in the preadipocytes treated with evodiamine in contrast to the stimulation of ERK phosphorylation. In addition, the Akt inhibition was restored by a MAPK kinase inhibitor, which profoundly blocked ERK phosphorylation in the cells, suggesting a connection between PI3K/Akt and ERK/MAPK pathways in the preadipocytes. Taken together, evodiamine may inhibit adipogenesis by suppressing insulin-stimulated Akt phosphorylation through the activation of ERK signaling. Similar effects of evodiamine on Akt and ERK phosphorylation were detected in the WAT from the UCP1-KO mice in the diet study. Although we could not detect a significant effect of evodiamine on ERK phosphorylation in the WAT from the control mice, the effects of evodiamine on ERK and Akt signaling in vivo were supported from the evidence that an injection of evodiamine to wild-type mice considerably stimulated ERK phosphorylation and reduced Akt phosphorylation in the WAT. Because Akt has important roles in growth (34), the decrease in Akt phosphorylation by evodiamine might inhibit mitotic clonal expansion in preadipocytes. Takada et al. (38) recently reported that evodiamine inhibits Akt activation in tumor cells.

We presently do not know how ERK regulates Akt phosphorylation in preadipocytes. Because the Akt activity is regulated negatively by several phosphatases such as the phosphatidylinositol 3′ lipid phosphate (39) or protein phosphatase type 2A (40), we examined the effect of evodiamine on the phosphorylation level of these phosphatases. However, evodiamine did not affect the phosphorylation levels of phosphatidylinositol 3′ lipid phosphate and protein phosphatase type 2A in the presence of insulin (Wang, T., unpublished data). Therefore, the contribution of these phosphatases to Akt inactivation in 3T3-L1 preadipocytes stimulated with evodiamine appears to be low, so other molecules may be involved in the negative cross talk of ERK signaling for the regulation of Akt activity in adipocyte differentiation.

In summary, our results indicate that evodiamine has the previously unrecognized action of inhibiting adipogenesis by a mechanism in which the stimulation of ERK/MAPK signaling down-regulates the expression of adipocyte transcription factors and insulin-induced Akt signaling. Because evodiamine clearly showed an antiobesity effect in UCP1-deficient mice, this compound may offer a new approach to circumvent the development of diet-induced obesity, especially in animals lacking UCP1 thermogenesis including adult humans; however, further details of the inhibitory mechanism and the effects on insulin sensitivity remain to be clarified.

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