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TC-E 5003, a protein methyltransferase 1 inhibitor, activates the PKA-dependent thermogenic pathway in primary murine and human subcutaneous adipocytes

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We previously reported the involvement of protein arginine methyltransferase 1 (PRMT1) in adipocyte thermogenesis. Here, we investigate the effects of PRMT1 inhibitors on thermogenesis. Unexpectedly, we find that the PRMT1 inhibitor TC-E 5003 (TC-E) induces the thermogenic properties of primary murine and human subcutaneous adipocytes. TC-E treatment upregulates the expression of *Ucp1* and *Fgf21* significantly and activates protein kinase A signaling and lipolysis in primary subcutaneous adipocytes from both mouse and humans. We further find that the thermogenic effects of TC-E are independent of PRMT1 and beta-adrenergic receptors. Our data indicate that TC-E exerts strong effects on murine and human subcutaneous adipocytes by activating beige adipocytes *via* PKA signaling.

Keywords: lipolysis; PKA; PRMT1; thermogenesis; UCP1

Thermogenic adipocytes found in adult humans [1–3] are heterogeneous. A considerable portion of cells resembles murine beige adipocytes [4,5], although some of the other cells are similar to murine brown adipocytes [6,7] Murine beige adipocytes are distinct from the classic brown adipocytes and reside in subcutaneous (SQ) white adipose tissue (WAT). These adipocytes consume energy in the form of heat by breaking down the proton gradient across the inner mitochondrial membrane by using uncoupling protein 1 (UCP1) without the generation of adenosine triphosphate (ATP) [8]. Hence, the understanding of the mechanism underlying the activation of beige adipocytes is important to overcome obesity and associated metabolic disorders.

Beige adipocytes are primarily activated by cold-mediated sympathetic activation, resulting in the release of norepinephrine from the nerve ending innervated in the WAT. One of the well-characterized subtypes of cold-activated adrenergic receptors of murine adipocytes is the β3-adrenergic receptor that activates adenylyl cyclase which catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP) [8]. An increase in the level of intracellular cAMP promotes the activation of protein kinase A (PKA), which subsequently increases *Ucp1* transcription. Thus, the signaling cascade, β3-adrenergic receptor–adenylyl cyclase–cAMP–PKA, is the canonical pathway involved in the activation of thermogenic adipocytes [9].

Abbreviations

AP1KO, adipocyte-specific PRMT1 knockout mice; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CARM1, coactivator-associated arginine methyltransferase 1; eWAT, epididymal white adipose tissue; HSL, hormone-sensitive lipase; iBAT, interscapular brown adipose tissue; ISO, isoproterenol; iWAT, inguinal white adipose tissue; PKA, protein kinase A; PRMT1, protein arginine methyltransferase 1; SQ, subcutaneous; SVF, stromal vascular fraction; TC-E, TC-E 5003; UCP1, uncoupling protein1; WAT, white adipose tissue.

Using genetic approaches, we recently demonstrated the strong involvement of protein arginine methyltransferase 1 (PRMT1) in beige adipocyte thermogenesis [10]. As PRMT1 is also associated with the progression of many types of cancers, diverse drugs that inhibit PRMT1 activity have been discovered [11,12]. However, their effects on beige adipocyte thermogenesis have not been elucidated. In the present study, we tested four different inhibitors of PRMT1 and found that one of them, TC-E 5003 (TC-E), exerted strong effects by activating beige adipocytes *via* PKA signaling.

Materials and methods

Reagents

TC-E 5003 (Tocris [#5099], Bristol, UK), furamidine dihydrochloride (Tocris [#5202]), AMI-1 (Cayman [#13965], Ann Arbor, MI, USA), MS023 (Cayman [#18361]), isoproterenol (ISO; Sigma [#I6504], St. Louis, MO, USA), cAMP (Sigma [#D0627]), and H-89 (Cayman [#10010556]) were purchased.

Primary murine cell cultures

To obtain primary cells, murine inguinal WAT (iWAT) or interscapular brown adipose tissue (iBAT) was isolated, minced with sharp surgical scissors, and digested in a 37 °C shaking (200 r.p.m.) water bath using collagenase D/dispase II (Sigma [#11088882001, #4942078]) or collagenase B/dispase II (Sigma [#11088831001]) solution, respectively. After filtration with a 100-μM mesh, the separated cells were centrifuged. The pellet obtained was resuspended and filtered again with 40-μM mesh before another centrifugation step. The pellet containing the stromal vascular fraction (SVF) was resuspended in DMEM/F12 glutaMax (Thermo Fisher [#10565042], Waltham, MA, USA) supplemented with 15% FBS and penicillin/streptomycin and seeded on collagen-coated plates. For adipogenic differentiation, the cells seeded on 12-well collagen-coated plates were stimulated with modified differentiation media (DMEM/F12 glutaMax instead of DMEM) for 2 days and then maintained in modified maintenance media (DMEM/F12 glutaMax instead of DMEM). The medium was changed every other day. On day 6, the cells were used for experiments. To obtain primary epididymal WAT (eWAT) cells, SVF was obtained from murine eWAT in a manner similar to that for iWAT SVF. The cells were seeded on collagen gel, and differentiation was induced as previously described [13].

Primary PRMT1 knockout iWAT cells were prepared from adipocyte-specific PRMT1 knockout (AP1KO) mice that were generated by breeding a $PRMT1^{fl/fl}$ mouse, that were kindly provided by S.-H. Koo (Korea University), with an adiponectin(AQ)-Cre recombinase mouse (Jackson Laboratory [#028020], Bar Harbor, ME, USA). Cells isolated from $PRMT1^{fl/fl}$;$AQ^{+/+}$ were used as wild-type (WT), while those obtained from $PRMT1^{fl/fl}$;$AQ^{Cre/+}$ served as AP1KO. $PRMT1^{fl/fl}$ mice used in this study were backcrossed to 6J background for more than 10 generations. The β-less primary iWAT cells were isolated from β-less mice that were kindly provided by B. Lowell (Harvard Medical School). Animals were maintained in accordance with the protocol reviewed and approved by the University Committee on Care and Use of Animals at the University of Michigan. Detailed methods are provided in Appendix S1.

Human cell cultures

Human adipose precursor cells isolated from the SQ fat were obtained as previously described [14]. All specimens were collected under the protocols reviewed and approved by the University of Michigan Medical School Institutional Review Board (IRBMED). Undifferentiated cells were cultured in MesenPRO RS medium (Thermo Fisher [#12746012]) supplemented with penicillin/streptomycin. For adipogenic differentiation, the cells were stimulated with DMEM/F12 glutaMax supplemented with 10% FBS, penicillin/streptomycin, 0.5 $\mu g \cdot mL^{-1}$ insulin, 0.5 mM IBMX, 5 μM dexamethasone, 5 μM rosiglitazone, 33 μM biotin (Sigma; B4639), and 17 μM pantothenic acid (Sigma; P5155) for 4 days. The cells were then maintained in DMEM/F12 glutaMax supplemented with 10% FBS, penicillin/streptomycin, 0.5 $\mu g \cdot mL^{-1}$ insulin, 1 μM rosiglitazone, 33 μM biotin, and 17 μM pantothenic acid. The medium was changed every other day. Fully differentiated cells (after 10 days) were used for experiments.

Results

TC-E treatment increases the expression of *Ucp1* mRNA in primary iWAT cells

To evaluate the thermogenic activity of beige adipocytes, the stromal vascular fraction (SVF) isolated from mouse inguinal WAT [iWAT; the largest subcutaneous (SQ) WAT] was differentiated into adipocytes (primary iWAT cells) and then treated with isoproterenol (ISO; nonselective β-adrenergic receptor agonist) or four kinds of PRMT1 inhibitors, namely AMI-1 [15], MS023 [16], furamidine [17], and TC-E [18]. As expected, *Ucp1* expression was robustly increased following ISO treatment but not after AMI-1, MS023, or furamidine treatment (Fig. 1A). However, unexpectedly, TC-E exposure resulted in a strong upregulation of *Ucp1* expression to a level comparable

with that observed following ISO treatment (Fig. 1A, B). Since the half maximal inhibitory concentration (IC50) of TC-E is approximately 1.5 μM [18], we tested the effect of TC-E at a concentration of 1, 3, or 10 μM in primary iWAT cells. TC-E greatly increased the expression of *Ucp1* at a dose of 10 μM, and the effect was absent at lower doses (1 or 3 μM) (Fig. 1C). Moreover, 10 μM TC-E treatment led significant increase in *Ucp1* mRNA and protein expressions up to 24 h (Fig. 1C,D). To examine the depot-specific effect of TC-E, the SVFs isolated from iWAT, iBAT (classic brown adipocytes), and eWAT (classic visceral white adipocytes) were treated with TC-E (Fig. 1E). The effect of TC-E on *Ucp1* mRNA expression in primary iWAT cells was abrogated in primary iBAT cells, whereas *Fgf21* (encoding fibroblast growth factor 21) expression was induced by TC-E in both cell types. Gene expression pattern of eWAT cells was similar to that of iWAT cells, although the increase by TC-E treatment was marginal (Fig. 1E). Given the increased *Ucp1* expression, we examined if TC-E treatment increases thermogenesis of the cells using OLTAM [ODD-Luc (luciferase fused with the oxygen-dependent degradation domain)-based Thermogenic Activity Measurement] system that we recently developed and reported to measure thermogenic activity of brown or beige adipocytes [14]. The result showed that TC-E treatment increased ODD-Luc activity to a level comparable with that observed following ISO treatment, suggesting that TC-E enhances thermogenesis (Fig. 1F). However, mRNA levels of the genes related to mitochondria and the protein levels of OXPHOS complexes were not altered by TC-E treatment (Fig. 1G,H). These observations suggest that TC-E strongly augments thermogenesis through increase in UCP1 expression (proton leak) without changing mitochondrial content in primary iWAT cells.

TC-E treatment activates the downstream molecules of PKA signaling

As PKA signaling is relevant to thermogenesis, we examined the dose- and time-dependent effects of TC-E treatment on PKA activity in primary iWAT cells. Consistent with the effects on *Ucp1* expression (Fig. 1C), 10 μM TC-E significantly increased PKA activity. This observation was evident from western blotting results using antibodies against phosphorylated PKA substrates as well as phosphorylated p38, one of the substrates of PKA, in a time-dependent manner (Fig. 2A,B). The TC-E-induced *Ucp1* and *Fgf21* expression in primary iWAT cells markedly reduced following treatment with the selective PKA inhibitor, H-89, indicating that TC-E activates the thermogenic pathway by inducing PKA activation (Fig. 2C). We also examined whether TC-E treatment induces lipolysis, as activated PKA not only upregulates the thermogenic pathway but also promotes lipolysis through the activation of hormone-sensitive lipase (HSL) and Perilipin1 [9]. The PKA-dependent phosphorylation of HSL at serine 563 and 660 increased following TC-E treatment, as observed with ISO treatment (Fig. 2D). In addition, the PKA-independent phosphorylation of HSL at serine 565, which leads to HSL inactivation, was decreased [19]. Further, the PKA-dependent phosphorylation of Perilipin1 at serine 522 was upregulated (Fig. 2D,E), suggesting that TC-E treatment increases lipolysis. Activation of lipolysis was further supported by the reduced size of lipid droplets and increased release of glycerol, the split form of triacylglycerol, after ISO or TC-E treatment (Fig. 2F,G).

The effects of TC-E are independent of PRMT1 and beta-adrenergic receptors

The thermogenic effects of TC-E were not anticipated as the thermogenic activity in iWAT of adipocyte-specific PRMT1 knockout (AP1KO) mice was clearly impaired after cold or β3-adrenergic receptor stimulation [10]. To clarify the discrepancy between the effects of TC-E and the phenotype of AP1KO mice, primary iWAT cells from WT or AP1KO mice were incubated with TC-E (Fig. 3A). As a result, we found that the observed effects of TC-E (increased *Ucp1* and *Fgf21* expression, PKA activity, and lipolysis) were unaltered by PRMT1 deficiency (Fig. 3B–D). These results suggest that effects of TC-E treatment were mediated in a PRMT1-independent manner.

Considering the canonical pathway of thermogenesis (β3-adrenergic receptor–adenylyl cyclase–cAMP–PKA), we speculated whether the TC-E-induced activation of PKA could be mediated by the direct stimulation of β-adrenergic receptor. Therefore, we isolated primary iWAT cells from β-less mice deficient for β1, β2, and β3 adrenergic receptors [20] and incubated them with ISO, cAMP, or TC-E. Given the lack of β receptors, no effects were observed following ISO treatment; however, cAMP, a downstream molecule of β3-adrenergic receptor, increased the expression of *Ucp1* and *Fgf21*, as expected (Fig. 3E). TC-E induced *Ucp1* and *Fgf21* expression even in β receptor-deficient cells (Fig. 3E), indicating that it does not directly stimulate β receptors. The significant increase in the intracellular level of cAMP following ISO treatment was unaltered by TC-E treatment (Fig. 3F).

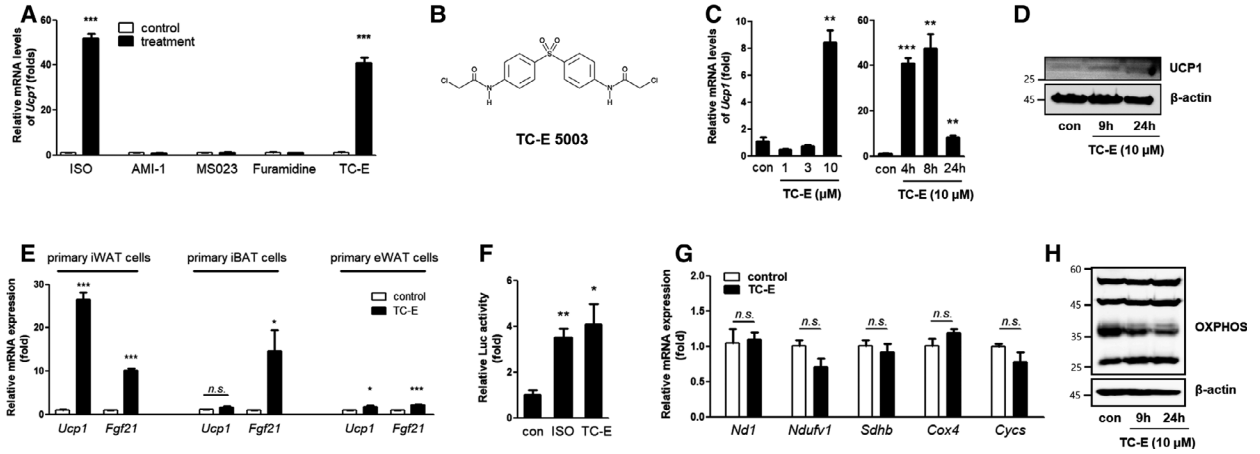

Fig. 1. TC-E increases *Ucp1* and *Fgf21* mRNA expression in primary iWAT cells. (A) Primary iWAT cells were incubated with ISO (10 μM), AMI-1 (10 μM), MS023 (10 μM), furamidine (100 μM), or TC-E (10 μM) for 4 h, and the expression of *Ucp1* mRNA was measured ($n = 3$). (B) Molecular structure of TC-E. (C, D) Cells were incubated with TC-E for 4 h or indicated time intervals. (C) The expression of *Ucp1* mRNA was measured ($n = 3$). (D) UCP1 protein expression was analyzed. β-Actin was used as the loading control. (E) The TC-E–induced expression of *Ucp1* and *Fgf21* mRNAs was determined in primary iWAT, iBAT, and eWAT cells (4 h treatment). (F) Adenoviral-OLTAM-transduced primary iWAT cells were incubated with 10 μM ISO for 5 h or 10 μM TC-E for 24 h, and then, luciferase activity was measured ($n = 4$). (G) Primary iWAT cells were incubated with 10 μM TC-E for 4 h, and the mRNA expression of genes related to mitochondria was measured ($n = 3$). (H) Primary iWAT cells were incubated with 10 μM TC-E for indicated time intervals. Protein expressions of OXPHOS complex were analyzed. β-Actin was used as the loading control. All values are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. n.s., not significant.

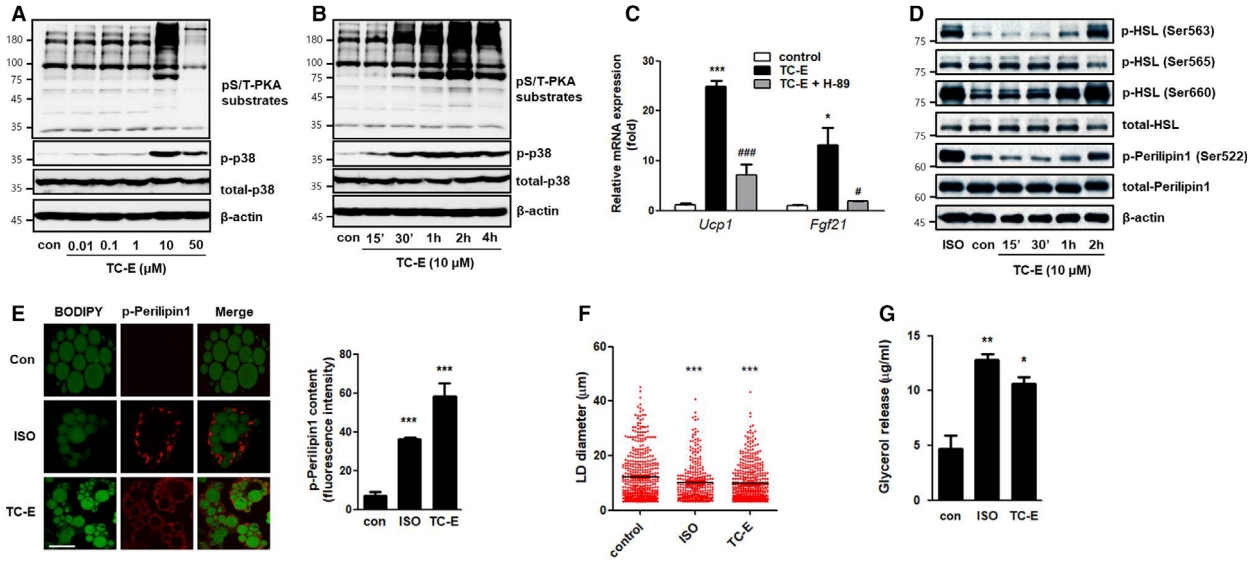

Fig. 2. TC-E activates the PKA signaling and lipolysis pathway in primary iWAT cells. (A, B) Primary iWAT cells were incubated with TC-E (A) at different doses for 4 h or (B) 10 μM concentration of TC-E for different time intervals. Protein levels were analyzed. β-Actin was used as the loading control. (C) Cells were preincubated with H-89 (50 μM) or vehicle for 1 h and treated with TC-E or vehicle for 4 h. mRNA levels were measured ($n = 4$). $^{*}P < 0.05$ and $^{***}P < 0.001$, control versus TC-E; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, TC-E versus TC-E + H-89. (D) Protein levels in primary iWAT cells incubated with TC-E, ISO (10 μM), or vehicle were analyzed. (E, F) Immunofluorescence analysis of primary iWAT cells incubated with ISO (10 μM for 1 h), TC-E (10 μM for 4 h), or vehicle. Signals for phospho-Perilipin1 are shown in red, and counterstaining of lipid droplets using BODIPY is shown as green (scale bar = 20 μm). (E) Content of phospho-Perilipin1 and (F) size of lipid droplets were quantified. (G) Glycerol levels in the medium of primary iWAT cells incubated with ISO (10 μM for 1 h), TC-E (10 μM for 1 h), or vehicle were measured ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control.

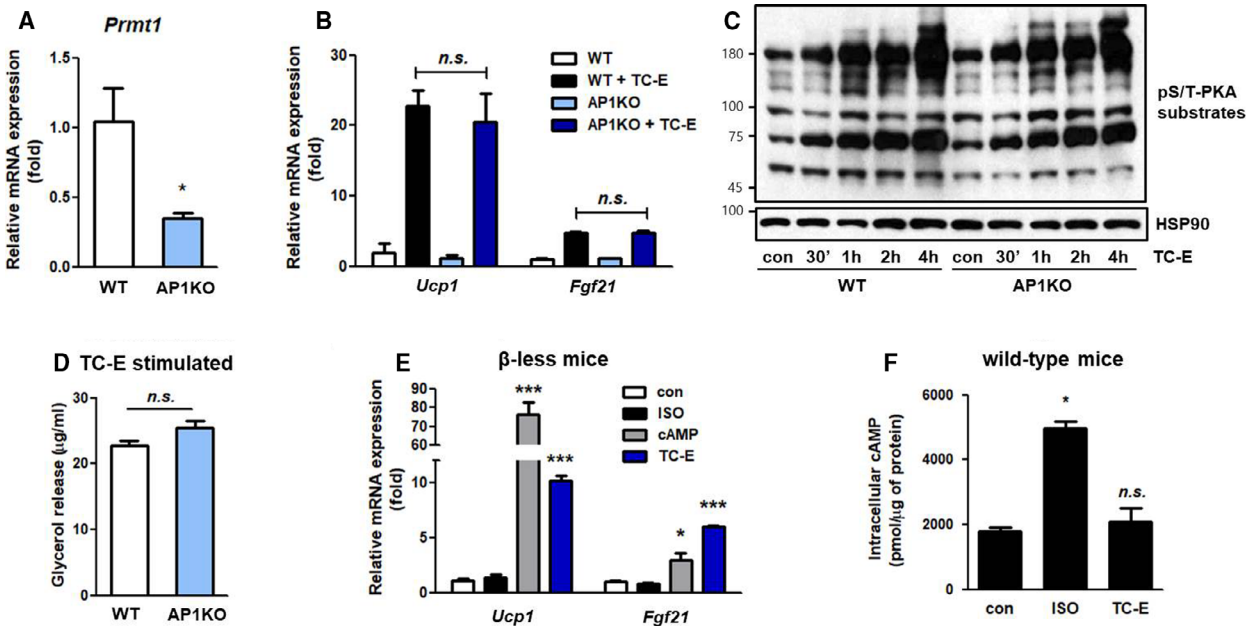

Fig. 3. Thermogenic properties of TC-E in primary iWAT cells are independent of PRMT1 and β-adrenergic receptor. (A, B) The mRNA levels were measured in primary iWAT cells of wild-type (WT) or adipocyte-specific PRMT1 knockout (AP1KO) mice ($n = 3$). (B) Primary iWAT cells treated with 10 μM TC-E or vehicle for 4 h were analyzed ($n = 3$). (C) Protein levels in primary iWAT cells incubated with 10 μM TC-E were analyzed. HSP90 was used as the loading control. (D) Glycerol levels in the medium of primary iWAT cells incubated with TC-E (10 μM for 4 h) were measured ($n = 3$). (E) Primary iWAT cells were isolated from β-less mice and incubated with ISO (10 μM), cAMP (500 μM), or TC-E (10 μM) for 4 h. The mRNA levels were measured ($n = 3$). (F) Primary iWAT cells were incubated with ISO (10 μM), TC-E (10 μM), or vehicle for 10 min. Intracellular cAMP levels were analyzed ($n = 3$). $^*P < 0.05$ and $^{***}P < 0.001$. n.s., not significant.

TC-E potently works in human SQ cells

We investigated the effects of TC-E on primary human adipocytes differentiated from SQ adipose precursor cells. In human SQ cells, as seen in murine primary iWAT cells, TC-E treatment increased *UCP1* and *FGF21* expression, PKA activity, and p38 and Perilipin1 phosphorylation (Fig. 4A,B). We further examined the effects of TC-E on human SQ cells derived from two more different individuals and found that TC-E increased the expression of thermogenic genes, although *UCP1* induction was lower and not statistically significant in #3 (Fig. 4C,D).

Discussion

In the present study, we demonstrate that treatment with TC-E, one of the chemical inhibitors of PRMT1, results in the activation of the PKA-dependent thermogenic pathway in primary murine iWAT cells and human SQ cells. SQ fat is the most widely distributed fat tissue, and individuals with a high proportion of lower body SQ fats are metabolically healthy [21].

One of the important questions to understand human thermogenic adipocytes is if β3-adrenergic receptor is expressed in human SQ adipocytes [22–24]. Recent studies have shown that the β3 agonist-mediated uptake of glucose, the typical fuel for thermogenesis, is absent in human SQ depots [25,26]. However, thermogenic adipocytes seem to use not only glucose but also various other molecules as fuel [27,28], and a significant portion of the energy expenditure occurs in the SQ fats [29]. Furthermore, the expression of *UCP1* mRNA has been detected in human SQ depots [30], suggesting that the activation of SQ fat may be potentially useful for the treatment of obesity. However, the uncertainty related to the presence of β3-adrenergic receptor on SQ adipocytes and the possibility of inducing catecholamine resistance by repeated β3 agonist treatment [31,32] have highlighted the discovery of the drugs that work directly inside SQ adipocytes. Interestingly, the activation of β3-adrenergic receptor and TC-E treatment has convergences of mechanism; (a) both activate the expression of thermogenic genes, (b) both activate lipolysis, and (c) both mediate PKA activation. Therefore, TC-E could be a potent activator of human SQ adipocytes. It is noteworthy that TC-E treatment activated the downstream signaling molecules of β3-adrenergic receptor even in primary iWAT cells isolated from β-less mice.

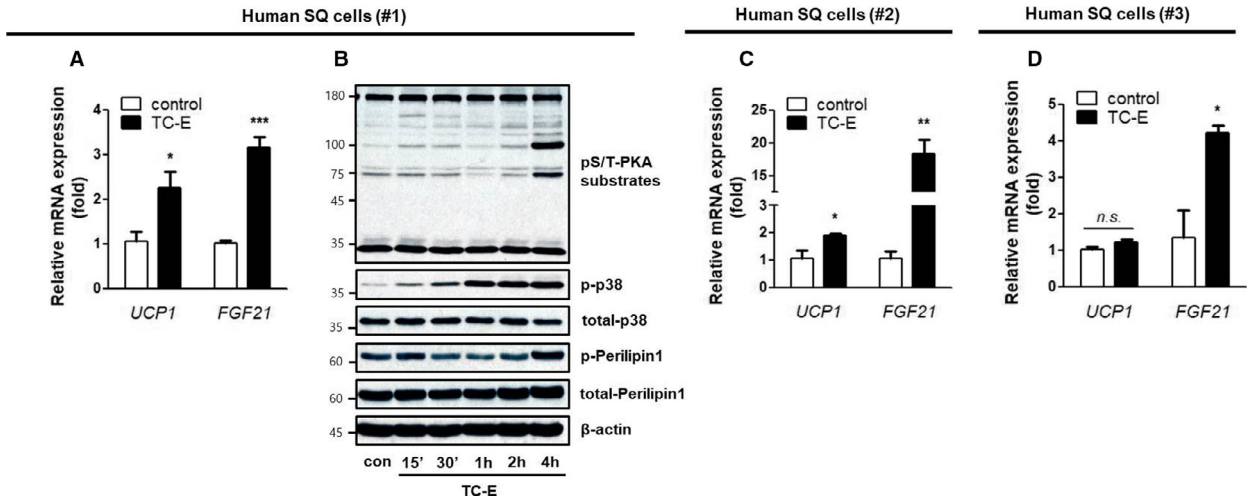

Fig. 4. TC-E increases thermogenic pathway in human SQ cells. (A, B) Human subcutaneous (SQ) cells (#1) were analyzed. (A) Human SQ cells were incubated with 10 μM TC-E or vehicle for 4 h, and the mRNA levels were measured ($n = 3$). (B) Human SQ cells were incubated with 10 μM TC-E for indicated time intervals. Protein levels were measured. β-Actin was used as the loading control. (C, D) mRNA levels were measured in human SQ cells (#2 or #3) ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. n.s., not significant.

As PRMT1 is required for the activation of thermogenic fat [10], we anticipated the downregulation of the thermogenic program by PRMT1 inhibitors. However, TC-E treatment paradoxically increased *Ucp1* and *Fgf21* mRNA expression in primary iWAT cells, whereas marginal effects were observed with AMI-1, MS023, and furamidine. In addition, the increase in *Ucp1* and *Fgf21* mRNA expression following the treatment of wild-type iWAT cells with TC-E was comparable to that in PRMT1 knockout iWAT cells, indicating TC-E exerts thermogenic activity presumably not through PRMT1 inhibition. As PKA activity increased from 15 min in primary iWAT cells following TC-E treatment, we measured the intracellular levels of cAMP, which activates PKA. However, unlike ISO, TC-E treatment did not alter cAMP level, indicating that TC-E activates PKA in a cAMP-independent manner. Studies have revealed quite a few mechanisms related to cAMP-independent PKA activation [33–36]. Future studies will, therefore, be needed to reveal the mechanism underlying the TC-E-mediated increase in PKA activity.

TC-E, furamidine, and AMI-1 are highly selective PRMT1 inhibitors, whereas MS023 is a type I PRMT inhibitor with affinity in the order of PRMT6 > PRMT8 > PRMT1 > PRMT4 > PRMT3. PRMT8 is exclusively expressed in the brain but not in the adipocytes [37]. PRMT4, also known as a coactivator-associated arginine methyltransferase 1 (CARM1), and PRMT6 regulate adipogenesis by interaction with peroxisome proliferator-activated receptor γ (PPARγ) [38,39]. We observed that MS023 treatment in our condition, failed to alter the expression of thermogenic genes probably because PRMT4 and PRMT6 are not involved in the thermogenic program or owing to the composite effects of MS023. Given the crucial role of type I PRMTs in various cellular processes, MS023 function is worth to be more investigated in the metabolisms of adipocytes.

In summary, TC-E treatment increases the expression of thermogenic genes most abundantly in primary murine iWAT cells. TC-E also upregulates the PKA signaling pathway and lipolysis, independently of PRMT1. The TC-E–induced expression of thermogenic genes and activation of related signaling pathways are also observed in primary human SQ cells. Thus, TC-E may contribute to the understanding of human SQ adipocyte thermogenesis and reveal the potential therapeutic implication.

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Author contributions

M-JP, JL and D-IK designed and performed the experiments. M-JP, JL and D-IK analyzed and discussed results. M-JP and D-IK wrote the manuscript. D-IK supervised the project.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supplementary Methods.