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Cilostazol induces mitochondrial fatty acid β-oxidation in C2C12 myotubes

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ABSTRACT

Cilostazol is a drug licensed for the treatment of intermittent claudication. Its main action is to elevate intracellular levels of cyclic monophosphate (cAMP) by inhibiting the activity of type III phosphodiesterase, a cAMP-degrading enzyme. The effects of cilostazol on fatty acid oxidation (FAO) are as yet unknown. In this study, we report that cilostazol can elevate complete FAO and decrease both triacylglycerol (TAG) accumulation and TAG secretion. This use of cilostazol treatment increases expression of PGC-1α and, subsequently, its target genes, such as ERRα, NOR1, CD36, CPT1, MCAD, and ACO. Expression of these factors is linked to fatty acid β-oxidation but this effect is inhibited by H-89, a specific inhibitor of the PKA/CREB pathway. Importantly, knockdown of PGC-1α using siRNA abolished the effects of cilostazol in fatty acid oxidation (FAO) and TAG metabolism. These findings suggested that the PKA/CREB/PGC-1α pathway plays a critical role in cilostazol-induced fatty acid oxidation and TAG metabolism.

1. Introduction

Fatty acid oxidation (FAO) in mitochondria (β-oxidation) is the prime pathway for the metabolism of fatty acids. It has been accepted as a key metabolic pathway for energy homeostasis in various organs including the liver, heart, and skeletal muscle [1]. Over the last decade, dysregulated fatty acid (FA) oxidation and its effector pathway have come to be associated with the pathophysiology of metabolic syndrome, atherosclerosis, cardiomyopathy, and diabetes mellitus [2]. Moreover, previous studies have found that an imbalance between circulating and cytosolic FA levels, which results in excessive intracellular accumulation of FAs and their derivatives (diacylglycerol, ceramides), is an underling cause of a broad spectrum of insulin-deficient states, including insulin resistance in metabolic syndrome, insulin resistance and relative insulin deficiency in type 2 diabetes (T2D), and absolute insulin deficiency in type 1 diabetes (T1D) [3]. Targeting the key molecules involved in FAO with the goal of balancing cellular FA concentration is considered a putative intervention that could serve to benefit patients with metabolic syndrome.

Cilostazol, 6[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3, 4-dihydro-2(1H)-quinolinone, is a licensed drug that was approved by the US Food and Drug Administration (FDA) in 1999 for use in the treatment of intermittent claudication [4]. Its main action is to elevate intracellular levels of cyclic monophosphate (cAMP) by inhibiting activity of type III phosphodiesterase, a cAMP-degrading enzyme [5]. Increased intracellular levels of cAMP lead to activation of the cAMP-responsive transcription factor CREB [6]. CREB has been reported to activate gluconeogenic and fatty acid oxidation programs by stimulating expression of the nuclear hormone receptor coactivator PGC-1α [7]. PGC-1α has been considered a major mediator in regulating metabolic pathways, including fatty acid oxidation, mitochondrial biogenesis, and mitochondrial oxidative phosphorylation (OXPHOS) [8]. Thus, we speculated that cilostazol could regulate fatty acid β-oxidation. In order to verify this hypothesis, we treated myotubes with cilostazol and investigated the alterations of fatty acid oxidation in myotubes.

2. Materials and methods

2.1. Cell culture

Murine C2C12 cells were grown in Dulbecco's modified low-glucose Eagle's medium (DMEM) containing 10% FBS (v/v), 4.0 mM glutamine, and 1% penicillin and streptomycin. Cells were

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maintained in a humidified incubator with 5% CO_2 at 37 °C. The differentiation of myoblasts into myotubes was induced by switching the medium to DMEM containing 2% horse serum (GIBCO, Grand Island, NY, USA) for 72 h. The PGC-1α and negative control small interfering RNAs (siRNAs) oligo were from Sigma, USA. siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's instructions.

2.2. Rates of fatty acid oxidation

Intracellular fatty acid oxidation (FAO) was performed as previously described [9]. Briefly, following serum starvation, C2C12 myotubes were incubated with ^{14}C-labeled FAO reaction medium consisting of 0.25 μCi/ml [1-^{14}C] palmitate, 0.25 μCi/ml [1-^{14}C] oleate, 50 μM palmitate, and 50 μM oleate at 37 °C for 3 h. For measurement of the ratio of complete FAO to CO_2, the $^{14}CO_2$ was driven from the media aliquot by addition of perchloric acid and trapped in NaOH, which was collected and analyzed by liquid scintillation counting. The acidified medium was collected, refrigerated, and centrifuged with 16,000*g* at 4 °C. An aliquot was analyzed using liquid scintillation counting for determination of the acid-soluble metabolites (ASMs) of FAO. Cells were lysed with SDS lysis buffer and the protein concentration of the lysate was determined by BCA assay.

2.3. TAG quantification

SDS cell lysates were used to isolate cellular and media lipid fractions by a modified chloroform–methanol extraction method, as previously described [10]. Briefly, 1 volume of aliquots from the SDS cell lysate and starvation medium was mixed with four volumes of chloroform–methanol–acetic acid (2:1:0.15). After centrifugation at 50*g* for 10 min at 10 °C, the bottom chloroform layer was pipetted off and filtered into a new tube. After being blown dry under N2, lipids were resuspended in chloroform–methanol (2:1) and resolved by TLC (hexane–diethyl ether–acetic acid, 70:30:1). Individual lipid species were visualized on a chromatography plate with 2, 7-dichlorofluorscein and collected in glass tubes. Lipid species were scraped from the TLC plate and then methylated by incubation with toluene-0.5 M sodium methoxide (methanol) (1:2) for 1 min and separated in isooctane. Gas chromatography was used to analyze fatty acid methyl esters.

2.4. Western blot analysis

Cells were lysed in cellular lysate buffer (Cell Signaling, USA) and supplemented with complete protease inhibitor and phosphatase inhibitor cocktail (Roche, USA) according to the manufacturer's instructions. Protein concentration was determined by BCA assay. Protein lysates were then subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA), as previously described [11]. The transferred PVDF membranes were blocked with 5% non-fat milk for 2 h at RT. After having been washed 2 times with TBST, membranes were sequentially incubated with primary antibodies for 3 h and horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature (RT). Bands were visualized using Beyo ECL Plus reagent (enhanced chemiluminescence) according to the manufacturer's protocol.

The following antibodies were used in this study: rabbit monoclonal antibody for PGC-1α from Calbiochem, USA; rabbit monoclonal antibodies for p-CREB, CREB and β-actin from Cell Signaling, USA.

2.5. Real time PCR

In order to determine the expression of target genes at the mRNA level, fluorescence-based Real time PCR was performed as described previously. Briefly, total RNA was extracted from cells using trizol as previously described [12]. 1 μg of cellular RNA

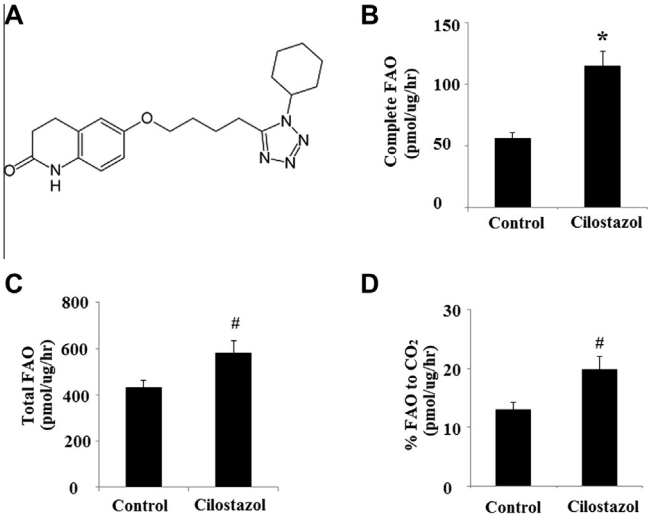

Fig. 1. Treatment with 5 μM cilostazol for 48 h increases fatty acid oxidation (FAO) in C2C12 myotubes. FAO rates of 14C-radiolabeled palmitate–oleate (1:1) were determined in both the presence and absence of cilostazol. (A) Molecular structure of cilostazol. (B) Complete FAO to CO_2 was elevated in cilostazol-treated C2C12 myotubes ($n = 5$, $*p < 0.01$ vs. control group). (C) Total FAO was greater in cilostazol treated C2C12 myotubes than in control cells ($n = 5$, $\#p < 0.05$ vs. control group). (D) FAO efficiency (percentage of FAO to CO_2) was greater in cilostazol treated C2C12 myotubes than in control cells ($n = 5$, $\#p < 0.05$ vs. control group).

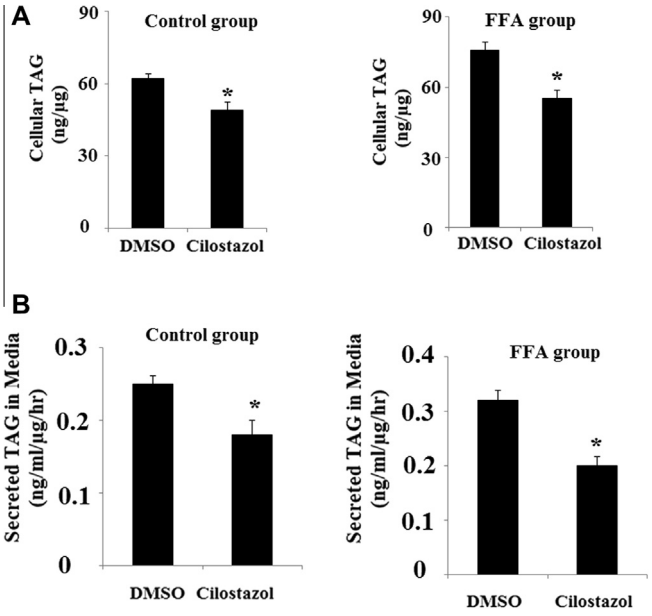

Fig. 2. Decreased triacylglycerol (TAG) accumulation and secretion in cilostazol treated C2C12 myotubes. TAG accumulation and secretion in C2C12 myotubes were determined following overnight treatment with either control or free fatty acids (FFA; 50 μM palmitate–50 μM oleate). (A) Cellular accumulation of TAG was decreased in cilostazol treated cells as compared to non-treated cells in both the control state and in free fatty acids treatment group ($*p < 0.05$. student-*t* test. $n = 5$). (B) TAG secretion into the medium was decreased in cilostazol treated C2C12 myotubes in both the control state and free fatty acids treatment group ($*p < 0.05$. student-*t* test. $n = 5$).

was then used as a template for reverse transcription PCR to synthesize cDNA. cDNA was used for real time PCR performed on an ABI PRISM 7700 Sequence Detection System with a TaqMan Universal PCR Master Mix reagent (Applied Biosystems) according to the manufacturer's instructions. Values in all samples were normalized to the expression level of the endogenous control, GAPDH.

2.6. Statistical analysis

All results are expressed as means ± standard error of means (SE). Fisher's least significant difference (LSD) post hoc test was performed to determine differences between groups with SPSS. Student's *t*-test was used to make statistical comparisons within groups. One-way analysis of variance (ANOVA) was used to assess the statistical significance of differences among treatment groups. The results were considered significantly different at $p < 0.05$.

3. Results

The structure of cilostazol is shown in Fig. 1A. C2C12 myotubes were treated with cilostazol. FAO in C2C12 myotubes was determined by monitoring the oxidation of [^{14}C] palmitate–[^{14}C] oleate (1:1). Complete FAO to CO_2, acid-soluble metabolites (ASMs) production, and total FAO (complete oxidation ASMs) was determined. As is shown in Fig. 1B, treatment with 5 μM cilostazol led to a nearly twofold increase in complete FAO to CO_2, which represents the oxidation of fatty acids from β-oxidation through the TCA cycle ($^{*}p < 0.01$; Fig. 1B). A portion of β-oxidation of fatty acids products (ASMs) do not undergo further β-oxidation cycles or oxidation via the TCA cycle and are excreted from mitochondria and cells. Our data display that treatment with cilostazol also leads to a significant increase in production of these ASMs (data not shown). These increases in complete FAO and ASMs resulted in 35% higher total FAO in cilostazol treated cells $p < 0.05$; Fig. 1C). The percentage FAO to CO_2 (the ratio of complete FAO and total FAO) has been considered as a marker of the overall efficiency of FAO. Importantly, we found that the percentage of FAO to CO_2 was higher in cilostazol treated cells than in control cells ($p < 0.05$; Fig. 1D), thereby suggesting greater FAO efficiency.

To determine whether cilostazol treatment in C2C12 myotubes altered lipid storage or secretion patterns, TAG levels were quantified using gas chromatography on extracts from cell lysate and starvation media samples in cells treated overnight with FFA (50 μM palmitate-50 μM oleate). Under control conditions, treatment with cilostazol led to decreased cellular TAG levels as compared with non-treated cells (21%, $p < 0.05$; Fig. 2A). Lipid exposure resulted in 25% less cellular TAG accumulation in cilostazol-treated cells than in control cells ($p < 0.05$). Importantly, 100 μM overnight lipid exposure produced an increase in cellular TAG accumulation in control cells (22%, $p < 0.05$), while no change in TAG accumulation was observed in cilostazol treated cells. Also, the secretion rate of TAG into the media was 29% lower in the cilostazol treated C2C12 myotubes than in control cells under control

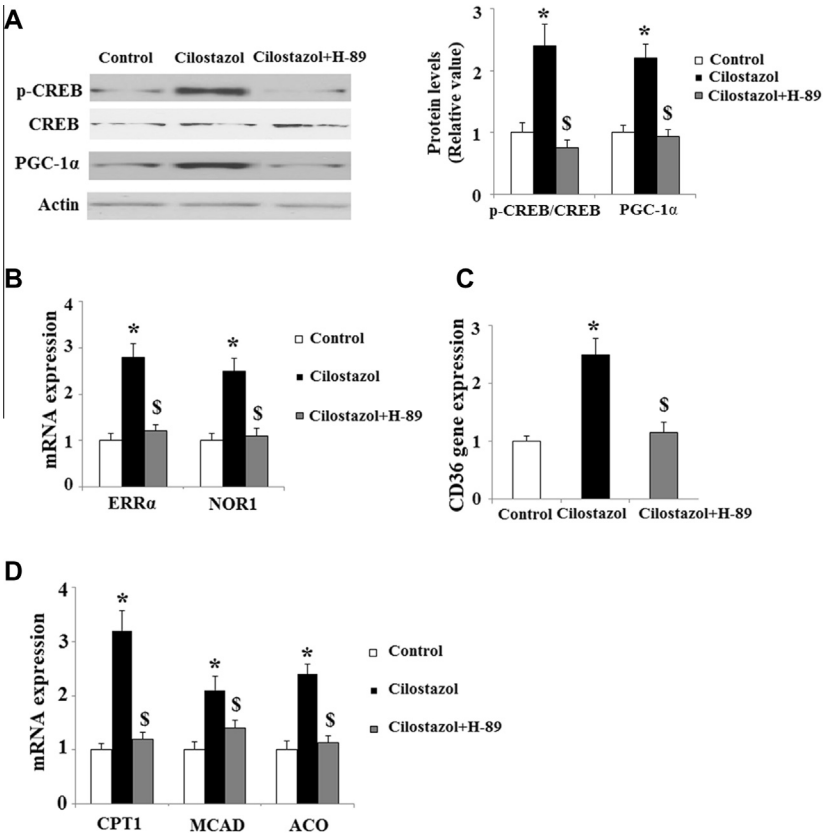

Fig. 3. Effects of cilostazol on expression of genes linked to fatty acid oxidation in cultured muscle cells is dependent on the PKA/CREB/PGC-1α pathway. After having been treated with 5 μM cilostazol in the presence or absence of PKA/CREB inhibitor H-89, mRNA levels of target genes were investigated using real time PCR. (A) Treatment with cilostazol upregulates the ratio of p-CREB/CREB and the expression level of PGC-1α, which is abolished by PKA/CREB inhibitor H-89 ($n = 4$, $*p < 0.01$ vs. control group; $\$p < 0.01$ vs. cilostazol treatment group); (B) Treatment with cilostazol upregulates fatty acid oxidation transcriptional regulatory genes ERRα and NOR1, which is abolished by PKA/CREB inhibitor H-89 ($n = 4$, $*p < 0.01$ vs. control group; $\$p < 0.01$ vs. cilostazol treatment group); (C) Treatment with cilostazol upregulates fatty acid transport gene CD36, which is inhibited by H-89 ($n = 4$, $*p < 0.01$ vs. control group; $\$p < 0.01$ vs. cilostazol treatment group); (D) Cilostazol treatment increases expression of all three mitochondrial b-oxidation genes, CPT1, MCAD, ACO, an effect that is abolished by H-89 ($n = 4$, $*p < 0.01$ vs. control group; $\$p < 0.01$ vs. cilostazol treatment group).

and lipid-treated conditions ($p < 0.05$; Fig. 2B). Similarly, overnight exposure to FFA resulted in 25% increases in secretion rates of TAG in cilostazol treated cells, though not in control cells ($p < 0.05$).

PGC-1α is a transcription cofactor that interacts with numerous transcription factors and has been shown to be a potent activator of mitochondrial biogenesis and FAO in skeletal muscle in response to increased physical activity [13]. The principal action of cilostazol is to elevate intracellular levels of adenosine 3′, 5′-cyclic monophosphate (cAMP). cAMP is the activator of PKA, which can lead to activation of transcriptional factor CREB [14]. Multiple lines of evidence have shown that CREB can regulate levels of PGC-1α [15]. Thus, we further investigated whether PGC-1α is involved in the effects of cilostazol on fatty acid oxidation. As is shown in Fig. 3A, western blot analysis results reveal that 5 μM cilostazol significantly increases phosphorylated CREB at Ser133 as well as total protein levels of PGC-1α in C2C12, which is inhibited by PKA inhibitor H-89. However, the total level of CREB did not change. Fatty acid oxidation is regulated by a broad range of genes which are, in turn, regulated by PGC-1α: (i) transcriptional regulatory genes, ERRα and NOR1; (ii) fatty acid transport genes, CD36/FAT; (iii) mitochondrial β-oxidation genes, CPT1, MCAD, and ACO. Being that cilostazol is able to promote fatty acid oxidation, we then investigated the effects of cilostazol on these genes. Firstly, as is shown in Fig. 3B, administration of cilostazol leads to a significant increase in fatty acid oxidation transcriptional regulatory genes, such as ERRα and NOR1, an effect that is abolished by H-89. Secondly, treatment with cilostazol caused a complete change in expression of the fatty acid transport gene CD36, an effect that was eliminated by H-89 (Fig. 3C). Thirdly and most importantly, cilostazol increased all three mitochondrial β-oxidation genes, including CPT1, MCAD, and ACO, all of which were inhibited by H-89 (Fig. 3D).

To assess whether increased expression of PGC-1α plays a causal role in increased fatty acid oxidation in C2C12 myotubes, we investigated the effects of knockdown of PGC-1α in the fatty acid oxidation promoted by cilostazol. C2C12 myotubes were transiently transfected with PGC-1α siRNA. Successful silencing of PGC-1α, as confirmed by western blot analysis, is shown in Fig. 4A. 48 h after transfection, complete FAO and TAG levels were determined. Our results reveal that the effects of cilostazol on complete FAO (Fig. 4B) and TAG (Fig. 4C) can be abolished by knockdown of PGC-1α.

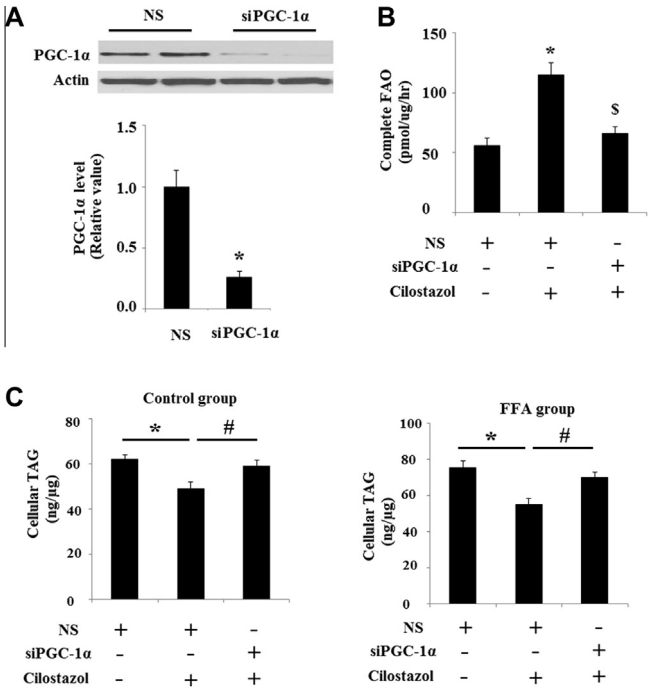

Fig. 4. Knockdown of PGC-1α abolished the effects of cilostazol in fatty acid oxidation (FAO) and triacylglycerol (TAG) accumulation. NS, non-specific small RNA; siPGC-1α, PGC-1α small RNA. (A) Expression of PGC-1α was knocked down using small RNA interferences; western blot analysis confirmed successful knockdown of PGC-1α ($n = 4$, $*p < 0.01$ vs. NS group); (B) After transfection with siPGC-1α, cells were treated with cilostazol. Complete FAO was determined ($n = 4$, $*p < 0.01$ vs. NS group; $\$p < 0.01$ vs. cilostazol treatment group); (C) After transfection with siPGC-1α, cells were treated with cilostazol. Cellular TAG levels were determined in both control group and FFA treated group (*, $\#p < 0.05$, $n = 4$, ANOVA).

4. Discussion

As a clinically prescribed drug, cilostazol has been widely used in clinical studies. The beneficial effects of cilostazol on serum lipid profiles have been reported in patients with intermittent claudication and type 2 diabetes. Randomized, double-blind, placebo-controlled trials report that cilostazol has beneficial effects on serum lipid profiles and fatty acid composition in plasma. It was concluded that cilostazol may be useful in the prevention of atherosclerosis progression by ameliorating lipid and fatty acid metabolism [16]. Takazakura et al. report that cilostazol reduces serum triglyceride (TG) concentrations and increases serum high-density lipoprotein-cholesterol (HDL-C) concentrations in diabetic patients [17]. Elam et al. also report on the beneficial effects of cilostazol on serum lipids in patients with intermittent claudication (IC) [18]. Moreover, the beneficial effects of cilostazol were demonstrated on the serum lipid profiles of type 2 diabetic patients with peripheral vascular disease [19]. Recently, Tani et al. reported that cilostazol reduces the activity of lipoprotein lipase in rats [20]. However, the molecular mechanisms and intracellular pathways underlying the effects of cilostazol on fatty acid reduction are still unknown. In this study, we found that cilostazol treatment increases the rate of fatty acid β-oxidation and reduces levels of TAG accumulation and secretion. Importantly, activation of PGC-1α through the PKA/CREB pathway was verified to participate in this process.

The main action of cilostazol is to elevate intracellular levels of cAMP. On one hand, elevated intracellular cAMP levels have been shown to promote the release of lipoprotein lipase from rat adipocytes [21], which can reduce serum TG concentrations. On the other hand, elevated intracellular cAMP can activate transcriptional factor CREB, which has been proven to induce expression of PGC-1α in a variety of tissues [22]. PGC-1α is a transcription cofactor that interacts with numerous transcription factors and has been shown to be a potent activator of mitochondrial biogenesis and FAO in skeletal muscle in response to increased physical activity [13]. PGC-1α has been observed to functionally control FAO efficiency in skeletal muscle. Koves et al. suggest that increased PGC-1α expression produces coordinated increases in fatty acid β-oxidation and downstream mitochondrial pathways, resulting in elevated FAO to CO_2 levels [23]. Correspondingly, it has been demonstrated that cilostazol promotes mitochondrial biogenesis through activating expression of PGC-1α in human umbilical vein endothelial cells (HUVECs) [24], which is in agreement with our study.

In conclusion, our findings demonstrate that cilostazol promotes mitochondrial β-oxidation and reduces TAG through activating PGC-1α. This suggests that cilostazol may be useful in improving dysregulated fatty acid (FA) oxidation and being administered as a preventative or therapeutic agent for metabolic dysfunction disease, such as obesity and insulin resistance.

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