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Eicosapentaenoic acid promotes thermogenic and fatty acid storage capacity in mouse subcutaneous adipocytes

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ABSTRACT

In this study, we determined if eicosapentaenoic acid (EPA) promotes beneficial metabolic activities of subcutaneous adipocytes. Stromal-vascular (SV) cells were isolated from inguinal adipose tissue of C57BL/6 mice and induced to differentiate into adipocytes. EPA effect on thermogenic and mitochondrial gene expression and oxidative metabolism were assessed in inguinal adipocytes. When added to SV cell cultures during 8 day differentiation, EPA significantly increased the expression of thermogenic genes UCP1-3, CIDEA and VEGFα. Moreover, EPA increased mitochondrial DNA content and the expression of genes involved in mitochondrial biogenesis including PGC1α, Nrf1 and COXiv. However, this effect was not perceived when EPA was added to mature inguinal adipocytes for 24 h, suggesting that EPA exerts its browning effect via recruiting brite adipocytes. Consistently, long-term EPA treatment also upregulated AMPKα phosphorylation and CPT1 expression and increased glucose uptake and GLUT4 mRNA expression, suggesting improved mitochondrial oxidation. Additionally, EPA-treated adipocytes had enlarged lipid droplets and increased expression of triglyceride synthesis genes GPAT1 and GPAT3, while significantly decreased glycerol release and down-regulation of HSL and ATGL gene expression. We conclude that EPA enhances energy dissipation capacity by recruiting brite adipocytes to stimulate oxidative metabolism and reduces fatty acid release by facilitating fatty acid storage in subcutaneous adipocytes.

1. Introduction

Obesity is a serious health problem in the United States. Adipose tissue dysfunction, which occurs in obesity, is the major contributor to obesity-associated metabolic complications such as hypertension, cardiovascular disease, inflammation, insulin resistance, and type 2 diabetes. Two types of adipose tissues exist and exert opposite metabolic functions. Brown adipose tissue (BAT) is the major depot that plays a role in thermogenesis and energy expenditure, while white adipose tissue (WAT) mainly stores lipids and secretes metabolic regulators of adipokines and cytokines.

In recent years, researchers found that brown-like adipocytes can be recruited in white adipose tissue in response to cold and β-adrenergic stimulation [1–3]. This browning process is regulated by multiple transcription factors, co-activators, and other molecular regulators, including PR domain containing 16 (PRDM16), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), peroxisome proliferator-activated receptor (PPARs), cell death-inducing DFFA-like effector a (CIDEA) and CCAAT-enhancer-binding protein-beta (C/EBPβ) [4–7]. More importantly, the browning process also potentially protects against obesity and insulin resistance [8,9]. It has long been known that there exist depot differences in metabolic functions of WAT. Recent studies suggest that subcutaneous white adipose tissue (SC-WAT) has higher lipid storage capacity as well as thermogenic potential when compared with visceral WAT [7,10]. These characteristics of SC-WAT may contribute to its metabolic benefits [11].

Mitochondrion is a key organelle responsible for substrate oxidation and energy dissipation. Recent studies have emphasized the importance of mitochondrial function, especially in SC adipocytes in the development of insulin resistance [12]. In obesity, mitochondrial dysfunction in adipocytes leads to the production of toxic lipid species, inflammation and insulin resistance [13,14]. Therefore, enhancing mitochondrial function and fatty acid oxidation, as well as increasing lipid storage capacity of adipocytes could lead to decreased release of free fatty acids (FFAs) from adipocytes, thereby preventing the circulating FFA elevation and subsequently metabolic deterioration.

Polyunsaturated fatty acids (PUFAs) of the *n*-3 series have been known for their beneficial effects in regulating adipose tissue function. Three major members of *n*-3 FAs include α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid

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(DHA). Previous studies on the metabolic effects of *n*-3 FAs showed that *n*-3 FAs are able to lower serum triglyceride (TG), reduce chronic inflammation, and increase insulin sensitivity in animals and human subjects [15–18]. The activation of PPARα, PPARγ and AMP-activated protein kinase (AMPK) could be the pathways that mediate the effect of *n*-3 FAs [19–22]. However, most of previous studies were performed in animal models or 3T3-L1 cell line without distinguishing the role of *n*-3 FAs in different fat depots.

In this study, we specifically investigated the metabolic effect of EPA on SC adipocytes and determined if EPA promotes beneficial metabolic activities in primary differentiated SC adipocytes. We found that EPA treatment during the process of inguinal adipocyte differentiation significantly increased the expression of thermogenic genes and mitochondrial biogenesis/function as well as TG storage.

2. Methods and materials

2.1. Animals

Animal handling followed the U.S. National Institutes of Health guidelines, and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee. C57BL/6 mice purchased from Jackson Laboratory were housed in specific pathogen-free facility at the University of Minnesota. Mice were allocated into groups (3 or 4 mice/cage) and fed a regular chow diet (RCD) with free access to water.

2.2. Cell culture

Stromal-vascular (SV) cells were isolated from inguinal WAT of C57BL/6 mice as described previously [23]. After mincing, fat pads were digested with collagenase (2 mg/ml solution) in Krebs–Ringer bicarbonate HEPES (KRBH) buffer. After 1.5 h digestion, SV cells were separated by centrifugation at 1500 rpm for 10 min and then cultured in growth medium (DMEM plus 10% FBS) until confluence. Cells were then treated with a differentiation cocktail containing 10% fetal bovine serum, 115 μg/ml methylisobutylxanthine, 390 ng/ml dexamethasone, and 1 μg/ml insulin for 3 days. Three days later, the differentiation cocktail was replaced with DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin/streptomycin, 1 μg/ml insulin, and cultures were continued for another 5 days. During 8 days of differentiation, 200 μM EPA (EMD Chemicals, NJ, USA) was added to the cultures as fatty acid/fatty acid-free bovine serum albumin (BSA) complexes. The molar ratio of fatty acid to BSA was 4:1. FA-free BSA was used as a control.

2.3. Western blotting

Proteins were extracted from cell samples in RIPA buffer (Sigma, St. Louis, MO, USA) containing protease inhibitors. Equal amounts of proteins were separated on 10% SDS–PAGE gel and transferred to polyvinylidene fluoride membrane. The membranes were probed with Phospho-AMPKα (Thr172), AMPKα or β-Actin (Cell Signaling, MA, USA) antibodies according to the recommendations of the manufacturers. ECL Western Blotting Detection Systems (GE Healthcare BioSciences, Piscataway, NJ, USA) were used to detect antibody reactivity. The density of bands was quantified by Image J software, and the results were normalized to β-Actin of each corresponding samples.

2.4. Quantitative real-time RT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Real-time PCR was performed using SYBR Green qPCR Master Mix (SABioscience, Frederick, MD, USA) with an ABI Step One Plus real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Table S1. Results are normalized to β-actin using the $\Delta\Delta C_t$ method and presented as levels of expression relative to that of controls.

2.5. Mitochondrial DNA content

Total DNA was extracted as previously described [24]. The samples were digested in lysis buffer (0.01 M Tris–HCl, 0.25 mM EDTA, 0.5% SDS, pH 8.3) containing 100 μg/ml proteinase K and extracted with chloroform and 8 M potassium acetate. The supernatant was collected and precipitated with isopropyl alcohol and ethanol. mtDNA content was determined by detecting DNA levels of cytochrome C oxidase 2 (COX2) using real-time PCR. The value was normalized to the levels of nuclear genome DNA RIP140.

2.6. Glucose uptake assay

Uptake of [^{3}H] deoxy-D-glucose (Amersham Biosciences, Piscataway, NJ, USA) was measured as previously described [25,26]. Briefly, cells were serum starved in KRH buffer supplemented with 0.5% BSA and 2 mmol/l sodium pyruvate (pH 7.4) for 3 h at 37 °C. Glucose uptake was initiated by adding 100 μmol/l [^{3}H]-deoxy-D-glucose. After 5 min, and the cells were solubilized with KRH buffer containing 1% Triton X-100 after three washes with ice-cold PBS buffer. Incorporated radioactivity was determined by scintillation counting.

2.7. Measurements of glucose, lactate and glycerol levels

Glucose and lactate levels in cell culture medium were detected with Autokit Glucose (Wako, VA, USA) and lactate Assay Kit (Sigma–Aldrich, MO, USA), respectively. media glycerol levels were detected with Free Glycerol Reagent (Sigma Aldrich, Saint Louis, MO) following the instruction provided by manufactures.

2.8. Triglyceride content measurement

On day 8 of differentiation, adipocytes were fixed in cold Baker's Formalin for 30 min at 4 °C, followed by staining in Oil Red O for 10 min. After rinsed the wells with water, isopropanol was added to each well to elute the dye for 15 min. Then the solvent was added into a 96 well plate and the OD was read at 540 nm.

2.9. Statistical analysis

Results are expressed as means ± SEM. Differences in the parameters between groups were evaluated using Student's *t*-test with a 0.05 2-sided significance level. A value of $P < 0.05$ was considered significant.

3. Results

3.1. EPA regulates thermogenic gene expression in inguinal adipocytes

As shown in Fig. 1A, 8 day of EPA treatment had no evident effect on inguinal adipocyte differentiation. This morphological result was confirmed by the gene expression of PPARγ (Fig. 1B). Interestingly, 8 day treatment of EPA during the adipocyte differentiation process significantly increased gene expression of UCP1-3 (Fig. 1C). Similar effect was observed in differentiated brown adipocytes (Fig. 1D). In addition, EPA significantly increased CIDEA but not PRDM16 and C/EBPβ expression in inguinal

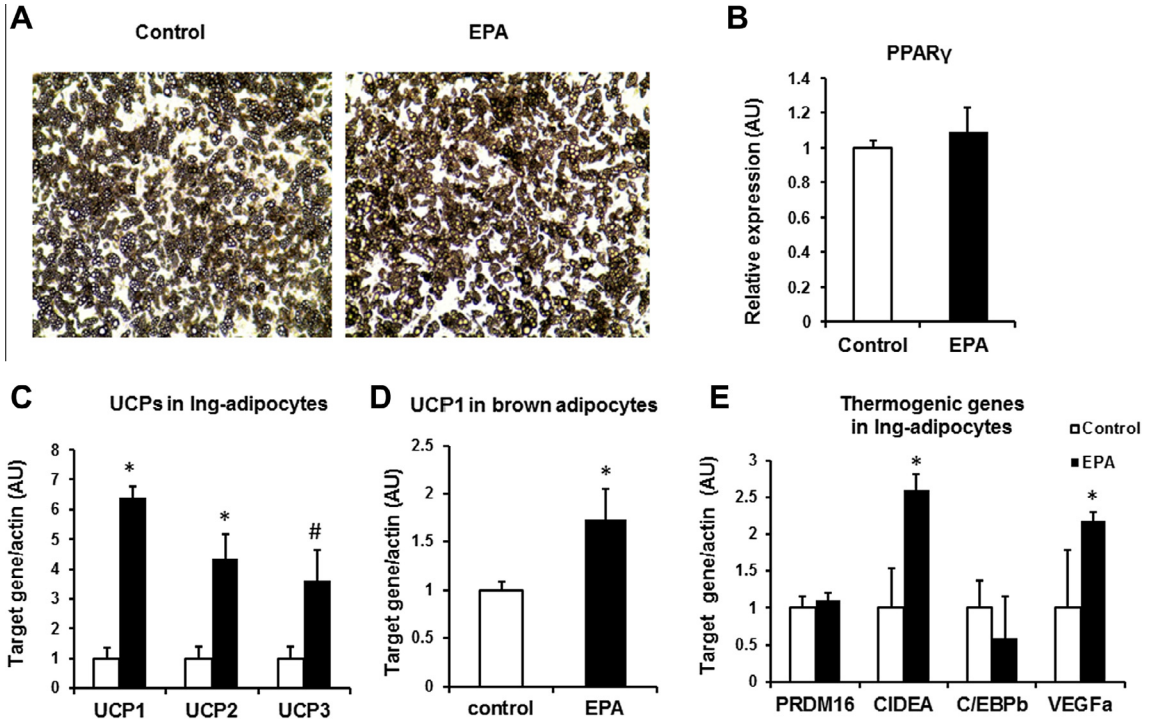

Fig. 1. EAP effect on thermogenic gene expression in inguinal and brown adipocytes. Morphology of inguinal adipocytes (4× magnification) (A). Gene expression of PPARγ (B) and UCPs in inguinal adipocytes (C) and brown adipocytes (D) with EPA treatment. Expression of thermogenic genes in inguinal adipocytes with EPA treatment (E). The results represent 2–3 independent experiments with different sets of mice (4–5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. ($n = 3$ per group) $^{*}p < 0.05$, $^{\#}p < 0.1$, EPA vs. control.

adipocytes (Fig. 1E). Moreover, our results showed that there was a 2-fold increase in vascular endothelial growth factor α (VEGFα) in EPA-treated inguinal adipocytes (Fig. 1E). VEGFα is a key pro-angiogenic factor and its upregulation is an essential process for thermogenic activation of BAT [27]. Taken together, our data suggest a browning effect of EPA in inguinal adipocytes.

3.2. Effect of EPA on mitochondria biogenesis and oxidation in brown and inguinal adipocytes

To evaluate the effect of EPA on mitochondrial biogenesis and oxidation capacity, mitochondrial DNA (mtDNA) content and mitochondrial oxidation gene expression were determined in brown and inguinal adipocytes. EPA treatment during the adipocyte differentiation process significantly increased mtDNA content in inguinal and brown adipocytes (Fig. 2A). Consistently, the expression of genes involved in mitochondrial biogenesis and oxidation such as PGC1α, Nuclear respiratory factor 1 (Nrf1) and cytochrome c oxidase iv (COXiv) were significantly up-regulated in inguinal adipocytes when EPA was added during 8 day of differentiation (Fig. 2B).

Mitochondrial function is an important factor that determines the fate of glucose metabolism, i.e., through mitochondrial oxidation or glycolysis. Therefore, lactate levels can be indicative of glucose oxidation level and mitochondrial function. As illustrated in Fig. 2C, EPA-treated inguinal adipocytes in the presence of insulin for 24 h had increased glucose uptake by approximately 30% compared to control cells. We also showed that the gene expression of GLUT4, but not GLUT1 was markedly upregulated by EPA treatment (Fig. 2D), suggesting that EPA increases glucose uptake by increasing GLUT4 expression. The glucose concentration in 24 h cultured conditional medium was significantly lower in EPA-treated inguinal and brown adipocytes compared to control cells (Fig. 2E), which consistently reflects increased uptake of glucose by the cells. However, lactate levels in the media were similar between control and EPA-treated inguinal and brown adipocytes (Fig. 2F). This implies that the additional glucose that has been taken-up into the cells is metabolized possibly through the oxidation pathway, supporting increased mitochondrial oxidation by EPA in adipocytes.

To provide additional evidence supporting the enhanced mitochondrial function by EPA, we examined the EPA effect on FA oxidation. As shown in Fig. 3A and B, EPA treatment led to the upregulation of lipoprotein lipase (LPL), Cluster of Differentiation 36 (CD36), Carnitine palmitoyltransferase I (CPT1) gene expression, while down-regulation of acetyl-CoA carboxylase-alpha (ACCα) expression. Moreover, EPA treatment increased the phosphorylation of AMPKα at Thr172 residue (Fig. 3C). Together with an increase in PGC1α expression, all of these changes indicate an enhanced FA oxidation in inguinal adipocytes with 8 day treatment of EPA.

3.3. EPA treatment during the differentiation process increases lipid storage capacity in inguinal adipocytes

We next determined the EPA effect on lipid storage capacity in inguinal adipocytes. As shown in Fig. 4A, EPA-treated inguinal adipocytes had significantly larger lipid droplets than control cells, which is consistent with the increased triglyceride content in EPA-treated adipocytes (Fig. 4B). Moreover, the expression of Glycerol-3-Phosphate Acyltransferase 1 (GPAT1) and GPAT3 genes was upregulated by ~80% (Fig. 4C), suggesting an increase in TG synthesis in EPA-treated adipocytes. In addition to TG synthesis, lipolysis determines the TG content and lipid droplet size. Interestingly, EPA treatment reduced glycerol release into medium by 70% (Fig. 4D). This inhibitory effect of EPA on lipolysis was only observed under the basal condition; EPA had no effect on norepinephrine-stimulated lipolysis (data not shown). In agreement

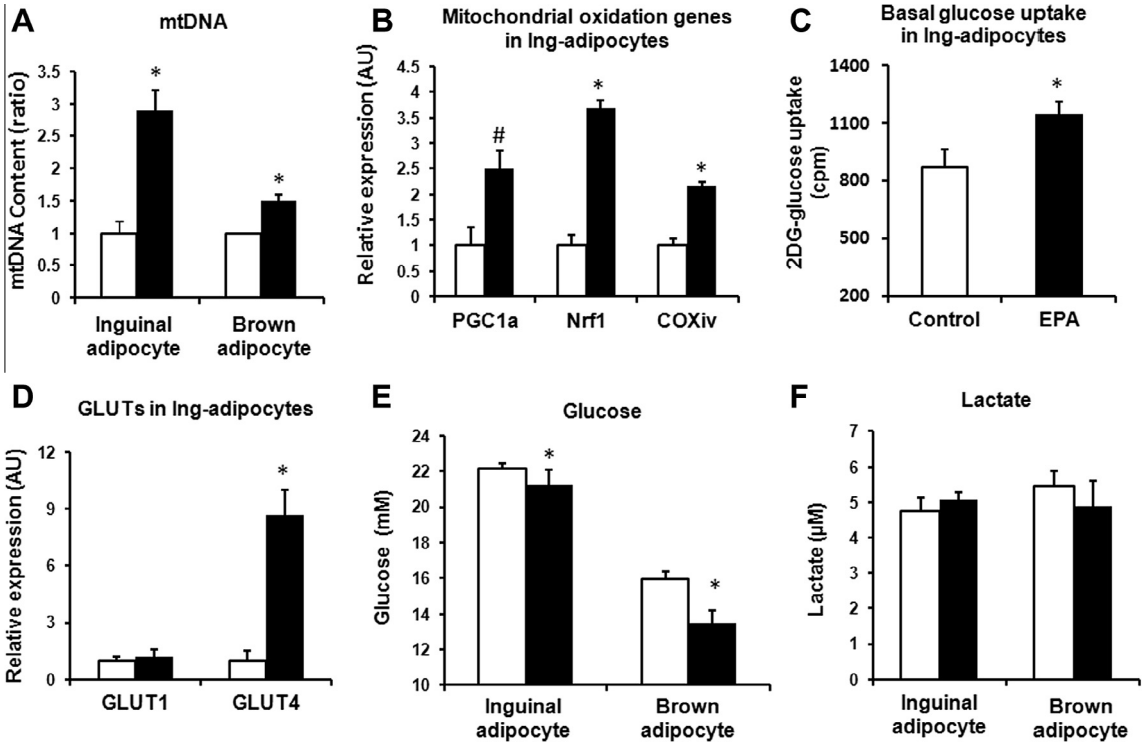

Fig. 2. EPA effect on mitochondria oxidation and glucose metabolism in inguinal and brown adipocytes. Mitochondrial DNA content in inguinal and brown adipocytes EPA treatment (A). Expression of mitochondrial oxidation genes (B), uptake of [^{3}H]-2 deoxy-D-glucose (C), and expression of GLUT1 and GLUT4 genes (D) in inguinal adipocytes with EPA treatment. The levels of glucose (E) and lactate (F) in the culture medium of inguinal and brown adipocytes with EAP treatment. The results represent 2–3 independent experiments with different sets of mice (4–5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. $^*p < 0.05$, $^\#p < 0.1$, EPA vs. control.

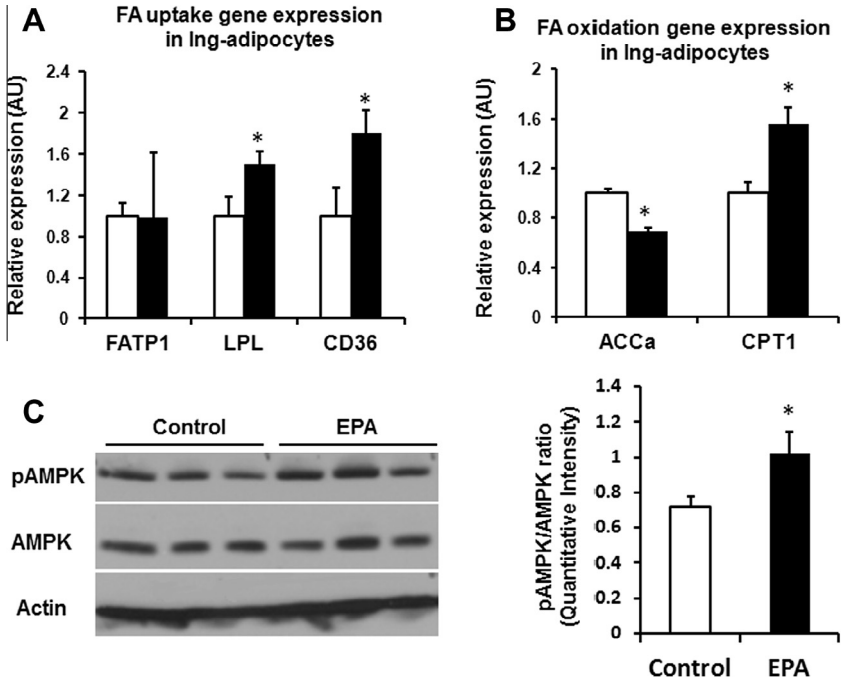

Fig. 3. EPA effect on lipid metabolism in inguinal adipocytes. Expression of genes involved in fatty acids uptake (A) and fatty acids oxidation (B) in inguinal adipocytes with EPA treatment. AMPK phosphorylation in inguinal adipocytes (C). The results represent 2–3 independent experiments with different sets of mice (4–5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. ($n = 3$ per group) $^*p < 0.05$, EPA vs. control.

with decreased glycerol release, EPA significantly inhibited the expression of HSL (hormone sensitive lipase) and ATGL (adipose triglyceride lipase) genes (Fig. 4E). However, EPA had no effect on perilipin1 gene expression (Fig. 4E). These data suggest that EPA promotes lipid storage capacity by increasing TG synthesis and decreasing lipolysis in inguinal adipocytes.

3.4. Palmitate and DHA had no effects on thermogenic gene expression in brown and inguinal adipocytes

Our results have clearly suggested that EPA increases mitochondrial function, FA oxidation, and lipid storage capacity in inguinal adipocytes. In terms of the specificity of EPA beneficial effect, we performed similar experiments using saturated fatty acid such as palmitate. Since chronic (8 day) treatment of palmitate is toxic to cells, we only looked at the effect of palmitate treatment for 24 h in brown adipocytes. Unlike EPA, 24 h treatment of palmitate had no significant effect on the expression of thermogenic genes including UCP1, PRDM16, C/EBPβ, and PGC-1α, while 24 h EPA treatment significantly increased the expression of UCP1, PRDM16, and C/EBPβ in brown adipocytes (Fig. S1A). Furthermore, we conducted a similar experiment to examine the effect of DHA. Different from EPA, treatment of DHA during 8 day of adipocyte differentiation had no effect on thermogenic gene expression (Fig. S1B). DHA significantly inhibited adipocyte differentiation (Fig. S1C); this result is in line with a previous study in 3T3-L1 adipocytes [28].

4. Discussion

Recent studies have demonstrated that brown-like adipocytes can be recruited in white adipose tissue, particularly in subcutaneous fat depot, which holds an against obesity potential [9]. Herein, we sought to investigate the effect of EPA on metabolic functions of subcutaneous adipocytes focusing on thermogenesis and lipid storage properties. We found that EPA treatment during inguinal adipocyte differentiation increases thermogenesis, mitochondrial oxidation, and lipid storage capacity in inguinal adipocytes.

N-3 PUFAs have been reported to increase the expression levels of UCPs in multiple tissues, such as brown adipose tissue, liver, and muscle [29–32]. However, few studies were focused on the browning effect of EPA on SC-WAT depot. In this study, we found EPA treatment increases the gene expression of UCP1-3 in inguinal adipocyte when added during the differentiation process. This, together with the up-regulation of CIDEA, VEGFα and PGC-1α expression, suggests the EPA enhancement of thermogenesis and energy expenditure in SC adipocytes.

Mitochondrial function in white adipocytes determines lipid homeostasis and insulin sensitivity [12]. It directly regulates glucose and fatty acid oxidation, and also impacts other metabolic pathways, such as lipogenesis, lipolysis and FA re-esterification. Hence, we determined the EPA effect on mitochondrial biogenesis and oxidation capacity in adipocytes. Our results showed that EPA increased mtDNA content and the expression of mitochondrial oxidation genes, such as PGC1α, Nrf1 and COXiv. Consistently, changes in ACCα and CPT1 expression, along with increased AMPK phosphorylation, suggest that FA oxidation is enhanced in EPA-treated adipocytes. Our results are in agreement with the previous reports that mice fed a diet rich in DHA and EPA had enhanced FA oxidation and mitochondrial function in adipose tissue [33]. Additionally, our result of glucose metabolism implies that EPA enhances glucose oxidation, which may result from the improved mitochondrial oxidation.

The impairment of lipid storage function of white adipose tissue has been directly associated with the development of insulin resistance. The release of excessive fatty acids into the circulation due to decreased lipid storage capacity could be an important contributor to the ectopic fat accumulation and insulin resistance [11]. On the other hand, an increase of fat accumulation capacity in subcutaneous adipose tissue has been associated with the improvement of insulin sensitivity [34,35]. In this study, we found that EPA promotes lipid storage capacity of inguinal adipocytes by increasing triglyceride synthesis and inhibiting lipolysis, which is consistent with previous reports showing that diet rich in fish oil reduces lipolysis and improves TG storage in adipose tissue [18,19]. Taken together, our results suggest that stimulating thermogenic and oxidative metabolism of subcutaneous adipocytes, as well as

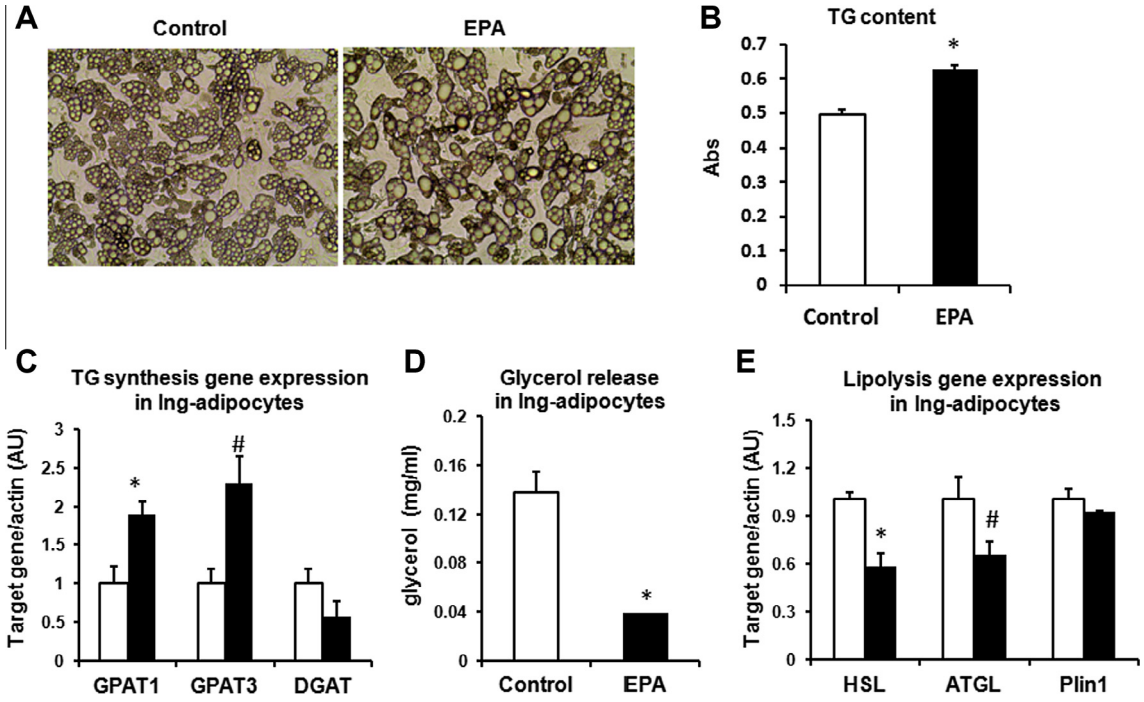

Fig. 4. EPA effect on lipid storage in inguinal adipocytes. Morphology (10× magnification) (A) and TG content (B) of inguinal adipocytes with EPA treatment. Expression of genes involved in triglyceride synthesis (C) in inguinal adipocytes with EPA treatment. The levels of glycerol in the culture medium of inguinal adipocytes with EAP treatment (D). Expression of genes involved in lipolysis (E) in inguinal adipocytes with EPA treatment. The results represent 2–3 independent experiments with different sets of mice (4–5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. $^{*}p < 0.05$, $^{\#}p < 0.1$, EPA vs. control.

increasing their lipid storage capacity, are some of the beneficial metabolic effects of EPA.

It seems contradictory that EPA inhibits lipolysis and increases FA oxidation simultaneously in the same adipocyte. However, we hypothesize that EPA may affect lipid storage and thermogenesis in two different types of adipocyte, i.e., white adipocyte and brown-like adipocyte (brite adipocyte), respectively in subcutaneous SV cell cultures. On the one hand, EPA increases thermogenic capacity by increasing the recruitment of brite adipocytes, and our data supports this notion. For instance, the effect of EPA on thermogenic gene expression was perceived only when EPA was added during SV cell differentiation, but not in fully differentiated inguinal adipocytes treated with EPA for 24 h (data not shown). On the other hand, EPA promotes lipid storage in white adipocytes by inhibiting lipolysis and increasing TG synthesis. Further investigations are needed to test this hypothesis.

In summary, our study provides the evidence on the beneficial metabolic effect of EPA in inguinal adipocytes. Our results show that EPA induces mitochondria biogenesis, increases glucose uptake, and promotes oxidation of FAs and glucose. On the other hand, EPA enhances fatty acid uptake, lipid storage, but inhibits lipolysis, leading to decreased FA release. All of these changes together could be the mechanism by which EPA reduces inflammation and inhibits ectopic fat accumulation, thereby preventing insulin resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.010.

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