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Eicosapentaenoic Acid Reduces Adiposity, Glucose Intolerance and Increases Oxygen Consumption Independently of Uncoupling Protein 1

Mandana Pahlavani¹, Latha Ramalingam¹, Emily K. Miller¹, Shane Scoggin¹, Kalhara R. Menikdiwela¹, Nishan S. Kalupahana^{1,2}, William T. Festuccia³, and Naima Moustaid-Moussa¹

¹Department of Nutritional Sciences, and Obesity Research Cluster, Texas Tech University, Lubbock, TX.

²Department of Physiology, Faculty of Medicine, University of Peradeniya, Sri Lanka

³Department of Physiology & Biophysics, Institute of Biomedical Sciences, University of São Paulo, Brazil

Abstract

Scope: Brown adipose tissue (BAT) dissipates energy through uncoupling protein 1 (UCP1) and has been proposed as an anti-obesity target. We previously reported that high fat (HF) diet enriched in eicosapentaenoic acid (EPA) significantly increased UCP1 and other thermogenic markers in BAT. We hypothesized that these effects are mediated through UCP1-dependent regulation.

Methods and Results: Wild type (WT) and UCP1 knockout (KO) B6 male mice were housed at thermoneutrality and fed HF, without or with EPA-enriched fish oil. HF-fed KO mice were heavier and had higher BAT lipid content than other groups. Protective effects of EPA in WT, previously observed at 22°C (reduced adiposity, improved glucose tolerance, and increased UCP1), disappeared at thermoneutrality. Mitochondrial proteins, COX I, II and IV were reduced in the KO mice compared to WT. Unexpectedly, EPA attenuated weight and fat mass gain, improved glucose tolerance in the KO mice. Finally, EPA increased BAT PGC1 α protein and gene expression, and whole-body oxygen consumption in KO mice, consistent with increased mtDNA/nucDNA ratio.

Corresponding author: Naima Moustaid-Moussa, Ph.D., FTOS, FAHA Professor, Nutritional Sciences & Director, Obesity Research Cluster Texas Tech University, 1301 Akron Street. Lubbock, TX 79409 Tel (O): 806-834-7946 naima.moustaid-moussa@ttu.edu. Author contributions

The authors' responsibilities were as follows N.M.M. designed the study in collaboration with NSK and LR; M. P. conducted most experiments in this paper, assisted by E.K.M, L.R; and S.S. Authors M. P; N.M.M. L.R. K.M., and N.S.K. were involved in data analyses and interpretation; WF made significant intellectual contributions and discussions of this work, and critically edited the manuscript. M. P. drafted the first version of this paper, which was first revised and edited by N.M.M, then all coauthors edited and approved the final version; N.M.M provided the facilities and materials for this research and has primary responsibility for this research and final content of the manuscript.

Conflict of interest

There is no conflict of interest for this study.

Conclusions: EPA rescued the weight gain and glucose intolerance in UCP1 KO mice at thermoneutrality, independent of UCP1; these effects of may be mediated in part via increased oxygen consumption and BAT PGC1 α .

Graphical abstract

The goal of this study is to determine whether protective effects of EPA in obesity and brown adipose tissue thermogenesis is mediated in part through UCP1.

Wild type (WT) and UCP1 knockout (KO) B6 male mice were housed at thermoneutral conditions (28–30°C) and fed a HF, either without or with EPA-enriched fish oil for 14 weeks. Metabolic phenotyping was conducted during this time, and blood and brown adipose tissue were collected at the end of the feeding period and analyzed. BAT exhibited white adipose tissue-like morphology with greater lipid content in the KO compared to the WT. EPA reduced lipid content in BAT in both the WT and KO with more pronounced effects in the WT. Mitochondrial proteins, COX I, II and IV were all reduced in the KO mice compared to WT. EPA increased PGC1 α gene, protein in BAT, and whole-body oxygen consumption in the KO mice. Overall, EPA rescued the excessive weight gain and glucose intolerance in UCP1 KO mice (not shown in graphical abstract); and these effects may be mediated in part via increased PGC1 α and oxygen consumption in BAT.

Keywords

Brown adipose tissue; Omega 3 fatty acids; Obesity; Thermoneutrality; Uncoupling protein 1

1. Introduction:

Obesity and its associated metabolic disorders have become a major health problem worldwide [1]. Obesity associated comorbidities include insulin resistance/type II diabetes, dyslipidemia, cardiovascular diseases, and certain types of cancers [2]. During obesity, white adipose tissue (WAT) stores excess energy as triglycerides and is also a major source of inflammation [3]. In contrast to WAT, brown adipose tissue (BAT) is known for its critical

role in energy balance through thermogenesis, an effect which is mediated by uncoupling protein 1 (UCP1) [4]. Two types of UCP1 positive adipocytes exist in humans and rodents: classical brown adipocytes located at the interscapular area and beige adipocytes primarily located in subcutaneous depots of WAT under certain conditions such as cold exposure [5].

BAT thermogenesis is crucial for maintaining body temperature in infants, and is therefore considered more essential in prenatal and infant stages of development [6, 7].

Thermogenesis is mainly mediated by UCP1, a protein located in the inner membrane of BAT mitochondria, through activation of catecholamine-mediated lipolysis or cold stimulation [8]. UCP1 uncouples oxidative phosphorylation from ATP synthesis, dissipating energy as heat. This process protects against hypothermia and obesity [9]. Moreover, UCP1 in BAT also contributes to decreased production of reactive oxygen species (ROS) by mitochondria [10]. This is important in obesity, as there are increased circulating levels of glucose and lipids, steering excessive substrates into metabolic pathways, which in turn leads to increased ROS [11]. Transgenic mice overexpressing UCP1 in skeletal muscle show upregulation of fatty acid oxidation markers indicating anti-obesity potential for UCP1 [12]. Moreover, ectopic UCP1 overexpression in WAT improves insulin sensitivity [13]. Additionally, UCP1 content in differentiated adipogenic cells is significantly higher in cells originating from lean than obese individuals [14].

Cold is one of the most potent activators of BAT and UCP1 activity; and environmental temperature is a critical factor that defines the amount of energy used for thermogenesis [15]. At 22°C (common mouse housing temperature), mice are exposed to chronic thermal stress [15]. To maintain body temperature, they increase their heat production and thereby energy expenditure. Thermoneutrality is the temperature for rodents at which energy expenditure is kept minimal to preserve body temperature. Couple of studies have shown that inactivation of UCP1 induces obesity and increases adiposity especially at thermoneutral (28–30°C) environments [16, 17].

With the identification of functional BAT in adults in 2007 [18, 19], several studies have shown that activation of BAT thermogenesis may be an efficient strategy to combat obesity [6, 20, 21]. Results obtained from 18-FDG-PET imaging indicate that exposing humans to a cooler temperature (15°C) significantly increases BAT activity in lean men compared to obese subjects [22]. Another study showed that UCP1 protein content after cold exposure in overweight subjects was much higher in supraclavicular fat compared to abdominal subcutaneous fat [23]. Furthermore, guanosine diphosphate (GDP) which is an inhibitory ligand of UCP1, represses mitochondrial respiration in BAT but not in WAT [23]. These results confirm the critical role of UCP1 in thermogenesis and existence of a functional BAT in human subclavicular region [24].

Long-chain omega 3 polyunsaturated fatty acids (ω -3 PUFA) such as eicosapentaenoic acid (EPA; 20:5; n-3) and docosahexaenoic acid (DHA; 22:6; n-3) are the main components in fish oil [25]. They have beneficial anti-inflammatory, hypotriglyceridemic, cardioprotective, and in some cases anti-obesity properties [26, 27]. We have previously reported that EPA significantly increased BAT UCP1 protein level as well as expression of other master regulators of thermogenesis such as cell death-inducing DFFA-like effector (Cidea),

peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Pgc1 α) and PR domain containing 16 (Prdm16) [28]. However, the precise mechanisms by which these dietary fatty acids induced these effects remains unknown. We hypothesized that these beneficial metabolic effects of EPA could be mediated through UCP1-induced thermogenesis.

Our main objective here was to investigate the involvement of UCP1 for the beneficial actions of EPA in glucose and energy homeostasis in mice maintained at a thermoneutral conditions. We demonstrated that (1) our previously reported beneficial effects of EPA in wild type (WT) mice, at 22°C, were attenuated at thermoneutral temperatures; and (2) UCP1 inactivation increased body weight, adiposity and glucose intolerance at thermoneutral temperatures, which was rescued by EPA supplementation.

2. Material and Methods:

2.1. Animal studies:

2.1.1. Genotyping—Heterozygous (HET) UCP1 C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME). Trio breeding was performed between HETs in our animal facility to produce homozygous UCP1 KO and WT littermates. To confirm the animal genotypes (WT, KO, or HET), we used WT and UCP1 mutant primers: Sequences for WT forward and reverse primers were TCGTCATCAATAAGGGGAAAC and CTTCTTCCCTGATGCTCCAT; respectively; and for KO genotypes, GGTGTTTGGAGCCTGCATTGC and CTT CCTGACTAGGGGAGGAGT respectively. Briefly, alkaline lysis reagent (Sigma-Aldrich, Louis, MO) was added to mice tails, followed by the neutralizing reagent to isolate DNA, which was subsequently used for PCR. Agarose gel electrophoresis was run to determine corresponding genotypes. WT and KO genotypes had only one specific band, while HET showed two bands (Figure 1).

2.1.2. Diets and study design—For experimental studies, WT and KO male mice at ~5–6 weeks were randomized into different groups (10–12 mice per group) and housed at thermoneutral temperature (28–30°C). Mice were either fed a HF diet (45%, 20%, and 35% of energy from fat, protein, and carbohydrate, respectively) or a HF diet supplemented with 36g/kg of AlaskOmega EPA-enriched fish oil (800mg/g), kindly provided by Organic Technologies (Coshocton, OH) for up to 14 weeks. Diet composition is provided in supplementary table 1. Animals were housed in individual cages with 12-hours light/dark cycle with free access to food and water. Food was replaced two to three times each week to avoid rancidity, and food intake was measured by assessing amount of food consumed. Glucose tolerance test (GTT), insulin tolerance test (ITT), body composition, and energy expenditure were performed during the feeding period, as described below. At the end of the 14 weeks, all animals were euthanized after a 5-hour fast using CO₂ inhalation method. Interscapular BAT, located on the dorsal side, between the shoulder blades, as well as other tissues, were collected, snap-frozen in liquid nitrogen and stored at –80°C until further analyses. BAT and epididymal adipose tissue (fat pads) were weighed and serum prepared from collected blood. All animal protocols were approved by the Institutional Animal Care and Use Committee of Texas Tech University (TTU-16011–04).

2.1.3. Glucose and Insulin Tolerance Tests—Mice were fasted for 5 hours for GTT, previously established as an appropriate duration of fasting in rodents for insulin resistance studies [29]. Blood glucose levels were measured at 0, 30, 60, 90, and 120 min after intraperitoneal (i.p.) injection of glucose (2 g/kg body weight), using OneTouch Ultra Glucose Meter (AlphaTrack, North Chicago, IL). After 12 weeks of dietary intervention, we conducted ITT, following 5 hours fasting: mice were injected with 1 U/kg insulin (Humulin, Indianapolis, IN). Blood glucose levels were then measured at 0, 15, 30, 45, 60, and 90 mins. Area under the curve was calculated using trapezoidal method.

2.1.4. Body composition—Body fat and lean mass percentage determination was performed using Echo-MRI instrument (EchoMRI LLC, Houston, TX) during the 11th week of dietary intervention.

2.1.5. Energy expenditure—Using a metabolic monitoring system (Omnitech, Columbus, OH), total energy expenditure was measured during 12:12 hours light and dark cycles by monitoring oxygen consumption and carbon dioxide production over 48 hours. Oxygen consumption was calculated in absolute terms relative to body weight.

2.1.6. RNA isolation and quantitative real-time PCR—BAT was homogenized in QIAzol solution using tissue homogenizer (TissueLyser LT, Qiagen, Valencia, CA). Total RNA was isolated using RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using the Maxima kit (Thermo Scientific, Grand Island, NY). Gene expression was performed by quantitative polymerase chain reaction (qPCR). Data were normalized to the housekeeping gene TATA binding protein (TBP), (Sigma-Aldrich, St Louis, MO). Ct method was used to determine mRNA expression and fold changes. Primers used were designed using Oligoarchitect™ Online (Supplementary table 2), and purchased from Sigma-Aldrich (St Louis, MO). All primers were optimized prior to gene expression.

2.1.7. Immunoblotting—Proteins were extracted from BAT by lysing in modified RIPA buffer, as previously described [28]. Proteins were subjected to electrophoresis on gradient gels (Bio-Rad, Hercules, CA), then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated in blocking buffer for 1 hour at room temperature, followed by primary antibodies for UCP1 (Thermo Fisher Scientific, Rockford, IL) (1:1000), PGC1α (Abcam, Cambridge, MA) (1:1000), and total OXPHOS cocktail (Abcam, Cambridge, MA) (1:250). Alpha-Tubulin (1:1000), and GAPDH (Cell Signaling, Danvers, MA) (1:1000) were used as the housekeeping control. Next, membranes were incubated in secondary antibodies, rabbit polyclonal (1:20000) and mouse polyclonal (1:20000) respectively and blots were developed using Li-COR Imager System (Lincoln, NE).

2.1.8. Triglyceride assay—Triglycerides colorimetric assay kit (Cayman, Ann Arbor, MI) was used to determine the triglyceride content in BAT protein lysates.

2.1.9. Mitochondria mass quantification (mitochondria DNA)—DNA was isolated from BAT using gene Mammalian DNA Kit (Sigma-Aldrich, Louis, MO). Cytochrome b (Cytb), mitochondrially encoded NADH: ubiquinone oxidoreductase core subunit 1 (Nd1), and Nd2 were used as the mtDNA primers. Pparg and Ucp1 loci were used

as the genomic DNA primers. The mtDNA/nucDNA ratio was calculated individually for each primer pair using $2^{-(CT_{nucDNA}-CT_{mtDNA})}$.

2.1.10. Histological assessments—BAT was fixed in z-fix (Anatech Ltd, Battle Creek, MI), transferred to 70% ethanol, paraffin embedded at 5 μ m, and stained with hematoxylin and eosin (H&E) to detect morphology changes. Immunofluorescence was performed with UCP1 (Thermo Fisher Scientific, Rockford, IL) and COX IV (Abcam, Cambridge, MA) in BAT sections and visualized using EVOS XL Microscope (Thermo Fisher Scientific, Rockford, IL) at 20X and 10X magnification for H&E and immunofluorescence respectively.

2.1.11. Statistical analysis—All data are presented as mean $\pm$ SEM. For multiple comparisons, one-way ANOVA was performed, followed by Tukey's post-hoc test ($p < 0.05$). Two-way ANOVA was used to measure effects of diet, genotype, and genotype X diet interaction. All statistical tests were performed using GraphPad Prism software.

3. Results

3.1. Effect of EPA and UCP1 deficiency on food intake and weight gain

Feldmann et al. have shown that UCP1 KO mice fed a HF diet (45% Kcal fat) develop obesity at thermoneutral conditions (28°–30° C) [17]. Moreover, we have previously shown that EPA reduces obesity and increases BAT UCP1 levels in WT mice kept at 22°C [26, 28]. Thus, we tested whether these EPA effects were reproduced in mice kept at thermoneutrality and whether they were dependent on UCP1. Accordingly, we compared the effects of EPA-enriched HF diet on WT and UCP1 KO male mice, all placed at thermoneutrality, for its role in BAT function. Food intake was comparable between HF and EPA diets within each genotype and throughout the feeding period (Figure 2.A.). However, KO-HF group gained significantly more body weight compared to all other groups (Figure 2.B.).

3.2. Effects of EPA and UCP1 deficiency on glucose homeostasis

In order to determine the effect of UCP1 deficiency and EPA on glucose homeostasis, we performed GTT and ITT. Glucose intolerance was significantly higher in the KO-HF group compared to the WT-HF ($p < 0.05$) (Figure 3.A.). In WT groups, no difference in glucose tolerance was observed between the EPA and HF fed mice (Figure 3.A. and B.). However, in the KO mice, EPA significantly lowered glucose intolerance ($P < 0.05$) compared to KO mice fed HF (Figure 3.A.). In contrast to the findings of the GTT, there were no differences in ITT between WT and KO mice fed HF. Moreover, EPA did not alter ITT in WT mice (figure 3.C.). However, KO mice fed EPA displayed a significant reduction in glucose levels compared to KO mice fed HF diet ($P < 0.05$), confirming that EPA attenuates glucose intolerance and insulin resistance in UCP1 KO mice (Figure 3.D.).

3.3. Effects of EPA and UCP1 deficiency on body and fat pad weights and body fat percentage

When comparing final body weights at the end of the study, KO-HF had the highest body weight among all 4 groups ($P < 0.05$) (Figure 4.A). There was no effect of EPA on final body

weights in the WT group (Figure 4.A). More importantly, KO-EPA mice displayed significantly lower body weight compared to KO-HF mice ($P<0.05$). Epididymal fat pad and fat percentage were not different between WT mice fed HF or the EPA diet, however, KO mice fed EPA had significantly lower values in both these variables compared to KO mice fed HF diet (Figure 4.B. and C.). No difference was observed in lean mass percentage among all four groups (Figure 4.D.) Taken together, this suggests that EPA prevents weight gain and excessive adiposity in UCP1 KO mice. However, no interaction between genotype and diet was observed (Table 1).

3.4. Ablation of UCP1 causes whitening of brown adipose tissue

Since UCP1 is the master thermogenic protein in BAT, we examined the effect of UCP1 deficiency and EPA in thermoneutral environments on interscapular BAT weight and morphology. Histology images showed higher lipid deposition in the BAT of KO mice (figure 5.A.) which was confirmed by the significant ($p<0.05$) higher triglyceride levels in the KO mice compared to WT group (figure 5.B.). Overall, BAT mass was significantly higher in KO mice when compared to WT ($P<0.05$) (figure 5.C.) EPA did not affect BAT mass in the WT genotype. However, in KO mice, EPA significantly reduced BAT mass ($P<0.05$) compared to the HF diet. A significant interaction was observed between genotype and diet, for interscapular BAT weight (Table 1).

3.5. Effects of EPA and UCP1 deficiency on thermogenic markers

We previously reported that EPA increased UCP1 and other thermogenic markers such as *Prdm16* and *Pgc1 α* at 22°C [28]. In this study we examined effects of EPA and UCP1 deficiency on BAT mRNA levels of thermogenic markers. In the KO genotype, *Pgc1 α* mRNA expression was significantly higher in the EPA group. *Sirt1* showed significant higher expression in the WT-EPA group compared to the other three groups. Moreover, as expected, *Ucp1* mRNA was undetectable in the KO but was highly expressed in the WT, while *Ucp2* mRNA was expressed more in the KO compared to WT (Figure 6. A.–D). We also measured gene expression of other thermogenic markers such as *Cidea* and *Prdm16* and found no differences across groups (Supplementary Figure 1.A. and B.). Additionally, we measured UCP1-independent genes such as *klotho beta (Klb)* and sarcoplasmic/endoplasmic reticulum calcium ATPase 2b (*Serca2b*) and observed no differences. (Supplementary Figure 1.C. and D.). Furthermore, several mitochondria biogenesis markers were measured beside *Pgc1 α* such as *Pgc1 β* , *Sirt3*, receptor interacting protein 140 (*Rip140*), mitogen-activated protein kinase (*Mapk*), transcription factor A (*Tfam*), 5' AMP-activated protein kinase (*Ampk*), nuclear respiratory factor 1 (*Nfr1*), and *Nfr2*. No significant differences were observed across groups in these genes (Supplementary Figure 2.). It was recently shown that ablation of UCP1 in cold temperature reduced calcium buffering and calcium overload in mice [30]. Moreover, UCP1-independent thermogenic mechanisms which involve enhanced ATP-dependent calcium cycling have been discovered recently [31]. Thus, we measured the expression of several genes involved in calcium cycling. Transient receptor potential vanilloid 2 (*Trpv2*), a calcium cycling gene, was expressed higher in KO mice fed the HF diet when compared to WT mice fed HF, with no effect of EPA in both WT and KO genotypes (Figure 6.E.). We also measured other calcium cycling genes such as *sarcophilin*,

and ryanodine receptor 1 (Ryr), but the expression was lower than the detection limit (data not shown).

To confirm the changes identified in gene expression, we examined protein levels of UCP1 and PGC1 α . There was no difference in UCP1 levels between HF and EPA in the WT group, (Figure 7.A. and B.), and as expected, UCP1 was undetectable in both KO groups. PGC1 α protein levels were comparable between WT-HF and KO-HF (Figure 7.A. and C.). However, in the KO genotype, EPA significantly increased ($P<0.05$) PGC1 α at protein level compared to the HF diet.

3.6. Loss of UCP1 decreased oxygen consumption, which was rescued by EPA feeding

Omega 3 fatty acids have previously been reported to increase energy expenditure and oxygen consumption at 22°C in mice [32]. In our studies, oxygen consumption was significantly lower in the KO-HF compared to WT-HF mice ($P<0.05$). No effect of EPA was observed in the WT mice; however, EPA significantly increased oxygen consumption in the KO mice. These data demonstrate that at thermoneutrality, EPA did not increase oxygen consumption in the WT mice but increased it in the KO mice, demonstrating that effects of EPA may be independent of UCP1 (Figure 8.A.).

As BAT has tremendous amounts of mitochondria, we determined overall mitochondrial health based on mitochondrial/nucleic DNA ratio. WT mice fed EPA had no differences in mtDNA/nucDNA ratio compared to WT mice fed HF. However, KO mice fed EPA showed significant increase in mtDNA/nucDNA ratio compared to KO-HF group and levels were comparable to WT groups (Figure 8.B). Significant effect of genotype and diet interaction was observed for oxygen consumption, as KO genotype in both diets displayed significantly decreased oxygen consumption (Table 1).

3.7. Effect of EPA and UCP1 ablation on mitochondria markers in thermoneutral environment

The respiratory chain complexes (component I, II, III, IV, and V) were measured at thermoneutrality (Figure 9. A.–F). In both groups of KO genotype (HF and EPA), COX I and COX IV protein levels were significantly decreased compared to the WT genotype (Figure 9, B. and E.). For COX II and COX III, only KO-HF group showed significant decreased levels compared to WT mice fed EPA group. COX V levels did not differ between the 4 groups (Figure 9. C. D. and F.). No interaction between genotype and diet was observed (Table 2). We also performed immunofluorescent staining with UCP1 and mitochondrial marker COX IV to examine mitochondrial changes, which showed UCP1 and COX IV positive staining in BAT sections in both WT and KO genotypes with two different diets (HF and EPA) in Fig 10. A. and Fig 10.B.. WT genotypes showed stronger staining of UCP1 and COX IV compared to KO genotypes consistent with higher protein expression in wildtype mice.

4. Discussion

Activation of brown or beige adipocytes has been proposed as a promising approach to combat obesity. This is due to their ability to increase thermogenesis and energy

expenditure, which has been primarily shown in rodents [21]. Recent studies have also confirmed that a metabolically active BAT exists in adult humans [18, 33]. These findings provide new insights into mechanisms that control energy expenditure in humans and have identified novel targets to prevent and treat obesity [34]. With respect to thermogenesis-related energy expenditure, environmental temperature is a crucial factor that determines the amount of energy that is used for thermogenesis [35, 36]. By increasing environmental temperature, especially in winter, as a comfortable state in modern society, the amount of heat that the body dissipates to maintain temperature is minimized [37]. By contrast, lower ambient temperatures (15–16°C) can induce BAT/beige fat activation and enhance non-shivering thermogenesis and reduce weight in both mice and humans [37, 38]. Most studies have used cold exposure (4°C) to activate thermogenesis in BAT/beige fat [35, 39, 40]. However, the metabolic outcomes of thermoneutrality on brown or beige adipose tissue function and obesity are less clear.

UCP1 is the major master regulator of thermogenesis, responsible for cold-induced BAT activation and thermogenesis, and studies have shown that UCP1 deficiency in rodents induces obesity at thermoneutrality [15, 17]. Moreover, we have previously shown that EPA activates BAT at ambient temperature [28]. Whether these effects of EPA are UCP1 dependent have not been explored. Thus, to address these questions, we maintained WT and UCP1 KO mice at thermoneutrality and fed them a HF diet with or without EPA, to determine whether EPA-induced BAT activation requires UCP1 and whether this occurs at thermoneutrality.

Ablation of UCP1 caused significantly increase in weight gain, higher fat percentage compared to WT mice, despite their comparable food intake, indicating UCP1 KO mice to be energy efficient and have reduced energy expenditure, which is expected to be in part affected by the thermoneutral environment. Winn *et al.* have previously reported that UCP1 KO mice gained more weight compared to WT mice when fed western diets (45% kcal fat) or control diet, (10% kcal fat) consistent with our results, but those studies were performed at 25°C [41]. Therefore, it seems that loss of UCP1 causes weight gain regardless of environmental temperature. Moreover, a negative association between thermoneutrality and the thermogenic program was reported [15, 17]. Our findings demonstrate that UCP1 deficiency exacerbated the effects of HF diet at thermoneutrality, as KO mice gained more weight and adiposity than WT mice fed HF. Consistent with our results, UCP1 deficient mice fed HF diet had WAT-like unilocular adipocytes appearing in BAT depots at 25°C [41]. On the other hand, BAT in WT mice stained darker with H&E with higher mitochondrial content compared to KO mice [41, 42]. Previous studies have also shown that thermoneutrality increases BAT mass in obesity-resistant strain A/J male mice fed a HF diet (60% kcal fat) giving rise to a pale color, indicating that increased environmental temperature triggers BAT whitening [15]. In line with these studies, several studies have reported increased adiposity in HF-fed mice at thermoneutral conditions, [15, 17, 43], while others did not [44, 45]. In respect to human studies, combination of HF diet with high indoor temperature, often used in developed countries, especially in winter, may be important factors that contribute to obesity [36, 39]. Tibetan subjects living at higher altitude with lower temperature (10–15°C) showed lower BMI, waist circumference, and waist-to-height compared to subjects living in lower altitude with higher temperature (22–33°C) [46]. Our

results showed that UCP1 KO mice fed EPA had significant increase in Pgc1 α expression, a factor important for mitochondrial biogenesis. Other studies have shown that UCP1 deficiency is compensated through increase in levels of other thermogenic genes to protect against energy imbalance [41, 47, 48]. Furthermore, as we reported here, ablation of UCP1 significantly decreased oxygen consumption in other studies, which were conducted at 22–25° suggestive of lower fat oxidation [30, 41, 49, 50].

Oxidative phosphorylation (OXPHOS) enzymes produce the majority of cellular energy in the form of ATP in mitochondria [51]. UCP1 KO mice fed HF diet at thermoneutrality showed a significant decrease in protein levels of COX I, COX II, and COX IV compared to WT in BAT which is consistent with our data [30, 41, 52]. Furthermore, while EPA did not significantly increase COX II protein levels in the KO mice compared to KO fed HF diets, it brought the levels up to that of the WT mice. These findings suggest potential protective effects of EPA on these mitochondria markers in the absence of UCP1. COX V have not shown any difference across the groups which is consistent with other studies [30, 41].

While BAT primary function is thermogenesis, several studies in animals and humans have reported its involvement in regulating glucose homeostasis and insulin sensitivity [53, 54]. BAT content and its activity are inversely associated with insulin resistance and type II diabetes [54]. One interpretation is that lower amount of BAT will lead to reduced glucose uptake, thus increasing the risk for type II diabetes [55]. UCP1 is required for glucose uptake by BAT and its deficiency in the KO mice prevents adrenergic stimulation of glucose utilization [56]. These findings are supported by results from Thoonen *et al.* showing that glucose intolerance in UCP1 KO mice was restored by BAT transplantation [57]. In line with these studies, we demonstrated that KO HF fed mice exhibited impaired glucose clearance compared to WT mice. Given the excessive whitening of BAT in KO mice, this glucose intolerance may be mediated by white fat in the BAT [41].

Ablation of UCP1 causes reduced calcium buffering and is highly sensitive to mitochondria permeability transition by reactive oxygen species (ROS) and calcium overload in mice at cold temperature [30]. As a surrogate for calcium influx, we measured expression of Trpv2 expression. TRP ion channels consist of major class of calcium-permeable channels (TRPV1, TRPV2, TRPV3, TRPV4) [58]. Intake of fish oil activates Trpv1 and UCP1 in BAT and WAT of mice and enhances energy expenditure at ambient temperature [32]. In our current study, we measured the expression of Trpv2, since it is highly expressed in mouse brown adipocytes and involved in thermogenesis. Trpv2 mediates calcium influx by β -adrenergic receptor activation [58]. We observed significant upregulation of Trpv2 in KO-HF group which is in line with the study which show upregulation of Trpv2 expression in BAT from HF diet induced obesity suggesting that Trpv2 induces thermogenesis at ambient temperature [59].

mtDNA number is a fundamental element of overall mitochondrial health and a component of the electron transport chain (ETC), which is critical for oxidative phosphorylation (OXPHOS) [60]. Kazak *et. al.* have shown that mtDNA-encoded transcripts were significantly reduced in BAT of UCP1-KO mice after cold exposure, however, at 30°C, no change was observed between WT and KO genotypes [30]. This data is consistent with our

findings which show no differences in mtDNA observed between two genotypes (WT and KO). However, UCP1 KO mice fed EPA had significant higher levels of mtDNA compared to UCP1 KO mice fed HF, suggesting protective effect of EPA in UCP1 knockout mice. This corroborates with Pgc1 α data, as well as oxygen consumption, which was also upregulated in the UCP1 KO mice fed EPA compared to KO mice fed HF. Nathan et al, have investigated the effect of diets (western and low fat diets) and genotypes (WT and UCP1 KO) and their interaction in mice [41]. They found a significant effect of diet on final body weight, VAT weight and fat percentage and significant effect of diet and genotype interaction on BAT weight and energy expenditure. Also, they found significant effect of genotype only in OXPHOS enzymes [41]. All of these results are consistent with our findings, however, it is worth noting that they used ambient temperature.

In previous studies, EPA and DHA increased UCP levels in tissues such as BAT, liver and muscle [61–63]. Moreover, recently we showed that HF diet enriched in EPA (> 90% purity) significantly increased BAT UCP1 protein as well as expression of several other thermogenic markers in mice maintained at ambient temperature, with no detected browning of subcutaneous fat [28]. Kim *et al*, also reported that mice supplemented with fish oil at 23°C induced UCP1 in both brown and beige adipocytes, however, they used fish oil (EPA 28%, DHA 12% or DHA 25%, EPA 8%) instead of pure EPA [32]. In line with this study, Ghandour *et al*., showed that the thermogenic activity of brown and beige adipocytes is also influenced by the dietary intake of omega 3 fatty acids in mice [64]. However, all of these studies were performed at ambient temperatures ranging from 22 to 25°C, rather than thermoneutral environment (28–30°). used in our study.

In our current study, we demonstrated that at thermoneutral environment, most metabolic effects of EPA were abolished in the WT mice but not in the KO mice. One interpretation is that EPA exerts its protective effects to reduce weight and increase thermogenesis at 22°C, which induces thermal stress in rodents [15]. In contrast, EPA significantly improved glucose homeostasis in KO groups, confirming that the protective effect of EPA against glucose intolerance is independent of UCP1 and environmental temperature. Furthermore, while UCP1 inactivation reduced oxygen consumption compared to WT, EPA significantly rescued this oxygen consumption to the level observed in the WT mice. Given that these EPA protective effects only occurred in KO groups, it is plausible that alternate mechanisms mediating regulation of BAT by EPA in absence of UCP1 may operate, possibly through increasing PGC1 α and UCP2 in adipose or muscle [41, 65].

In some studies, fish oil intake prevented the development of obesity in rodents [66, 67]. It also reduced body fat accumulation in rodents more effectively than other dietary oils [68], through different mechanisms, such as proliferation and differentiation of fat cells [69], and metabolic changes in adipose tissue [64], and small intestines [70]. Bargut et al. have reported that C57BL/6 male mice fed HF diet supplemented with fish oil showed less body mass, triglycerides, CD36 and elevated thermogenic markers such as Pgc1 α and Ucp1 in BAT in ambient temperature, compared to diet without fish oil [61]. Another study showed that fish oil intake elevated oxygen consumption, Ucp1, Pgc1 α , Cpt1b, Cidea, Prdm16, Fgf21 at gene levels in BAT and inguinal WAT in mice to attenuate fat storage, body weight gain and increase energy expenditure at ambient temperature [32].

Previous studies from our lab demonstrated that feeding mice high-fat (HF) diets enriched with EPA, 36 g/kg (same amount as current study), decreased inflammation, adiposity and insulin resistance in mice maintained at 22°C, independent of energy intake [26]. This amount is equivalent to 6.75% of energy intake in diet. In comparison, the intakes of EPA and DHA in the United States are low for omega 3 long chain PUFAs ranging between ~0.1–0.2 g/d [71] and the current recommendations vary from 1 g/d to 4 g/d [72] with the upper levels necessary to reduce hypertriglyceridemia. Higher doses of PUFAs have been used in human studies ranging from 4.2 g/day [73] to 15 g/day as reported in a meta-analysis study [74, 75]. However, in these human studies, the effect of fish oil on obesity and related metabolic conditions such as insulin resistance is inconsistent. Some factors responsible for these inconclusive results include usage of lower doses in human studies compared to animal studies, genetic variabilities in humans compared to animal models and different types of fatty acids and treatment durations, used for clinical research [76, 77].

5. Conclusion

In summary, our study for the first time provided detailed analysis of the metabolic phenotype of the UCP1 KO compared to WT male mice housed in a thermoneutral environment, while fed isocaloric HF or HF-enriched EPA diets. Ablation of UCP1 aggravated HF diet-induced glucose intolerance in UCP1 KO mice. Notably, we found that the beneficial effects of EPA in obesity and insulin resistance are independent of UCP1.

WT-HF mice had comparable body weight, BAT weight, oxygen consumption, glucose tolerance, and thermogenic markers including *Ucp1* and *Pgc1α*, compared to WT-EPA mice. Thus, response of WT mice to EPA was attenuated at thermoneutrality. By contrast, in UCP1 KO mice, EPA significantly decreased body weight, BAT weight, glucose intolerance, while increasing thermogenic markers, such as *Pgc1α*, mtDNA/nucDNA ratio, and oxygen consumption. These effects may reflect compensatory mechanisms that the body developed when UCP1 is absent and demonstrate UCP1-independent protective effects of EPA. Additional cell and molecular studies are necessary to dissect the mechanisms underlying these differential effects of EPA, and compensatory mechanisms of BAT in UCP1 deficient mice, as well as those mechanisms mediating the response of BAT metabolism and function at thermoneutrality. It would be worthwhile to determine the effects of DHA, omega 3 fatty acid lipid mediators vs whole fish oil on thermogenesis at thermoneutral environment and in absence of UCP1; and whether these findings can be translated in clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

2-DG	2-deoxyglucose
Ampk	5' AMP-activated protein kinase
AUC	area under the curve
BAT	brown adipose tissue
Cidea	cell death-inducing DFFA-like effector A
COX	cyclooxygenase
Cytb	cytochrome b
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ETC	electron transport chain
FDG	fluorodeoxyglucose
GDP	guanosine diphosphate
GTT	glucose tolerance test
HET	heterozygote
HF	high-fat
IL	interleukin
ITT	insulin tolerance test
Klb	Klotho Beta
KO	knockout
Mapk	mitogen-activated protein kinase
mtDNA	mitochondria DNA
Nd	mitochondrially encoded
NADH	ubiquinone oxidoreductase core subunit
Nrf	nuclear respiratory factor 1
nucDNA	nuclear DNA
PET	positron emission tomography
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1 alpha
Prdm16	PR domain containing 16

PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
Rip140	receptor interacting protein 140
ROS	reactive oxygen species
Ryr	ryanodine receptor 1
TBP	TATA binding protein
Tfam	transcription factor A, mitochondrial
Trpv2	transient receptor potential vanilloid 2
Serca2b	sarcoplasmic/endoplasmic reticulum calcium ATPase 2b
Sirt1	sirtuin 1
SNS	sympathetic nervous system
UCP	uncoupling protein
VAT	visceral adipose tissue
WAT	white adipose tissue
WT	wild type

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Figure. 1. Genotyping of WT and UCP1 KO mice:
Positive/negative PCR products were measured by gel. Agarose electrophoresis of (A) WT
(B) KO products.

Figure 2. Body weight and food intake in WT and UCP1 KO mice fed a HF diet vs. EPA. A: weekly food intake. B: weekly body weight. Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$; $n = 10-12$ mice per group.

Figure 3. The effect of UCP1 and thermoneutral environment on glucose homeostasis in WT and UCP1 KO mice fed a HF diet vs. EPA.

A: glucose tolerance test. B: Area under the curve of GTT. C: insulin tolerance test D: Area under the curve of ITT. Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$; $n = 10-12$ mice per group.

Figure 4. Final body weight, epididymal fat pad weight, fat percentage, and lean percentage in WT and UCP1 KO mice fed a HF diet vs. EPA.

A: final body weight. B: epididymal fat pad weight. C: fat percentage. D: lean percentage.

Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$; $n = 10-12$ mice per group.

Figure. 5. Histology of interscapular BAT, triglyceride content, and BAT weight in WT and UCP1 KO male mice fed a HF diet vs. EPA.

A: histology images (20X) of interscapular BAT, B: triglyceride content, C: interscapular BAT weight. Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$; n 6–10 mice per group.

Figure 6. Gene expression of thermogenic and calcium cycling markers in interscapular BAT in WT and UCP1 KO mice fed a HF diet vs. EPA. Thermogenic markers:

A: Pgc1 α , B: Sirt1, C: Ucp1, D: Ucp2, E: Trpv2. Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$; $n = 10$ mice per group.

Figure. 7. Interscapular BAT protein content in WT and UCP1 KO mice fed a HF diet vs. EPA.

A: UCP1 and Pgc1 α protein content. B and C: Blots were normalized to α -Tubulin. Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$; $n = 6$ mice per group.

Figure. 8. (A) Oxygen consumption rate, (B) mitochondria DNA (mtDNA) measured as a ratio of mtDNA to nuclear DNA (nucDNA) in WT and UCP1 KO mice fed a HF diet vs. EPA. Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$; $n = 4-6$ mice per group.

Figure 9. Interscapular BAT OXPHOS protein content in WT and UCP1 KO mice, fed a HF diet vs. EPA.

(A) COX I, II, III, IV, V protein content. (B-F) blots were normalized to GAPDH. Data are expressed as mean $\pm$ SEM. WT, wild-type; KO, knock out; HF, high fat. Groups represented with same letter indicate no difference. $p < 0.05$, $n = 6$ mice per group.

Figure. 10. Immunofluorescence analysis (10×) of UCP1 and COX IV expression of interscapular BAT in WT and UCP1 KO mice sections, fed a HF diet vs. EPA in thermoneutral environment. WT, wild-type; KO, knock out; HF, high fat, n=3.

Table 1.
Effects of diet, genotype, and interaction on body weight and metabolic characteristics in male mice in thermoneutral environment.

WT, wild-type; KO, knock out; HF, high fat. Significant P values (0.05); n 5–10 mice per group.

Variable	P-value		
	Geno (WT&KO)	Diet (HF&EPA)	Geno X Diet
Final body weight (g)	0.0001	0.0005	NS
Epididymal fat pad (g)	NS	0.0002	NS
Fat percentage	0.0001	0.0001	NS
Interscapular BAT weight (g)	0.0001	0.0002	0.01
GTT (area under the curve) (AU)	0.002	0.0009	NS
Insulin (pg/ml)	0.0006	0.001	NS
Oxygen consumption (Vo2/ml kg-1- H-1)	0.0001	0.0001	0.0007
mtDNA/nucDNA	NS	0.03	NS

Table 2.
Effects of diet, genotype, and interaction on gene and protein expression in male mice in thermoneutral environment.

WT, wild-type; KO, knock out; HF, high fat. Significant P values (0.05); n 5–10 mice per group.

Variable	P-value		
	Geno (WT&KO)	Diet (HF&EPA)	Geno X Diet
Pgc1α (mRNA)	NS	0.001	NS
Ucp1 (mRNA)	0.0001	NS	NS
Ucp2 (mRNA)	0.0001	NS	NS
Sirt1 (mRNA)	0.0001	0.01	NS
Fgfr1 (mRNA)	0.0001	0.04	NS
Trpv2 (mRNA)	0.005	NS	NS
PGC1α (protein)	NS	0.01	NS
UCP1 (protein)	0.0001	NS	NS
COX I (protein)	0.0003	NS	NS
COX II (protein)	0.008	NS	NS
COX III (protein)	0.002	NS	NS
COX IV (Protein)	0.0001	NS	NS