

**Effects of N-3 PUFA-Derived Epoxides Combined with
sEH Inhibition on Brown Adipose Tissue and Brown
Adipocytes in Obesity**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
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**Yang Yang
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Abstract

Brown adipose tissue (BAT) has become a promising target for obesity treatment and prevention. However, effective dietary factors to promote BAT mass and function have not been identified. 17,18-epoxyeicosatetraenoic acid (17,18-EEQ) and 19,20-epoxydocosapentaenoic acid (19,20-EDP) are two prominent epoxy fatty acids (EpFAs) produced from n-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively. EpFAs are quickly metabolized to less active diols by soluble epoxide hydrolase (sEH). In this dissertation, the effects of an sEH inhibitor (*t*-TUCB) (which stabilizes EpFAs) alone or combined with n-3 EpFAs on thermogenic activity in the BAT and brown adipocytes in treating/preventing diet-induced obesity are investigated.

Using the mini osmotic pump delivery system, the effects of *t*-TUCB were studied in the obese mice. *t*-TUCB decreased serum triglycerides and increased perilipin protein expression in the BAT. Next, the effects of 19,20-EDP alone or combined with *t*-TUCB were investigated in diet-induced obese mice. 19,20-EDP alone or combined with *t*-TUCB did not improve body weight but improved the cold tolerance in the obese mice. We further investigated the effects of *t*-TUCB alone or combined with 19,20-EDP or 17,18-EEQ on BAT activation in preventing diet-induced obesity in mice. We found that 19,20-EDP or 17,18-EEQ combined with *t*-TUCB decreased the fasting glucose and serum triglycerides, in part through differentially regulating the thermogenic and lipid metabolic protein expression and inflammatory pathways in the iBAT of mice fed a high-fat diet. Finally, we investigated the effects of n-3 epoxides combined with *t*-TUCB on brown adipogenic differentiation and thermogenic capacity in murine brown adipocytes. We found that 19,20-EDP or 17,18-EEQ combined with *t*-TUCB promoted murine brown adipogenesis and mitochondrial respiration and uncoupling *in vitro*, which were accompanied by PPAR γ activation and suppression of NF κ B activation. Moreover, when

combined with *t*-TUCB, 17,18-EEQ and 19,20-EDP differentially promote the thermogenic function of mature brown adipocytes.

In summary, sEH inhibition alone or combined with n-3 EpFAs may be beneficial in obesity-associated metabolic disorders through improving BAT activity by modulating thermogenic and lipid metabolic protein expression in mice. Moreover, 17,18-EEQ is more potent than 19,20-EDP in promoting brown adipocyte activity both *in vitro* and *in vivo*.

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List of Abbreviations

12,12-DiHOME	12,13-dihydroxy-9Z-octadecenoic acid
17,18-EEQ	17,18-epoxyeicosatetraenoic acid
¹⁸ (FTHA) PET/CT	¹⁸ fluoro-6thia-heptadecanoic acid-mediated positron emission tomography/Xray computed tomography (PET/CT)
¹⁸ FDG	¹⁸ Fluoro-deoxyglucose
¹⁸ FDG-PET/CT	¹⁸ Fluoro-deoxyglucose positron emission tomography/Xray computed tomography
19,20-EDP	19,20-epoxydocosapentaenoic acid
AC	Adenylyl cyclase
ACC	Acetyl-coenzyme A carboxylase
acetyl-CoA	Acetyl-coenzyme A
ACS	acyl-CoA synthetase
ALA	Alpha-linolenic acids
AMPK	AMP-activated protein kinase
<i>ApoE/apoE</i>	Apolipoprotein E
ARA	Arachidonic acid
AT	Adipose tissue
ATF2	Activating transcription factor 2
ATGL	Adipocyte triglyceride lipase
AUC	Areas under the curve
BMI	Body mass index
BMP7	Bone morphogenetic protein 7
C/EBP β	CCAAT/enhancer-binding protein-beta
cAMP	Cyclic adenosine monophosphate

<i>Cd36/CD36</i>	Cluster of differentiation 36
ChREBP	Carbohydrate response element-binding protein
Cidea	Cell death-inducing DNA fragmentation factor alpha-like effector A
CLAMS	Comprehensive lab animal monitoring system
CM	Chylomicron
COVID-19	Coronavirus disease 2019
COX	Cyclooxygenase
COX1	Cytochrome c oxidase subunit 1
CPT1	Carnitine palmitoyltransferase I
CREB	cAMP response element-binding protein
CRF	Corticotropin releasing factor
CT	Computed tomography
CYP450	Cytochrome P-450
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DIT	Diet-induced thermogenesis
DMEM	Dulbecco's modified eagle's medium
DTA	Diphtheria toxin A
EBF2	Early B-cell factor 2
ECAR	Extracellular acidification rate
EET	Epoxyeicosatrienoic acid
EHMT1	Euchromatic histone-lysine N-methyltransferase 1
En1	Homeobox transcription factor gene engrailed
EPA	Eicosapentaenoic acid

EPSTI1	Epithelial stromal interaction 1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
EVA1	Epithelial V-like antigen 1
eWAT	Epididymal white adipose tissue (WAT)
EWSR1	Ewing sarcoma breakpoint region 1
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-ptrifluoromethoxyphenylhydrazone
FGF21	Fibroblast growth factor 21
GP	Guinea pepper
GPD	glycerol 3-phosphate dehydrogenase
GPR120	G-protein coupled surface receptor 120
G _s αS	Alpha subunit of G _s protein
H&E	Hematoxylin-eosin
HDL-c	High-density lipoprotein cholesterol level
HNF-4α	Hepatocyte nuclear factor-4 alpha
HSL	Hormone-sensitive lipase
iBAT	Interscapular BAT
IκBα	Nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB) inhibitor alpha
ISO	isoproterenol
ITT	Insulin tolerance test
iWAT	Inguinal white adipose tissue (WAT)
JNK	c-Jun NH2-terminal kinase

LA	Linoleic acid
LCFA	Long-chain fatty acid species
LDL-c	Low-density lipoprotein (LDL) cholesterol
<i>Ldlr</i> /LDLR	Low-density lipoprotein receptor
LHX8	LIM homeobox eight protein
LOX	Lipoxygenase
LPL	Lipoprotein lipase
LXR	Liver X receptor
MAPK	p38 mitogen-activated protein kinase
MED1	Mediator of RNA polymerase II transcription subunit 1
Mfn1	Mitofusin-1
mGPD	Mitochondrial GDP
mRNA	Messenger ribonucleic acid (RNA)
miRNA	Micro RNA
MPC	Mitochondrial pyruvate transporters
MRI	Magnetic resonance imaging
mtDNA	Mitochondrial deoxyribonucleic acid (DNA)
mtTFA	Mitochondrial transcription factor A
Myf5	Myogenic factor 5
N-3 EpFA	N-3 polyunsaturated fatty acids-derived epoxy fatty acid
NE	Norepinephrine
NEFA	Non-esterified fatty acids
NFE2L2	Nuclear factor erythroid 2-related factor 2
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cell
NIRTRS	Near-infrared time-resolved spectroscopy

NRF	Nuclear respiratory factor
NST	Non-shivering thermogenesis
OCR	Oxygen consumption rate
OGTT	Oral glucose tolerance test
OETF	Otsuka long evans tokushima fatty
ORO	Oil red O
Pax7	Paired box
PDH	Pyruvate dehydrogenase
PDK4	Pyruvate dehydrogenase 4
PEG400	Polyethylene glycol 400
PGC-1 α	Peroxisome proliferator-activated receptor gamma-coactivator- 1 alpha
PI3K α	Phosphoinositide 3-kinase alpha
PIC	Preinitiation complex
PKA	Protein kinase A
PLIN	perilipin
PLTP	Phospholipid transfer protein
PPAR γ	Peroxisome proliferator-activated receptor gamma
PRDM16	PR domain containing 16
PUFA	Polyunsaturated fatty acids
QD-TRL	Fluorescent nanoparticle-labeled triglyceride-enriched lipoproteins
RER	Respiratory exchange ratio
RT	Room temperature
RXR α	Retinoid X receptor alpha

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
sEH	Soluble epoxide hydrolase
SIRT1	Sirtuin 1
SNS	Sympathetic nervous system
SREBP	Sterol regulatory element-binding proteins
sWAT	Subcutaneous WAT
<i>t</i> -AUCB	Trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid
<i>t</i> -TUCB	Trans-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy-benzoic acid
TBX1	T-box 1
TC	Total cholesterol
TG	Triglycerides
TMEM26	Transmembrane protein 26
TR β	Thyroid hormone receptor beta
TRLs	Triglyceride-enriched lipoproteins
TRP	Transient receptor potential
TRPM8	Transient receptor potential (TRP) melastatin 8
TRPV1	Transient receptor potential (TRP) potential cation channel subfamily V member1
TUPS	1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea
UCP1	Uncoupling protein 1
WAT	White adipose tissue
YBX1	Y-box-binding protein 1

ZFP516	Zinc finger protein 516
ZIC-1	Zinc finger transcription factor
β -AR	Beta-adrenergic receptor

Chapter I Introduction

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This chapter has been revised from the published article in the following ways: the material and method, result, and discussion sections were excluded and the introduction sections of these two articles were combined and modified to fit with this dissertation content; font were altered to fit with this dissertation format.

Obesity remains one of the biggest public health challenges worldwide [1]. It is associated with various complications, such as type 2 diabetes, dyslipidemia, and cardiovascular diseases, leading to a shorter lifespan and higher medical cost [2, 3]. Recent studies have indicated that obesity is also associated with increased prevalence and severity of coronavirus disease 2019 (COVID-19), an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [4, 5]. Brown adipose tissue (BAT) has become a promising target to treat or prevent obesity due to its ability to increase energy expenditure through non-shivering thermogenesis [6-9]. Cold exposure has been reported to improve glucose and lipid metabolism in rodents and humans, associated with the cold-induced thermogenesis of BAT [10-12]. Therefore, strategies that activate BAT thermogenic and metabolic activity (i.e., BAT activation) may confer beneficial effects in diet-induced obesity and associated metabolic comorbidities.

N-3 polyunsaturated fatty acid (PUFA)-derived epoxy fatty acids (n-3 EpFAs) are bioactive metabolites generated by cytochrome P-450 (CYP450) epoxygenases from n-3 PUFAs [13, 14]. 17,18-epoxyeicosatetraenoic acid (17,18-EEQ) and 19,20-epoxydocosapentaenoic acid (19,20-EDP) are the major EpFAs derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), respectively. These EpFAs modulate angiogenesis, vascular dilation, inflammation, and cell growth and differentiation and are implicated in tumor growth and metastasis, cardiovascular disease, diabetes, metabolic syndromes, and pain [15-17]. Because these epoxides are unstable and quickly metabolized to less active diols by soluble epoxide hydrolase (sEH), a cytosolic enzyme encoded by the *Ephx2* gene [14], many of the beneficial effects of these EpFAs were potentiated by co-administration of an sEH inhibitor [14, 16, 17].

Recent studies indicate that higher sEH activity was associated with obesity and metabolic diseases in rodents [18]. Further, sEH expression in the subcutaneous white adipose tissue of

obese adults was significantly higher than that of lean subjects [19]. Therefore, sEH ablation or inhibition has become a promising strategy to protect against obesity and associated metabolic disorders by stabilizing endogenous EpFAs. For example, potent sEH inhibitors were reported to improve systemic metabolism in rodents [20-23]. Interestingly, one study reported that an sEH inhibitor decreased the body weight in the high-fat and high-fructose induced obese mice, accompanied by increases in heat production and UCP1 protein expression in the interscapular BAT (iBAT) [22]. Another study found that the iBAT mass in the fat-1 mice was significantly increased by a different sEH inhibitor [23]. Fat-1 mice had the transgenic expression of n-3 desaturase, resulting in the accumulation of endogenous n-3 PUFA and resulting n-3 PUFA-derived EpFAs in the liver and adipose tissue[23]. It is worth noting that in some disease models, sEH inhibitor alone did not show strong beneficial effects unless it was combined with an EpFA [24] or enriched tissue levels of n-3 PUFAs [23].

We have previously demonstrated that a potent sEH inhibitor trans-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy-benzoic acid (*t*-TUCB) promoted brown adipogenesis *in vitro* [25]. However, whether an sEH inhibitor alone or combined with n-3 epoxides promotes brown adipogenesis, enhances iBAT activity, and improves metabolic dysfunction in diet-induced obesity has not been investigated.

In the current dissertation, the effects of sEH inhibitor by *t*-TUCB and 17,18-EEQ or 19,20-EDP alone or in combination on brown adipogenesis and iBAT activation were studied both *in vitro* and *in vivo*. We investigate (1) the effects of *t*-TUCB alone or combined with 19,20-EDP on obesity and associated metabolic parameters in diet-induced obese C57BL/6J mice (Chapter III and IV); (2) the effects of *t*-TUCB alone or combined with 19,20-EDP or 17,18-EEQ in preventing the development of diet-induced obesity and associated metabolic disorders in C57BL/6J mice (Chapter V); (3) the effects of 17,18-EEQ and 19,20-EDP combined with *t*-TUCB on brown adipogenic differentiation and thermogenic capacity in an *in*

vitro cell model (Chapter VI). The schematic diagrams of the study designs are shown in Figure 1.1 (see in the Appendix).

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Appendix

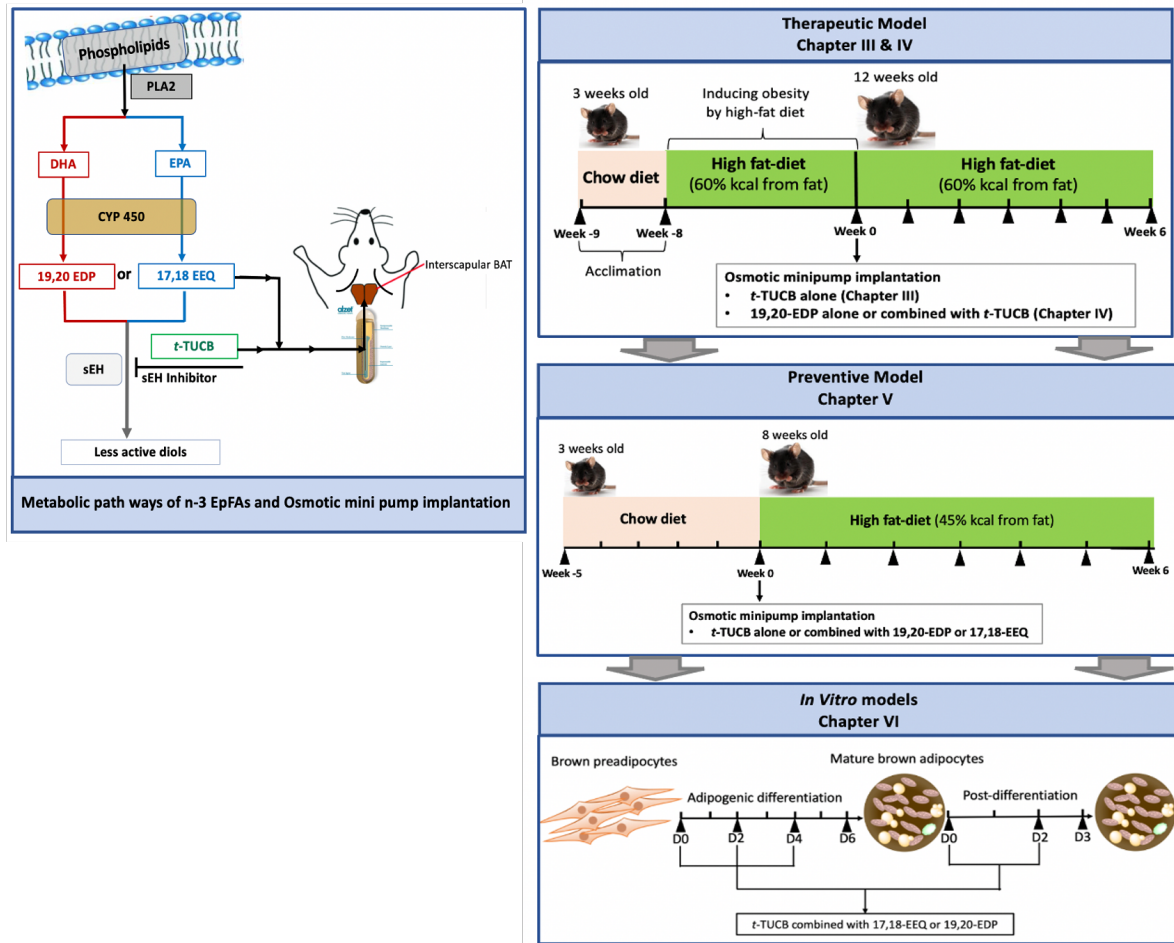


Figure 1.1. Schematic diagrams of the study designs used in the dissertation.

Chapter II Literature Review

2.1 Obesity prevalence and health risks: an overview

Obesity is a worldwide epidemic affecting nearly one-third population of the United States [1, 2]. Based on the most recent data of the National Health and Nutrition Examination Survey, obesity incidence in the adults was 42.4% in the United States in 2017-2018, showing a significantly increasing trend over the past twenty years [3]. Obesity is the major risk factor for multiple chronic disorders, leading to increased morbidity and mortality, responsible for 46 percent increased medical care costs in the United States [4-6].

Obesity is defined by the body mass index (BMI), which is significantly associated with total body fat content [7]. BMI is calculated as weight (kg)/height squared (m^2), and people with higher BMI and larger waist circumference are at increased risk for type 2 diabetes, hypertension, and cardiovascular diseases [7]. In addition, obesity is also associated with higher risks of certain types of cancer, such as breast cancer, colorectal carcinoma, and esophageal adenocarcinoma [8]. Moreover, obesity also affects outcomes of acute infectious diseases, such as coronavirus disease 19 (COVID-19) [9-11]. Obese patients with COVID-19 infection had increased risks for respiratory failure, resulting in higher demand for mechanical ventilation and higher mortality [10, 11].

Establishing effective methods to prevent and manage obesity is thereby critical for human health and well-being. However, many challenges exist in weight loss approaches that focus on changing personal lifestyles [4]. It is not always achievable to lose or maintain body weight by restricting energy intake or a well-balanced diet along with regular physical activity, especially in the aged group [4]. On the other hand, pharmacological or surgical treatments for obesity are often associated with risks of side effects, including hypoglycemia, hypotension, or malnutrition [4].

The causes of obesity are multifaced, including individual behavior, community and social environment, and genetics [4, 12, 13]. However, fundamentally, obesity is characterized by

excess fat accumulation in the body caused by imbalanced energy intake and expenditure [14, 15]. Understanding of adipose tissue holds great potential to prevent or treat obesity as adipose tissue is critical for modulating energy homeostasis [16].

2.2 Brown adipose tissue

2.2.1 Historical Perspective

Adipose tissue (AT) has been recognized as the major depot for energy storage and an endocrine organ that releases adipokines, such as adiponectin and leptin. White adipose tissue (WAT) and brown adipose tissue (BAT) are classified as two primary types of AT according to distinct morphology and function. One of the primary functions of WAT is to manage energy homeostasis by its lipid storage function and through release of free fatty acids (FFAs), mainly from subcutaneous WAT (sWAT), in response to hormonal signals [17]. In contrast, BAT regulates energy homeostasis by dissipating energy as heat through non-shivering thermogenesis (NST) in response to cold exposure or other stimuli.

BAT was first described and identified in the interscapular area of the marmot important for hibernation in the middle of the 16th century [18]. Four centuries later, after World War II, the physiology and biochemistry of BAT were investigated both *in vitro* and *in vivo*, which defined BAT as a thermogenic organ critical for the regulation of body temperature in hibernators and newborns [18, 19]. Thanks to the upsurge of studies of BAT in the 1970s and early 1980s, the more detailed understanding of the molecular and mechanistic basis for the thermogenic capacity of BAT was proposed and confirmed [19, 20]. *In situ* methods that assess tissue activity in the experimental animals revealed that BAT is primarily responsible for non-shivering thermogenesis in cold-adapted conditions [19]. Moreover, the identification of uncoupling protein 1 (UCP1) in the mitochondria of brown adipocytes uncovered the unique mechanism of BAT to increase energy expenditure [20]. Further, the involvement of BAT in diet-induced thermogenesis was proposed, suggesting the significance of BAT in multiple

types of metabolic inefficiency, such as obesity, at least in rodents [21]. Since thermogenic defect is recognized as an important cause for human obesity in some theories, BAT has become a potential target for human obesity [22]. Such a hypothesis is based on the findings that adult humans contain metabolically active BAT, similar to what had been observed in experimental small mammals.

It has long been believed that BAT is only functionally significant for newborn infants, evidenced by the requirement of an incubator for premature infants to survive due to poorly developed BAT [23]. The size and function of BAT are thought to drastically decrease with age as the physiological and environmental acclimation, such as the decreased surface-to-volume ratio and constant room temperature [20]. But the possibility that adult humans could maintain or recruit the thermogenic ability that occurred in infancy cannot be entirely excluded, even though the early evidence was controversial [23, 24]. In 2009, several studies successfully used scanning techniques in nuclear medicine, ¹⁸fluoro-deoxyglucose positron emission tomography/X-ray computed tomography (¹⁸FDG-PET/CT), to visualize BAT *in vivo* and demonstrated that human adults present a certain amount of BAT that could be activated by cold exposure [25-27]. In addition, these studies revealed that BAT activity was negatively correlated with BMI and body fat percentage, age, outdoor temperature, and the season [25-27]. Later, histological features and messenger ribonucleic acid (mRNA) and protein expression profiles of the speculated BAT in human adults through PET/CT scan were analyzed, which further confirmed the presence of functional BAT in adult humans [26, 28]. Since the rediscovery of metabolically active BAT in adult humans, BAT and its potential regulators have become a research focus in recent 12 years with extensive animal and epidemiological studies [29, 30]. Several pharmacological or dietary compounds have been reported to activate BAT to improve metabolic health in combating obesity and other metabolic disorders [31-33].

2.2.2 Anatomic and cellular identities

In rodents, BAT primarily exists in the interscapular area during fetal life and be present throughout life [34]. It also exists in the deep anterior cervical, subscapular, axillary, periaortic, and perirenal areas in rodents [35] (Fig. 2.1A see in the Appendix). In human infants, iBAT accounts for the largest depot of the total BAT, while axillary-deep cervical and perirenal area present only a small portion of the total BAT [24] (Fig. 1.1B). Recently, with PET/CT imaging techniques, BAT activities were detected in adult humans, mainly in the upper trunk, including cervical, supraclavicular, axillary, paravertebral, and pericardial area [25-27] (Fig. 1.1C).

As the major cell population in BAT depots, brown adipocytes are characterized by the distinct morphology compared to white adipocytes (Fig. 2.2) [36]. Brown adipocytes present multilocular cytoplasmic lipids and a higher density of UCP1-positive mitochondria, responsible for uncoupling adenosine triphosphate (ATP) synthesis from oxidative phosphorylation [36]. Although both BAT and WAT are associated with vascular and nerve networks, BAT is more densely vascularized and innervated than white fat depots [35]. The rich vascularity of the BAT and the high mitochondrial density in brown adipocytes are mainly responsible for the brown color of BAT seen even by gross inspection [35].

In recent years, discrete clusters of UCP1-expressing adipocytes were found to develop in anatomical white fat depots presenting similar thermogenic capacity and phenotype as brown adipocytes in response to thermogenic stimuli, such as cold and beta-adrenergic agonists [37]. Although it remains debatable whether these UCP1-positive adipocytes come from the *de novo* differentiation of precursors or stem cells or the trans-differentiation of pre-existing white adipocytes, these UCP1-positive adipocytes did not share identical genetical and functional signatures with brown adipocytes in rodents [37]. For example, the zinc finger transcription factor (ZIC-1), epithelial V-like antigen 1 (EVA1), LIM homeobox eight protein (LHX8), pyruvate dehydrogenase 4 (PDK4), and epithelial stromal interaction one (EPSTI1) are highly

expressed in brown adipocytes [38-40], whereas CD137, T-box 1 (TBX1), transmembrane protein 26 (TMEM26), and short stature homeobox 2 (SHOX2) are highly expressed in UCP1 positive adipocytes from WAT [40-42]. Moreover, UCP1 positive adipocytes expressed a significant lower level of UCP1 and did not show thermogenic capacity unless activated by certain stimuli, such as cold and beta-adrenergic agonists [40]. Further, additional UCP1 independent thermogenic processes were found in mouse and human beige adipocytes under cold or beta-adrenergic stimulation, affecting systemic energy homeostasis, such as the creatine cycle [43, 44]. Therefore, at least clearly applicable in rodents, brown adipocytes reside in anatomically defined BAT depots are defined as ‘classical’ brown adipocytes, whereas the UCP1-expressing adipocytes found in WAT are named as ‘beige’ or ‘brite’ cells, based on the distinct functional features and gene expression patterns (Fig. 2.2).

However, characterizations of the BAT at the upper trunk anatomical areas in humans based on gene expression profiles are mixed. Several studies reported a typical beige adipocytes expression pattern, but not a classical brown adipocyte expression pattern, in the supraclavicular BAT in neonates, children, and adult humans [40, 45]. Moreover, another study reported that human fetuses presented both classical brown adipocytes and beige cells in the different anatomical regions [42]. Furthermore, the deep cervical and supraclavicular fat of adults have been reported to contain adipocytes with identical gene expression patterns to those of classical BAT [41, 46]. Overall, it seems that there is heterogeneity in brown fat composition in humans, which may result from genetic differences between donors or different human brown adipocyte precursors [47]. Since the iBAT in mice and the brown adipocytes within the iBAT of mice are the major targets investigated in the current dissertation, the following content focuses on classical BAT.

2.2.3 The developmental origins and molecular controllers

A. Developmental origins of BAT

It is generally believed that, in rodents, WAT grows mainly after birth while BAT develops during embryonic formation and presents throughout life [34]. Brown adipocytes present in major BAT depots (the dorsal anterior region in mice) originate from precursors in the embryonic mesoblastic somite analyzed by fate-mapping studies (Fig. 2.3). The cells in the central dermomyotome expressing a homeobox transcription factor gene engrailed 1 (*En1*) were considered to form dorsal dermis and epaxial muscle in mice, which also give rise to interscapular brown fat [48-50]. The timing of *En1* expression in these precursors during the embryonic day is critical to their developmental fate. Cells expressing *En1* at early embryonic days 8.5 to 9.5 (E 8.5-9.5) are predominantly confined to brown adipocytes development [48]. In contrast cells expressing *En1* at a later embryonic stage can give rise to all three tissues including BAT, muscle, and dermis, presenting a multipotent capacity [48]. *En1*⁺ cells in embryonic somite that also express myogenic factor 5 (*Myf5*) and paired box (*Pax7*), two markers expressed in skeletal myogenic cells, were found to develop brown adipocytes or skeletal muscle cells [49-52]. Early *Pax7*⁺ cells preferentially undergo brown fat commitment while cells expressing *Pax7* at a later stage are confined to skeletal muscle lineage [50]. The above evidence suggests a similar developmental origin between BAT and skeletal muscle.

B. Molecular controllers of BAT development

The commitment or determination of *Myf5*⁺ and *Pax7*⁺ or *En1*⁺ precursors into either muscle lineage or brown fat lineage are regulated by multiple cell-fate controllers (Fig 2.3). Bone morphogenetic protein 7 (BMP7) is one of the critical regulators that induce the differentiation of somatic precursors into brown preadipocytes, which is transcriptionally regulated by ewing sarcoma breakpoint region 1 (EWSR1) and Y-box-binding protein 1 (YBX1) [53, 54]. Besides, the embryonic precursors that preferentially express a transcription factor early B-cell factor 2

(EBF2) by embryonic day 12 can uniquely differentiate into brown adipocytes [55]. However, upstream regulators to activate EBF2 expression in embryonic precursors remain elusive.

PR domain containing 16 (PRDM16) is a crucial transcriptional co-regulator that promotes brown fat lineage determination and suppresses muscle commitment [49, 56-58]. Ablation of PRDM16 in *Myf5*⁺ precursors impairs brown adipocyte development both *in vitro* and *in vivo* [49, 56, 58] while promotes muscle differentiation [49]. PRDM16 interacts with the transcriptional factor CCAAT/enhancer-binding protein-beta (C/EBP β) to form the PRDM16 complex to control brown adipocyte cell fate, mediated by an enzyme named euchromatic histone-lysine N-methyltransferase 1 (EHMT1) [57]. The specific knockout of *Ehmt1* in murine brown adipocytes *in vivo* led to histone demethylation at muscle-selective gene promoters, resulting in severe loss of brown fat identities and enhanced skeletal muscle gene expression in the BAT [57]. Moreover, the deletion of *Ehmt1* in *Myf5*⁺ myoblasts abolished the myogenic differentiation induced by PRDM16 [57]. These results indicate that EHMT1 is indispensable for PRDM16-mediated development of brown adipocytes and suppression of muscle lineage commitment from the common precursor cells.

Preadipocytes undergo morphological and gene expression changes before differentiated into mature adipocytes, which refers to as the adipogenic differentiation process (Fig. 2.3) [59]. Adipogenic differentiation of white preadipocytes and brown preadipocytes shares common molecular regulators [60]. The nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ) is one of the master transcriptional regulators for adipogenic differentiation [61, 62]. In brown adipogenesis, PPAR γ activate the expression of multiple downstream brown marker genes, including *Ucp1* [63]. The agonists of PPAR γ , such as rosiglitazone, significantly improve insulin sensitivity and lipid metabolism by increasing BAT differentiation and thermogenesis [64]. Dominant-negative mutation of *Ppar γ* led to BAT dysfunction, including decreased development and thermogenic ability, in parallel with increased white adiposity and

insulin resistance in a mouse model [65]. PPAR γ 's control of the transcriptional pathways in adipogenesis is facilitated by several co-activators [66-70]. PPAR γ -coactivator-1 alpha (PGC-1 α) co-activates PPAR γ through direct interactions to promote brown adipogenesis but was dispensable by complementary action of family member PGC-1 β [66, 67]. EBF2 also acts as PPAR γ co-activator to promote brown adipogenesis by recruiting PPAR γ to its binding sites in addition to serving as a selective marker of brown adipogenic precursors [68] (Fig 2.3). Similarly, in addition to brown adipose lineage determination, PRDM16 also promotes the PPAR γ -induced brown marker genes transcription through forming a promoter loop to interact with mediator of RNA polymerase II transcription subunit 1 (MED1), which in turn interacts with preinitiation complex (PIC) to start gene transcription of PPAR γ target genes in brown adipocytes [69, 70] (Fig 2.3).

2.2.4 Thermogenesis in brown adipose tissue

A. Adaptive thermogenesis

Energy homeostasis is a dynamic balance between energy intake and energy expenditure. Food intake constitutes almost entirely energy intake, while it is more complicated when analyzing the components of energy expenditure [71]. In general, total energy expenditure consists of three major elements: obligatory energy expenditure, energy expenditure from physical activity, and from adaptive thermogenesis [72]. Obligatory energy expenditure is primarily for the functional performance of cells and organs, which produces heat through exothermic forward reactions and contributes to more than half of total energy expenditure [72]. Energy expenditure from physical activity is variable due to personal lifestyle and social-cultural environments [72]. Adaptive thermogenesis-induced energy expenditure is thought to be heat production in response to diet or environmental temperature [72, 73]. All three components of total energy expenditure could be summarized as the conversion of stored energy (such as glycogen, fat, or protein), food, and oxygen to water, carbon dioxide, work

performance, and heat [72, 73]. Therefore, when at rest without physical activities, the energy expenditure of an organism could be directly represented as heat production by direct calorimetry, or indirectly measured by the amount of oxygen consumption as indirect calorimetry [72, 73].

B. Cold-induced adaptive thermogenesis in BAT

Environmental temperature affects thermogenesis intensely [74]. Skeletal muscle undergoes rapid contraction and relaxation to generate excessive heat in response to acute cold exposure, referred to as cold-induced shivering thermogenesis (acute thermogenesis) [75]. Although with high efficiency for heat production, cold-induced shivering thermogenesis could not be used in long-term cold acclimation since it is detrimental to muscle function [75]. Cold-induced NST, named in contrast to shivering thermogenesis, has evolved to help an organism survive in long-time cold environments and is also recognized as cold-induced adaptive thermogenesis [76].

In mammals, especially in rodents, brown fat is the major site of NST during cold exposure, primarily because of UCP1 expression [18-20]. As we described in the previous section, brown adipocytes in the defined BAT depots contain multilocular lipid droplets and high mitochondrial density with UCP1 expression. Due to the presence of UCP1 in the inner membrane of mitochondria, brown adipocytes can uncouple proton gradient from ATP synthase to generate heat, thereby increasing energy expenditure (Figure 2.4) [20].

a. Acute cold exposure

UCP1 activity increases within seconds upon cold stimulation, to confer a rapid proton leak to produce heat, which is modulated by the TRP-SNS-BAT axis both in rodents and humans [73]. Specifically, cold is sensed by transient receptor potential (TRP) channels expressed in sensor neurons on the body surface [77]. TPR then transmits temperature changes to the brain and activates the sympathetic nervous system (SNS) [77], leading to the release of

norepinephrine (NE) from the sympathetic nerve endings that innervate the BAT [20]. Released NE interacts with all three types of adrenergic receptors, including α_1 -, α_2 -, and beta-adrenergic receptors; however, only beta-adrenergic receptor (β -AR) is critical in stimulating BAT thermogenesis [20]. β -AR is a G protein-coupled membrane receptor, which couples with G_s subtype protein in brown adipocytes [78, 79]. Once being activated by NE, the α subunit of G_s protein ($G_s\alpha$) coupled with β -AR is released to activate the adenylyl cyclase (AC) activity, leading to increases in intracellular cyclic adenosine monophosphate (cAMP) levels, which consequently activates protein kinase A (PKA) to enhance lipolysis of intracellular lipid droplets [80, 81]. The PKA-stimulated intracellular lipolysis depends on proteins involved in mobilization and catabolism of triglycerides, including perilipin (PLIN), hormone-sensitive lipase (HSL), and adipocyte triglyceride lipase (ATGL) [82]. As a lipid-coating protein, PLIN protects triglycerides from lipolytic enzymes and is deactivated and dissociated from lipid droplets upon phosphorylated by PKA [83]. Furthermore, the phosphorylation of serine 517 residue at murine PLIN is essential to activate ATGL [84]. ATGL hydrolyzes sn-1 fatty acids in stored TGs to produce diacylglycerols (DAGs) [85], which are then hydrolyzed by activated HSL to generate monoacylglycerol (MAG) [86]. The resulting monoacylglycerols are further hydrolyzed by monoacylglycerols lipase [86]. In contrast to PLIN, phosphorylation by PKA stimulates HSL activity to translocate to lipid.

Increased FFAs resulting from the intracellular lipolysis are thought to fuel the mitochondrial oxidative phosphorylation by providing reducing equivalents through β -oxidation and stimulate UCP1 activity simultaneously [19, 20]. FFAs β -oxidation is enhanced in brown adipocytes in response to adrenergic stimulation partially due to increased expression of carnitine palmitoyltransferase I (CPT 1), a rate-limiting enzyme in FFAs β -oxidation [87].

b. Prolonged cold exposure

As cold exposure persists over hours and days, the thermogenesis of BAT is enhanced not only by stimulated UCP1 activity but also by increased UCP1 abundance and mitochondrial biogenesis, which is critical for maintaining cellular ATP levels at a proper level in response to enhanced mitochondrial proton leak [72]. PGC-1 α primarily regulates mitochondrial biogenesis induced by prolonged cold exposure in brown adipocytes [66, 88]. As a cold-inducible transcription coactivator, PGC-1 α induces the gene expression of two transcription factors, including nuclear respiratory factor 1 (NRF1) and NRF2, that regulate genes encoding the subunits of the mitochondrial respiratory chain [88]. Moreover, PGC-1 α binds to and activates the NRF1, resulting in an upregulation of mitochondrial transcription factor A (mtTFA), which is another transcription factor activating the mitochondrial deoxyribonucleic acid (DNA) replication and transcription [88].

Increased UCP1 abundance induced by chronic cold exposure generally results from upregulated UCP1 mRNA expression in both differentiation-dependent and -independent manners [72]. On the one hand, the cold-induced NE-cAMP signaling pathway promotes the differentiation of brown preadipocytes into mature brown adipocytes, thereby increasing total UCP1 expression through regulating several transcription factors and coactivators such as PPAR γ , PRDM16, and PGC-1 α [20]. On the other hand, UCP1 expression could be directly upregulated in mature brown adipocytes. One critical mechanism involves the cAMP-PKA-CREB/ATF2 pathway [89, 90]. cAMP response element-binding protein (CREB) or activating transcription factor 2 (ATF2) (both belong to ATF/CREB subfamily) is activated by p38 mitogen-activated protein kinase (MAPK) in response to cold-induced cAMP signaling pathway. It then binds to CRE2 located at the upstream enhancer region of the UCP1 gene, leading to UCP1 transcriptional activation [89, 90]. Moreover, CREB also induces zinc finger protein 516 (ZFP516), which also acts as a transcriptional activator to stimulate UCP1

expression [91]. Several other cold-inducible transcriptional activators for UCP1 are also identified, including nuclear factor erythroid 2-related factor 2 (NFE2L2) [89] and thyroid hormone receptor beta (TR β) [92, 93]. Moreover, persistent cold exposure also modifies the cell composition of BAT by promoting the proliferation of both brown preadipocytes and *Ucp1*⁺ mature brown adipocytes, contributing to BAT hyperplasia [94-95]. Since cold-induced NE-cAMP signaling pathway promotes the differentiation of brown preadipocytes into mature brown adipocytes, cold-induced hyperplasia leads to increases in the percentage of mature brown adipocytes and total UCP1 amount in BAT [94, 95].

Although FFAs from intracellular lipolysis are the major substrates for BAT in cold-induced NST, brown adipocytes can also uptake FFAs from circulation derived from WAT lipolysis or triglyceride-enriched lipoprotein (TRLs) [30]. Moreover, cold exposure also stimulates glucose uptake and utilization in an insulin-independent manner, indicating that glucose is an additional substrate for BAT in response to NE [96]. The substrate utilization of BAT is discussed in more detail in Section 2.3.2).

c. Diet-induced adaptive thermogenesis in BAT

Diet-induced thermogenesis (DIT) is one of the components of adaptive thermogenesis that is critical to energy homeostasis [97]. DIT generally refers to as a long-term overfeeding-induced adaptive increase in energy expenditure [97]. Firstly reported in 1979, BAT is found to be involved in DIT in rodents to increase energy inefficiency in response to increased energy intake [21], which may be under similar sympathetic catecholamines regulation as in cold-induced NST [98, 99]. Moreover, UCP1 ablation abolishes the DIT in mice fed with a normal diet or obesogenic diet at thermoneutral temperature [100, 101]. Further, ingestion of a single meal also has been reported to enhance BAT activity in rodents [102-104]. In humans, the evidence of diet-induced thermogenesis in BAT is controversial. The postprandial ¹⁸FDG uptake by the BAT was lower than that after overnight fasting or cold exposure, while no

difference in whole-body energy expenditure occurred. However, whole-body energy expenditure after meals is higher in healthy humans with a higher amount of BAT characterized by higher ^{18}F FDG uptake [105]. In addition to ^{18}F FDG-PET/CT, oxygen consumption and blood flow measured by ^{15}O [O_2]-PET has also been used to analyze BAT activity in humans [106]. After meals, the oxygen consumption and blood flow in the BAT of healthy adults increased to a similar extent as in cold stress [107]. The inconsistent results between ^{18}F FDG-PET/CT and ^{15}O [O_2]-PET may be due to the limitation of ^{18}F FDG-PET/CT [108]. Cold-induced ^{18}F FDG uptake is primarily increased in BAT in response to adrenergic activation. In contrast, ^{18}F FDG uptake is elevated in multiple tissues other than BAT, such as skeletal muscle, heart, and WAT, in response to insulin [108]. Therefore, BAT activity after meals measured by ^{18}F FDG-PET/CT may underestimate the BAT thermogenic capacity because of the competitive inhibition of ^{18}F FDG uptake by other insulin-sensitive tissues [108]. Collectively, these results suggest that BAT plays a role in DIT in both rodents and humans, but the significance of BAT-mediated DIT on energy homeostasis or systematic metabolism is still unclear [109].

2.2.5 Secretory function of brown adipose tissue

Traditionally, the beneficial effects of BAT have been attributed to its thermogenic function. However, recent studies have revealed that BAT also plays a secretory role by releasing multiple molecules, also known as batokines. These batokines can be categorized into complement elements, hormones, extracellular matrix proteins, and growth factors [110]. These batokines regulate the development and thermogenic recruitment of BAT in an autocrine manner and interact with other organs involved in systemic metabolisms, such as bone, liver, and heart, in a paracrine manner [111].

Collectively, the thermogenic and secretory function of BAT become new targets to combat obesity and obesity-related metabolic disorders.

2.3 Brown adipose tissue, obesity, and its associated metabolic disorders

2.3.1 The significance of BAT in obesity

With the rediscovery of BAT in adult humans and its role in energy expenditure, BAT has become a promising target to combat obesity [37]. The significance of BAT in obesity has been demonstrated in both rodents and humans in the past four decades [112].

Early studies using genetically obese models established a link between BAT and obesity. In response to cold stimulation, decreased blood flow and oxygen consumption in the iBAT were found in the genetically obese rodent models, such as *ob/ob* and *db/db* strains, suggesting a defective BAT thermogenesis in genetic obesity [113-116]. In addition, mature *ob/ob* mice showed significantly decreased UCP1 expression and reduced sympathetic activity in the BAT compared to their lean controls [117-119]. Moreover, adrenalectomy reduced body weight gain and energy efficiency in *ob/ob* mice fed a high-carbohydrate diet, partially associated with increased SNS-stimulated BAT thermogenesis [120]. The increased BAT thermogenesis in adrenalectomized *ob/ob* mice may be associated with increased responsiveness to corticotropin-releasing factor (CRF) by the BAT [120]. These results lead to the hypothesis that BAT dysfunction contributes to obesity and could be a target to combat obesity. Surgically denervating or removing BAT in mice thereby were used as experimental models to address this hypothesis. BAT denervation in mice led to increased energy efficiency and adiposity, indicating the important role of BAT in obesity development [121]. However, Sprague-Dawley rats with BAT denervation failed to gain more weight than sham denervation controls under a high-calorie diet [122, 123], which may be due to compensatory pathways that regulate the BAT metabolism and thermogenesis in response to a chronic defect in SNS [124]. Similarly, surgical removal of the BAT increased adiposity in rats [125, 126], but contradictory results have been reported, possibly due to the compensatory regeneration and hypertrophy of BAT from other depots [127, 128].

Genetic ablation and overexpression techniques have also been used in rodents to investigate the contribution of BAT in combating obesity. Transgenic mice with the expression of diphtheria toxin A (DTA)-chain driven by UCP1 promoter (UCP-DTA) showed decreased thermogenic response to cold exposure and β -AR agonist [129]. Moreover, these mice showed primary and persistent BAT deficiency and developed extreme obesity and associated metabolic abnormalities, including insulin resistance and hyperlipidemia [129]. Furthermore, UCP-DTA mice were resistant to leptin [130] and more susceptible to high-fat diet-induced obesity compared to their wild-type littermates [131]. Consistently, another transgenic mice model with specific expression of an attenuated mutant DTA driven by UCP1 promoter (UCP-176) had impaired BAT development and were extremely obese at an early age (0-2 weeks old) [129]. Interestingly, the BAT in these mice was nearly completely regenerated at 8 weeks old, followed by a reversal of obese phenotype, further demonstrating the therapeutic potential of BAT in obesity [129].

In addition to BAT ablation, the UCP1 knockout mouse model has also been used to investigate the link between BAT dysfunction and obesity. However, results from UCP1-null mice are unexpected. Although UCP1-null mice are susceptible to high-fat diet-induced obesity with age [132], no obesogenic effect of UCP1 ablation has been observed in younger mature mice [133, 134]. The discrepancy found between UCP1 knockout and BAT ablation may be due to alternative adrenergic thermogenesis developed in other thermogenic sites, such as muscle, in UCP1-null mice [133, 134]. Indeed, when housed at a thermoneutral condition (30 °C), mice with ablated UCP1 developed obesity even fed with standard chow diet and were much more vulnerable to high-fat diet-induced obesity [100]. Moreover, mice with deletion of PKA-RII β subunit presented a high amount of UCP1 protein expression, which are resistant to obesogenic stresses, including expression of obesity gene (*agouti* gene) and high-fat diet [135, 136]. In another gene knockout model, mice with specific ablation of cell death-inducing DNA

fragmentation factor- α -like effector A (*Cidea*), a protein expressed abundantly in BAT, showed enhanced UCP1 mRNA expression in the BAT, which might be partially responsible for the lean phenotype and the resistance to diet-induced obesity [137].

In humans, early studies using ^{18}F FDG-PET/CT demonstrated that functional BAT is present in adults [25, 27, 28]. Overweight or obese people showed decreased BAT activity compared to lean individuals [27]. In addition, BAT activity positively correlates with resting metabolic rate while negatively associates with adiposity and BMI [25]. Such findings suggest the association between BAT and obesity in humans, but questions remain as to how BAT significantly contributes to the whole-body energy expenditure and thus confer anti-obesity effect in humans. BAT volume in humans measured by ^{18}F FDG PET/CT varies from a few to hundreds of milliliters [138]. Some individuals present an amount of BAT up to 60 grams [28], which can consume around 4 kilograms of WAT within one year if fully activated [139]. Besides, it was speculated that nearly 20% of daily energy expenditure could be contributed from as little as 50 grams of BAT [140]. Although the ^{18}F FDG-PET/CT is the common method to quantify total BAT mass and activity, ^{18}F FDG uptake was found to be more stable to indicate insulin sensitivity of BAT, but not the BAT volume [139]. Thus, the contribution of BAT-mediated thermogenesis to whole-body energy expenditure may be largely underestimated based on BAT estimation by ^{18}F FDG uptake [139]. In addition, BAT volume could range from 510 to 2358 ml analyzed by radiological 3D mapping technology. Based on such volume, BAT thermogenesis could contribute up to 123 kcal energy expenditure per day even at room temperature and up to 211 kcal per day under mild cold conditions [141]. It is worth noting that even a small negative energy balance, as little as 50-100 kcal/day, is promising to induce weight loss in the overweight and obesity population and confers clinical benefits [142, 143]. In this context, combined with evidence from animal models, it is appropriate to believe that BAT holds a promising therapeutic potential in human obesity.

2.3.2 Metabolic impact of brown adipose tissue

Obesity is associated with multiple metabolic disorders, such as insulin resistance, hyperlipidemia, leading to increased morbidity and mortality [2]. The thermogenesis property of BAT drives it to consume a considerable amount of substrates from circulation, including triglycerides (TG), non-esterified fatty acids (NEFA), and glucose, which makes BAT a metabolic sink [20]. Therefore, BAT is considered to be beneficial in obesity-associated glucose and lipid disorders.

A. Substrates utilization by brown adipose tissue

a. Intracellular lipolysis derived FFAs

In rodents, it is believed that lipolysis of intracellular lipid droplets is drastically enhanced in cold-induced NST to release FFAs as the fuel for mitochondrial oxidative phosphorylation through β -oxidation and to directly activate UCP1 [20]. Intracellular lipolysis is regulated by multiple lipolytic enzymes, including ATGL, HSL, and MAG, as well as lipid coating protein PLIN [144]. In brown adipocytes, while both key enzymes ATGL and HSL are activated to enhance lipolysis in response to cold-induced adrenergic stimulation [82], ATGL may be more important in lipolytic regulation [145-149]. Inhibition of ATGL by the antagonist abolished 80% of UCP1-dependent proton leak in response to adrenergic stimulus isoproterenol (ISO), while HSL inhibition only induced a 30% reduction of UCP1-dependent proton leak [145]. In addition, although globally knockout of HSL (HSL^{-/-}) led to increased BAT lipid content, body weight and cold tolerance were not affected [146, 147]. In contrast, both global and adipose-specific ablation of ATGL in mice significantly reduced the TG hydrolysis activity in the BAT by 85% [148], leading to severely impaired BAT thermogenesis in response to cold exposure [148, 149]. Together, these results suggest that, at least in rodents, intracellular lipolysis is required for UCP1-mediated thermogenesis and is primarily dependent on ATGL activity in BAT during cold exposure.

In humans, intracellular lipids lipolysis in BAT were indirectly investigated by imaging techniques [150-153]. Radio-density measured by computed tomography (CT) scanning is an index inversely correlated with lipid contents in tissue [150, 151], whereas fat fraction measured by magnetic resonance imaging (MRI) represents lipid accumulation in tissue [152, 153]. It was found that the radio-density of BAT measured by CT scanning was increased, while the fat fraction of BAT measured by MRI was decreased in human participates exposed to cold, suggesting that intracellular lipolysis was increased to provide FFA for BAT thermogenesis in humans upon cold activation [150-153]. In addition, by microdialysis analysis, glycerol released from the BAT was increased to higher levels than the WAT when adult humans were exposed to cold, further suggesting cold-induced lipolysis in human BAT [154].

b. FFAs from circulation

In addition to FFAs derived from intracellular lipolysis, FFAs derived from circulating NEFA and TRLs are also important fuels for BAT thermogenesis in rodents [82]. The direct evidence for the utilization of circulating FFAs and TRLs by BAT in cold-induced NST has been reported [155, 156]. There was increased uptake of ^{14}C -triolein-derived FFAs and albumin-bound ^{14}C -oleic acid by the BAT during cold exposure [155, 156], higher than that for muscle in mice [155]. In addition, when injected with double isotope-labeled TRLs, H^3 -cholesteryl ether and C^{14} -triolein as markers for lipoprotein core and TG, respectively, into mice, cold exposure induced an increased accumulation of both H^3 and C^{14} radioactivity in the BAT [156]. Moreover, fluorescent nanoparticle-labeled TRL (QD-TRL) were observed to rapidly attach to endothelium and then internalized into the BAT in cold-exposed mice when visualized through intravital microscopy, which was attenuated by inhibition of lipoprotein lipase (LPL) activity [155]. LPL is responsible for hydrolyzing TRLs to release FFA [157]. Therefore, decreased uptake of TRLs induced by LPL inhibition suggests that BAT may take

up both holo-TRL-particles and TRL-derived FFA [155]. Analysis of gene expression profile of BAT after cold exposure showed significantly upregulated gene expression of cluster of differentiation 36 (*Cd36*), a membrane scavenger receptor responsible for FFA and lipoprotein uptake. CD36 upregulation is consistent with increases in TRLs internalization in BAT upon cold stimulation [155]. CD36 knockout mice presented severely impaired cold tolerance, slower TRLs turnover, decreased TRL uptake into the BAT, and increased plasma NEFA level, which further demonstrate the critical role of CD36 in TRLs-FFA uptake in the BAT [155, 158]. Interestingly, a later study found that BAT primarily takes up TRL-derived FA rather than entire TRL particles upon activation by injecting double radio-labeled TRL-mimicking particles [159], which is inconsistent with previous observation [155]. Such inconsistency may be due to the different size or apolipoproteins composition in TRL particles used in these two studies [160]. Collectively, these results demonstrate that, in rodents, BAT takes up circulating FFAs, TRL-derived FFAs, and possibly holo TRL particles upon activation, suggesting a hypolipidemic role of BAT.

Interestingly, several studies reported that the selective uptake of TRL-derived FFA by activated BAT in rodents might modulate subsequent cholesterol-enriched remnants clearance by the liver through the hepatic apoE-LDLR pathway [161, 162]. When low-density lipoprotein receptor knockout (*Ldlr*^{-/-}) or apolipoprotein E knockout (*Apoe*^{-/-}) mice were exposed to cold or treated with β_3 -AR agonist to activate BAT thermogenesis, both *Ldlr*^{-/-} mice and *Apoe*^{-/-} mice presented increased heat production as expected, but no reduction of plasma cholesterol was observed [161, 162]. Instead, cold exposure even exacerbated the hypercholesterolemia in both *Ldlr*^{-/-} mice and *Apoe*^{-/-} mice [161]. In contrast, in the hyperlipidemic *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice models, β_3 -AR agonist treatment significantly decreased plasma cholesterol levels due to, at least in part, activated BAT thermogenesis [162]. Although *E3L.CETP* mice are also hyperlipidemic; unlike *Ldlr*^{-/-} mice or *Apoe*^{-/-} mice, they expressed a

human apoE3 mutant that still had a binding affinity to LDLR even though lower than normal apoE [162]. It is believed that when circulating TRLs are hydrolyzed by LPL, the resulting cholesterol-enriched remnants are assembled with apoE that could interact with low-density lipoprotein receptor (LDLR) to facilitate the uptake of remnants by tissues, such as the liver [163]. In fact, activated BAT is responsible for the clearance of nearly half triglycerides from a meal measured by oral fat tolerance test [164]. Therefore, in this context, these results indirectly demonstrate the uptake of TRL-derived FFAs by the BAT upon activation in rodents may induce a reduction of plasma cholesterol level in the presence of apoE-LDLR interaction [160].

In humans, circulating FFA uptake by BAT during cold exposure is confirmed by radiotracer ^{18}F -fluoro-6-thia-heptadecanoic acid-mediated PET/CT (^{18}F THA) PET/CT [150, 165]. However, evidence for TRL-derived FFA uptake by activated human BAT is limited. FFA uptake by BAT was increased during cold exposure accompanied by increased LPL expression in human BAT, indirectly suggesting a link between FFA uptake in the activated BAT and LPL-mediated TRL hydrolysis in humans [166]. In addition, prolonged (4 weeks) BAT activation by β_3 -AR agonist mirabegron significantly increased the plasma high-density lipoprotein cholesterol (HDL-c) level in human adults [167]. When TRLs are hydrolyzed by LPL, HDL-c may be subsequently produced by transferring cholesterol from the resulting cholesterol-enriched remnants to HDL particles, possibly catalyzed by phospholipid transfer protein (PLTP) [168]. Therefore, BAT activation-associated increase in plasma HDL-c level in humans may indirectly suggest an enhanced TRL clearance by stimulated BAT thermogenesis [167]. Together, these results suggest a possible role of BAT activation in TRL clearance by uptake of TRL-derived FFA in humans.

De novo fatty acid synthesis in BAT is also enhanced under cold exposure and adrenergic stimulation, suggesting that it could be another source of FFA to fuel BAT thermogenesis [169-

171]. But this may be more relevant with glucose utilization by BAT during active thermogenesis [169], which is discussed below.

Collectively, FFAs derived from intracellular lipid droplets and circulating NEFA or TRLs may be important fuels for BAT thermogenesis in rodents and humans, which may lead to enhanced TG and cholesterol clearance. Therefore, BAT activation may be a promising approach for regulating obesity-related hyperlipidemia.

c. Glucose

Although FFA is the primary substrate for cold-induced NST of BAT in both rodents and humans, cold exposure also stimulates glucose uptake and utilization, indicating that glucose is an additional substrate for BAT in cold-induced thermogenesis[96].

In rodents, the investigation of glucose utilization in BAT may be due to early findings in the late 1970s that the hyperglycemic action of glucagon was attenuated in cold-acclimated rats [172]. After glucagon infusion for 30 minutes, glucose concentrations in venous drainage from the iBAT of rats at warm temperature were significantly increased. In contrast, such a glucagon-induced increase in glucose levels in venous blood near iBAT was markedly reduced by cold-acclimation [172]. In addition, later studies found that glucagon induced an increase in heat production, core body temperature, and BAT temperature in both warm- and cold-acclimated rodents, and such increases in thermogenesis were potentiated by cold acclimation while attenuated by warm acclimation [173, 174]. All these results suggest that glucose uptake may facilitate BAT thermogenesis during cold exposure.

Directly evidence of glucose utilization in rodents has been reported in the late 1980s and early 1990s through 2-deoxy-[³H]glucose method [175-177]. 2-deoxyglucose can only be phosphorylated by hexokinase and then trapped in the tissue, glucose utilization rate can then be measured by the amount of 2-deoxyglucose 6-phosphate [178]. The glucose utilization by BAT was significantly increased in cold-acclimated rats at fed state[175-177]. Moreover, after

rats have fasted for 18 hours at room temperature, glucose uptake significantly decreased in heart and skeletal muscles but not in BAT [176]. Further, cold exposure significantly induced an increase in glucose uptake by peripheral tissues in fasted rats, especially in BAT, with a 95-fold increase, suggesting that the glucose uptake induced by cold in tissues may be independent of the action of insulin [176]. Such insulin-independent glucose uptake by BAT upon cold stimulation was further demonstrated by a recent study [179]. The specific inhibitor of phosphoinositide 3-kinase alpha (PI3K α), a critical enzyme involved in insulin signaling pathways, did not affect glucose uptake by the BAT and systemic glucose clearance upon β -AR activation in mice [179].

The increased glucose uptake by BAT under cold exposure may be regulated by the glucose transporter system, which is dependent on SNS stimulation [175] [177, 180, 181]. By quantifying glucose transporters through [3 H]cytochalasin B-binding assays, it was found that the total number of glucose transporters was increased in the BAT in cold-acclimated mice, accompanied by increased glucose transporters on plasma membranes and decreased microsomal glucose transporters in the BAT [175, 177]. In addition, total GLUT4 expression, the predominant glucose transporter in mature brown adipocytes [182], was significantly increased in the BAT from cold-acclimated rodents but not in the WAT or skeletal muscle [180, 181]. Also, functional activity and amount of plasma GLUT1 in brown adipocytes treated with adrenergic stimuli were significantly increased [182, 183]. Moreover, surgically sympathetic denervation of BAT abolished the cold-induced increases in glucose uptake and glucose transporter numbers [177, 181]. These results suggest that BAT takes up glucose during cold exposure in an insulin-independent manner, which may be regulated by the SNS-dependent modulation of *de novo* synthesis, redistribution, and functional activity of glucose transporters in rodents.

In human adults, the rediscovery of functional BAT was, in fact, based on the hypothesis that human BAT consuming glucose similar to rodents upon activation. Through PET-CT imaging with the ^{18}F -fluoro-deoxyglucose (^{18}F FDG) tracer, human adults were found to present adipose tissue depots that actively take up ^{18}F FDG in response to cold, which were identified as classical BAT or beige fat later [25-28]. Moreover, human BAT, but not WAT, also takes up glucose during warm conditions, suggesting a higher metabolic activity of BAT independent of the thermogenic function [184].

After glucose is taken up into BAT, it can be metabolized in multiple ways. First, a significant portion of glucose is used to provide ATP in the cytosol, as evidenced by the increased expression of glycolytic enzymes in the BAT of mice in response to cold [185]. In addition, it was reported that in cold-acclimated rats, 33% of glucose taken up by BAT was released as lactate or pyruvate, suggesting a considerable portion of glucose undergoes cytosolic glycolysis [186]. Second, glucose can be used to synthesize FFAs in rodents. It was found that de novo fatty acid synthesis was significantly increased after cold stimulation, which was paralleled with increased activities and expression of enzymes involved in fatty acid synthesis, including acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FAS), elongase of very long-chain fatty acids (ELOVL), and fatty acid desaturase [20]. Such increase in fatty acid synthesis was markedly potentiated after glucose administration [169] and was paralleled with increased activation of carbohydrate response element-binding protein (ChREBP), a glucose sensor that activates glucose transportation [187]. Pyruvate generated from cytosolic glycolysis could be the precursors for de novo fatty acid synthesis by producing acetyl-coenzyme A (acetyl-CoA) as evidenced by the cold- or adrenergic stimuli-induced increase in the expression of glycolytic enzymes, mitochondrial pyruvate transporters (MPC), pyruvate dehydrogenase (PDH), and ACC both in vivo and in vitro [185, 187, 188]. FFAs synthesized from glucose are primarily incorporated into TG and simultaneously hydrolyzed

as FFAs to fuel thermogenesis rather than directly used as energy substrates or activate UCP1 [189]. Third, glucose may contribute to triglyceride synthesis. In adrenergic stimulated murine brown adipocytes, glucose-derived glyceraldehyde-3-phosphate is converted to glycerol-3-phosphate and then used as substrates for TG synthesis, but only accounts for a small portion [188]. However, evidence for the contribution of glycolysis in the cytosol and the subsequent metabolism upon BAT activation in humans is limited. One study reported that glucose uptake by the BAT during cold activation was around 11.5 $\mu\text{mol}/100\text{g tissue}/\text{min}$, and lactate release by the BAT during cold exposure was around 21 $\mu\text{mol}/100\text{g tissue}/\text{min}$, accounting for about 90% glucose uptake by BAT in human adults [184]. This result suggests that glucose uptake by BAT may primarily undergo cytosolic glycolysis pathway to generate lactate upon activation in humans, but more detailed evidence and underlying mechanisms need further study. Together, studies have suggested that BAT consumes glucose at a higher rate upon activation, which could benefit systemic glucose homeostasis.

In sum, it is clear that activated BAT may increase heat production and is involved in systemic lipid and glucose metabolism by taking up circulating FFAs and glucose. BAT, therefore, has become a target to combat obesity and its associated metabolic disorders through nutritional and pharmacological strategies.

2.3.3. Strategies targeting BAT to improve obesity and associated metabolic disorders

A. Cold exposure

Cold exposure (acute or chronic) is the most classical stimulus used to activate BAT to investigate its thermogenesis capacity and the precise mechanisms underlying BAT activation. Understanding how cold activates BAT helps researchers identify endogenous regulators or targets that could be used in BAT activation instead of the uncomfortable long-term cold exposure, especially in humans. Studies on cold exposure as a strategy to treat or prevent obesity and associated metabolic disorders are limited both in rodents and humans [155, 190,

191]. In rodents, chronic cold exposure (8 weeks) has been reported to prevent high-fat diet-induced obesity and improve glucose tolerance [190, 191]. Moreover, short-term cold exposure (24 hr) significantly increased the TRL clearance independent of insulin levels and insulin resistance, and decreased plasma TG levels in diet-induced obese mice [155]. Furthermore, 24 hour-cold exposure improved the glucose tolerance in diet-induced obese mice to a level comparable to that of lean control mice [155]. However, intermittent chronic cold exposure did not reduce body weight gain in mice fed a high-fat diet but instead promoted high-fat diet-induced obesity in mice [192, 193]. The possible reason is that intermittent cold exposure may not be strong enough, and the resulting increased energy expenditure thereby could be fully compensated by hyperphagia in response to chronic cold exposure [192, 193]. In humans, short-term cold acclimation (10 days) was able to increase the BAT volume and activity, leading to increased non-shivering thermogenesis in both healthy [194] and obese participants [195]. In addition, chronic (4-6 weeks) daily cold exposure (1-2 hours per day) increased BAT volume, activity, metabolic rate, and energy expenditure [151, 196, 197], paralleled with a decreased body fat mass in the healthy participants [196]. However, no current studies reported the effects of chronic cold exposure in the obese population on body weight, fat mass, or other systemic metabolic parameters. Moreover, severe and long-term cold exposure is intolerable in humans, making cold exposure impractical in human trials. Therefore, other strategies such as pharmacological compounds and food ingredients are investigated to target BAT in obesity treatment, either by mimicking cold exposure or acting on molecular regulators involved in BAT development.

B. Pharmacological Strategies

It is well believed that during cold-induced NST, NE released from the SNS acts on β -AR to activate BAT thermogenesis [20]. Since β_3 -AR is primarily, but not exclusively expressed, in brown adipocytes in both rodents and humans, pharmacological agonists of β_3 -AR have

been developed to target BAT as therapeutics against obesity [20]. Administration of β_3 -AR agonists, including BRL 26830A [198-200], BRL 35135A [201], or CL 316,243 [202-204], was reported to enhance BAT thermogenesis and increase energy expenditure and was partially responsible for decreased body weight gain [198, 201, 202], improved glucose tolerance [199-201, 203], insulin sensitivity [201, 203, 204], and hyperlipidemia [201] in diet-induced obese and genetically obese rodents. However, the anti-obesity effects of β_3 -AR agonists are complicated and controversial in humans. Non-selective β -AR agonist isoproterenol failed to increase thermogenesis and lipid utilization [205] or activate BAT measured by ^{18}F FDG uptake in humans [206]. Moreover, chronic administration of selective β_3 -AR agonists, such as CL 316,243 [207], ZD7114/ZD2079 [208], or L-796568 [209], failed to activate BAT, increase energy expenditure, or change body weight and composition in the healthy [207] or obese humans [208, 209]. Although a higher dose of L-796568 (1000mg compared to 358mg/day used in chronic administration) acutely increased lipolysis and energy expenditure in overweight men, it also caused significant increases in systolic blood pressure [210], which has hindered its therapeutic potential as an anti-obesity drug in humans.

Recently, single-dose administration (200 mg) of a selective β_3 -AR agonist mirabegron, an approved drug for overactive bladder treatment, has been reported to increase BAT metabolic activity and resting metabolic rate in healthy men with a minimal increase in systolic blood pressure compared to other β_3 -AR [211-213]. Moreover, chronic administration of mirabegron at the dose of 100 mg/day for 4 weeks significantly increased the BAT metabolic activity, resting energy expenditure, concurrently with improved glucose tolerance, insulin sensitivity, and increased plasma apolipoprotein A1 (apoA1) and HDL-c levels in the healthy women [167]. However, chronic mirabegron treatment in obese, insulin-resistant humans improved glucose homeostasis and insulin sensitivity mainly through the formation of beige

adipose in the subcutaneous WAT depot rather than improving BAT activity [214, 215]. Therefore, whether this high-affinity β_3 -AR mirabegron could be an effective anti-obesity drug in humans and its precise mechanisms need further study.

C. Bioactive compounds from foods

Due to the compliance issue or possible side effects from cold exposure and pharmacological agents, increasing studies have focused on identifying thermogenic food ingredients that activate BAT to improve obesity and its associated disorders [216, 217].

a. Resveratrol

Polyphenols have been reported to present many beneficial effects in metabolic syndrome in humans [32]. Resveratrol is one of the natural polyphenols, mainly found in the root of *polygonum cuspidatum*, *mulberries*, and grapes [218]. Resveratrol exerts anti-obesity effects [219], at least in part, through promoting BAT development [220].

In mice fed either with a high-fat diet [221, 222] or a standard diet [223], chronic (4-15 weeks) administration of resveratrol (0.1-0.4% w/w) reduced the body weight gain [221, 222], visceral fat mass [221, 223], and plasma glucose and total cholesterol levels [223], which were accompanied by increases in cold-induced thermogenesis [221] and basal energy expenditure [221, 223], possibly through upregulated mitochondrial biogenesis [221] and UCP1 expression [221-223] in the iBAT of mice. Similarly, resveratrol treatment in high-fat diet-fed sprague-dawley rats (30mg/kg/day for 8weeks) [224] or Otsuka long evans tokushima fatty (OLETF) rats (10mg/kg/day for 27 weeks) [225] improved the insulin sensitivity [225], which was associated with increased UCP1 expression and mitochondrial activity of the iBAT [224, 225]. The effects of resveratrol on BAT are likely through the modulation of AMP-activated protein kinase-Sirtuin 1-PGC-1 α (AMPK-SIRT1-PGC-1 α) axis to increase mitochondrial activity and biogenesis [221-224], as well as via the upregulation of the expression of PRDM16, BMP7,

and estrogen receptor alpha (ER α) expression to promote brown fat development and adipogenic differentiation [222, 223, 225].

Although positive effects of resveratrol on BAT to induce weight loss have been demonstrated in rodents, the effects of resveratrol in humans are mixed. Resveratrol administration (1-2 g/day for 4-6weeks) was reported to improve glycemic control in individuals with impaired glucose tolerance [226] or type 2 diabetes [227] but failed to improve glucose tolerance or insulin sensitivity in the obese but otherwise healthy humans [228]. None of these studies reported changes in energy expenditure, metabolic rate, adiposity, or body weight [226-228]. Another study reported that 1.5 g of trans-resveratrol ingestion for 12 weeks reduced the body weight, fat mass, waist circumference, and insulin secretion in subjects with metabolic syndrome, but with no changes in energy expenditure or metabolic rate [229]. It is worth noting that resveratrol treatment (150-500 mg/day) for 30 days in the obese participants improved mitochondrial respiration in muscle by activating AMPK-SIRT1-PGC-1 α axis [230] while decreased the size of subcutaneous white adipocytes possibly by upregulating autophagy-induced lipid breakdown [231] and expression of brown markers (UCP1, PRDM16, and PGC-1 α) [232] concurrently with improved glycemic and lipid profiles [230, 232]. All these results suggest that the effects of resveratrol may vary due to the different health condition of subjects and the dose or duration of treatment. The beneficial metabolic effects of resveratrol in obese subjects may come from the regulation of muscle and WAT activity rather than activating BAT, which was observed in the rodent studies. Therefore, it is not conclusive whether resveratrol is an effective anti-obesity compound in humans by increasing energy expenditure through BAT activation.

b. Capsaicin and capsinoids

Capsaicin is a worldwide-consumed spice from chili pepper, generating hotness and pungency sensation. Capsaicin has shown beneficial health effects, including anti-obesity

effects, in humans [233, 234]. Capsinoids, primarily found in red peppers, inducing much less pungency sensation than capsaicin, present similar chemical structures and confer similar beneficial effects as capsaicin [235]. Administration of either capsaicin or capsinoids can activate the gastrointestinal TRP potential cation channel subfamily V member 1-SNS (TRPV1-SNS) pathway to activate thermogenesis, energy expenditure, and fat oxidation [233, 236], making them promising in activating BAT thermogenesis and thereby fighting against obesity.

In rodents, capsaicin and/or capsinoids increased SNS activity in the BAT and increased core body temperature and whole-body energy expenditure, paralleled with decreased body fat mass [237-241]. These effects were attenuated by β -AR antagonists [237], parasympathetic denervation of jejunum [238, 239], TRPV1 ablation [238, 240], or UCP1 knockout [242]. In humans, a single dose of capsinoids increased energy expenditure only in the healthy participants with ^{18}F FDG-PET/CT-detectable BAT, suggesting the functional BAT is critical for capsinoid-induced beneficial effects in humans [243]. In addition, chronic capsinoid ingestion (6-12 weeks) was able to enhance cold-induced thermogenesis in healthy humans with low BAT activities [196] and induce a reduction of visceral fat in the obese participants [244], possibly due to BAT activation as confirmed by ^{18}F FDG-PET/CT and near-infrared time-resolved spectroscopy (NIRTRs) [245, 246]. The important role of TRPV1 in capsinoid-induced beneficial effect was demonstrated by the fact that fat-reducing effects of capsinoids were diminished in the participants with TRPV1 mutation [244], consistent with the results found in TRPV1 ablated mice [238, 240]. Importantly, either single ingestion of 30 mg [243] or chronic administration of 6-9 mg/day [196, 244] of capsinoids did not induce severe adverse effects in humans.

In sum, capsaicin and capsinoids may be a safe and practical strategies to combat obesity through the TRP-SNS-BAT axis in humans.

c. Other food ingredients targeting the TRP-BAT axis

Other than capsaicin and capsinoids, multiple food ingredients present TRPV1 activation capacity, such as piperine found in black pepper and gingerols and 6-paradol found in the gingers and Guinea peppers (GP). These food components may activate BAT thermogenesis similar to those of capsaicin and capsinoids [247]. However, evidence for these food ingredients is limited. Acute ingestion of the GP extract (40 mg) increased the cold-induced thermogenesis only in the subjects with metabolically active BAT [248], and chronic ingestion of the GP extract (30 mg/day for 4 weeks) in non-obese women decreased the visceral fat mass and increased whole-body energy expenditure [249]. However, further studies are needed to investigate whether the fat-reducing effects of the GP extract in non-obese women can be translated to the obese population.

In addition to TRPV1, another critical member of the TRP family is TRPM8, which is sensitive to lower temperatures. TRPM8 stimulation holds promise to activate BAT similar to cold exposure [216, 217]. Menthol, responsible for the cooling sensation of mint, is a representative agonist of TRPM8, which has been demonstrated to increase thermogenesis in a UCP1-dependent manner to prevent diet-induced obesity in mice [250]. However, such beneficial effects of menthol need to be further investigated in human trials.

d. Long-chain fatty acid (LCFA) species

An early study reported that voluntary over-eating stimulated the SNS and brown adipose tissue thermogenesis, suggesting a role of diet on BAT thermogenesis [251]. The degree of BAT thermogenesis differs in response to diet with different compositions of macronutrients [252]. Rodents fed on a high-protein diet present lower SNS-induced BAT thermogenesis than the rodents fed on a high-carbohydrate or high-fat diet [252-256]. Moreover, a high-fat diet has been reported to increase UCP1 content in the BAT in rodents, which was not affected by fat content but instead associated with the unsaturation index of dietary fat [257]. In the early

1990s, dietary polyunsaturated fatty acids (PUFAs) are considered to be the causative factor to induce BAT thermogenesis [258]. Later, several studies reported that fish oil, rich in EPA and DHA, decreased high-fat diet-induced body fat accumulation while increased the UCP1 mRNA expression in the iBAT of rats to a greater extent than safflower oil (rich in linoleic acids) [259, 260]. In addition, a low dietary n-6/n-3 ratio (3.7) has been reported to improve the thermogenic activity of the iBAT in mice in response to β_3 -AR agonist [261]. Consistently, a high ratio of n-6/n-3 PUFAs found in current western diets is associated with an increased risk of cardiovascular diseases, cancer, inflammatory diseases, and obesity in humans [262, 263]. N-3 PUFAs have been a particular focus in BAT activation to combat metabolic disorders in recent 20 years, which are discussed in the next section.

2.4 N-3 PUFA, BAT activation, and Obesity

2.4.1 Structure, nomenclature, and food sources

PUFAs are a family of fatty acids with two or more double bonds in the hydrocarbon chain, which could be classified as either n-6 or n-3 PUFAs based on the first double bond position relative to the methyl carbon [264, 265]. If counting the methyl carbon as the first carbon (n-1) of the acyl chain, PUFAs containing a double bond starting from the third carbon atoms (n-3) are defined as n-3 PUFAs, while PUFAs having a double bond beginning from the sixth carbon atoms are classified as n-6 PUFAs [265]. PUFAs are named according to the number of carbon atoms and the number of double bonds [265]. For example, linoleic acid (LA) and alpha-linolenic acids (ALA) are named as 18:2 n-6 and 18:3 n-3, respectively, which are the simplest n-6/n-3 PUFAs found only in plant sources and cannot be synthesized in animals [265]. Thereby, LA and ALA are essential FAs for humans [266]. LA and ALA could be converted to other n-6/n-3 PUFAs, such as arachidonic acid (ARA, 20:4n-6) from LA or EPA (20:5n-3) and DHA (22:6n-3) from ALA, by a series of elongation and desaturation [264].

Several plant seeds-derived oil and nuts are rich in ALA, such as flaxseeds and flaxseeds oil, rapeseed oil, and walnuts, containing more than half of the total fatty acids as ALA [267]. Other plant oil, such as corn oil, sunflower oil, and safflower oil, are critical food sources for LA which is the main PUFA in most western diets and is consumed in a large amount 5- to 20-fold greater than ALA [268]. Alternatively, other more unsaturated very-long-chain n-3 PUFAs could be obtained from marine sources. Fish, especially fatty fish, and seafood are good dietary sources for DHA and EPA [267]. The content of DHA and EPA in fish differs between fish species, season, water temperature, and the diet of fish [268]. One serving of salmon or mackerel, two typical fatty fishes, provides up to 3 grams of very-long-chain n-3 PUFAs, which is 10 fold of one serving of lean fish meal [268]. However, it has been reported that the typical intake of fatty fish was less than half of the intake of lean fish in North America, East Europe, the United Kingdom, and Australasia [268].

Currently, a typical western diet with a high ratio of n-6/n-3 PUFAs is associated with increased risk of cardiovascular diseases, cancer, inflammatory diseases, and obesity in humans [262, 263]. Over the past 20 years, DHA- and EPA-enriched foods, such as fish oil added spreads, yogurts or milk and meat, eggs or dairy products from the animals fed with n-3 fatty acids, are good sources to provide up to a hundred milligrams of DHA and EPA per day to help people increase n-3 PUFAs intake without significantly changing eating habits [266].

2.4.2 Metabolism of n-3 PUFA and its biological effects

A. ALA converted to EPA and DHA

Upon being absorbed through the small intestine, dietary n-3 PUFAs are primarily incorporated into TG-rich chylomicrons (CMs) entering into the lymphatic system and then blood circulation to be taken up by tissues [269]. Two major metabolic fates of ALA are β -oxidation and carbon recycling for *de novo* synthesis of fatty acids and cholesterol in mammals, including humans [270]. Also, it could be utilized as precursors to synthesize EPA and DHA

following several tissue-specific desaturations and elongations in humans [271]. Similarly, LA could also be converted to ARA by desaturation and elongation following the same enzyme pathways as ALA [271]. ALA competitively inhibits ARA synthesis from LA, thereby increasing the ratio of n-3/n-6 PUFAs ratio to confer beneficial effects [271].

B. EPA/DHA metabolisms

One of the important metabolic fates of dietary EPA/DHA is as substrates for cellular oxygenase to generate oxylipins, including cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) monooxygenases, all of which also catalyze the oxidation of ARA [272].

The major COX and LOX products of ARA, including various thromboxanes (TXs), leukotrienes (LTs), and prostaglandins (PGs), are potent modulators of platelet aggregation, smooth muscle contraction, and immune cell infiltration and adherence [273]. Excess production of ARA-derived PGs/LTs may lead to pathological processes resulting in certain diseases such as cardiovascular diseases (CVD) [273]. In contrast, EPA-derived PGs and LTs are far less potent in pathological processes [273]. Therefore, n-3 PUFAs could competitively reduce the level of ARA-derived eicosanoids to promote beneficial outcomes related to CVD [273]. Moreover, several COX- and LOX-generated oxylipins from n-3 PUFAs are anti-inflammatory modulators, such as resolving E_{1-3} (RvE $_{1-3}$) from EPA and resolving D_{1-6} (RvD $_{1-6}$) and maresin1 (MaR1) from DHA, which play protective roles in inflammatory diseases [274, 275].

The CYP450 enzymes produced metabolites include hydroxy fatty acids, dihydroxy fatty acids, and epoxy fatty acids, whose synthesis is thought to be intensely regulated in response to different dietary fatty acid compositions [276]. Among these derivatives, epoxides formed by CYP450 epoxygenases from ARA, namely epoxyeicosatrienoic acids (EETs), present well-documented beneficial effects on cardiovascular and renal diseases primarily through autocrine

and paracrine regulation [277]. In rodents and humans, CYP 2C and 2J subfamilies are two major CYP epoxygenases that produce epoxides from ARA but also efficiently epoxidized alternative substrates, such as n-3 PUFAs [276]. CYP epoxygenases metabolized EPA and DHA with higher efficiencies than ARA in the healthy participants when supplemented with n-3 PUFAs [278].

C. Biological effects of EPA/DHA

Intracellular free n-3 PUFAs, primarily DHA and EPA, are quickly esterified by acyl-CoA synthetase (ACS) to fatty acyl-CoA thioesters and then incorporated into complex lipids, including triglycerides and phospholipids [279]. N-3 PUFAs enriched phospholipids are critical for membrane structure and function in most cells, such as rods of the retina and neurons in the brain [268, 280, 281].

In addition, n-3 PUFAs bind to membrane surface receptors to regulate cell and tissues behavior[282]. One well-established cell surface receptor that is activated by n-3 PUFAs is G-protein coupled surface receptor 120 (GPR120) highly expressed in inflammatory macrophages and adipocytes [282]. Moreover, n-3 PUFAs modulate various transcription factors or nuclear receptors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), PPAR α , PPAR γ , sterol regulatory element-binding proteins (SREBPs), retinoid X receptor alpha (RXR α), liver X receptor (LXR), and hepatocyte nuclear factor-4 alpha (HNF-4 α) to modulate intracellular signal transduction, thereby influencing a wide range of cells and tissues functions [264, 279, 282].

In general, n-3 PUFAs present protective effects in cardiovascular diseases [283], neurodegenerative diseases [284], non-alcoholic fatty liver diseases[285], and chronic inflammatory diseases [286]. In recent years, dietary n-3 PUFAs have been reported to be beneficial effects in obesity treatment and prevention [287].

2.4.3 Anti-obesity effects of N-3 PUFA: the role on BAT

A. N-3 PUFA promotes BAT activity in obesity

Evidence from animal studies has shown that EPA and DHA supplementation may prevent high-fat diet-induced body weight gain, visceral WAT accumulation, and metabolic disorders [287-290]. Possible mechanisms underlying the fat-reducing effects of n-3 PUFAs include appetite regulation, anti-inflammation, inhibition of white adipocyte proliferation and adipogenesis, and regulation of gene expression involved in lipid metabolism and brain-gut-WAT axis [287-290]. The effects of n-3 PUFAs supplementation on weight loss and body fat composition in human trials are inconsistent, which may be due to the heterogeneity of study design, demographics of participants, dose, and duration of n-3 PUFA administration [263]. Overall, based on current evidence from rodents and human studies, n-3 PUFAs hold promising therapeutic potentials in obesity and associated metabolic disorders. Recently, n-3 PUFAs have been reported to prevent high-fat diet-induced adiposity in parallel with an increase in energy expenditure in rodents, suggesting the possible role of n-3 PUFAs on BAT [291].

It was firstly reported that 4-weeks of administration of a mixture of EPA and DHA prevented the high-fat diet-induced epididymal and retroperitoneal fat accumulation in rats, associated with an increase in total DNA content and UCP content in iBAT [292]. Such results suggest that BAT-related thermogenesis induced by the mixture may be responsible for their WAT-reducing effects in diet-induced obesity [292]. Consistently, later studies reported that fish oil consumption prevented the excessive WAT accumulation induced by a high-fat diet in rats, accompanied by an increase in total iBAT mass [293], UCP1 mRNA [259] and protein expression [293].

Similar results were reported in mouse models. Dietary n-3 PUFA supplementation in the forms of fish oil with different formulations [294-298] or pure DHA and/or EPA [299-303] prevented high-fat diet-induced body weight gain and abdominal WAT accumulation possibly

through enhanced BAT thermogenic function as indicated by increased core body temperature or oxygen consumption [294, 297, 298, 303], increased mitochondrial biogenesis [301], and upregulated expression of thermogenic genes, including β_3 -AR, UCP1, PGC-1 α , and PRDM16 [294-298, 300-303]. Moreover, dietary n-3 PUFA consumption improved glucose intolerance [296, 298], insulin resistance [297], and hypertriglyceridemia [294, 296, 299, 303] in high-fat diet-fed mice concurrently with upregulated mRNA expression of genes involved in glucose uptake (GLUT4), lipids uptake (LPL and CD36), and fatty acid beta-oxidation (CPT1B) in the iBAT [294, 296, 301]. These results suggested that the anti-obesity effects of n-3 PUFAs in rodents may be partially achieved by improving BAT thermogenic and metabolic function.

B. Mechanisms by which n-3 PUFA promotes BAT activity

The mechanisms by which n-3 PUFA potentiates BAT function are not well-understood, and several mechanisms have been reported. Since BAT thermogenesis is intensely regulated by SNS, it is possible that n-3 PUFA regulate BAT thermogenesis through SNS activation. DHA- or EPA-enriched fish oil administration prevented high-fat diet-induced obesity and hypertriglyceridemia in mice [294]. These effects were accompanied by increased energy expenditure, urine catecholamine levels, noradrenaline turnover rate, and β_3 -AR and UCP1 expression in the iBAT, suggesting an enhanced sympathetic activity in the iBAT induced by n-3 PUFA [294]. Moreover, such SNS activation effects were abolished in TRPV1 knockout mice, indicating that n-3 PUFAs may stimulate SNS- β_3 -AR pathways through TRPV1 activation [294], similar to those of TRPV1 agonists capsaicin and capsinoids discussed in the previous section (2.3.3. C).

In addition, adrenergic-independent pathways have been proposed recently. Mice fed a high-fat diet enriched with DHA and EPA showed significantly upregulated UCP1 protein expression in the iBAT compared to the controls, which was associated with upregulated mRNA levels of GPR120 and fibroblast growth factor 21 (FGF21) in the iBAT [301]. GPR120

is a lipid sensor that can be activated by n-3 PUFAs to induce FGF21 production in the brown adipocytes [304], which has been identified as a novel autocrine mediator in BAT thermogenesis [305, 306]. Moreover, phosphorylation of AMPK α was increased in the iBAT from n-3 PUFAs treated mice [301], which may be the downstream signaling pathway induced by FGF21 to regulate BAT thermogenesis [307]. Therefore, n-3 PUFAs may enhance BAT thermogenesis by activating GPR120-FGF21-AMPK α pathway to improve diet-induced obesity in mice. However, the effect of n-3 PUFAs in inducing FGF21 was inconsistent. Although administration of fish-derived n-3 PUFA concentrates in mice prevented high-fat diet-induced adiposity, hyperinsulinemia, and hypertriglyceridemia, neither plasma FGF21 level nor FGF21 mRNA expression in the iBAT and WAT were increased compared to the controls [299]. These results suggest that FGF21 induction may not be the major mechanism underlying the anti-obesity effects of n-3 PUFAs. Moreover, the importance of GPR120 in n-3 PUFAs-induced beneficial effects was challenged by the study using GPR120 knockout mice [308]. It was reported that effects of fish oil administration in GPR120 knockout mice on core body temperature, energy expenditure, and BAT mass did not differ from those in wild-type mice [308]. These results suggest that GPR120 activation is not required by n-3 PUFAs to confer anti-obesity effects, and alternative mechanisms may exist [308]. Indeed, in addition to upregulated GPR120 mRNA level, n-3 PUFAs supplementation in mice fed a high-fat diet significantly upregulated PPAR α and PPAR γ mRNA levels in the iBAT [295, 296, 301, 302]. PPAR γ is a well-documented transcription factor that regulates BAT development and function. PPAR α is another member of the PPARs family that contributes to the thermogenic activation of BAT by modulating genes involved in lipids metabolism and mitochondrial biogenesis [309]. Together, these results suggest that n-3 PUFAs may regulate BAT function to combat obesity through multiple distinct targets, such as GPR120, PPAR α , and PPAR γ .

A growing evidence suggests that n-3 PUFAs, especially DHA, can remodel mitochondrial inner membrane structure and composition associated with increased cardiolipins [310], a group of phospholipids synthesized by cardiolipin synthase one that is responsible for mitochondrial biology and energetic function [311, 312]. Recently, the lipidomic analysis found that cold adaptation significantly increased cardiolipins in the iBAT of mice [313, 314]. Moreover, cardiolipins are essential for UCP1-mediated thermogenesis in BAT by transcriptionally modulating UCP1 expression [313] and structurally supporting UCP1 activity [315, 316]. All these results suggest that cardiolipins may be a possible mechanism by which n-3 PUFAs promote BAT thermogenesis to fight against obesity. However, no studies have reported the changes of cardiolipin profiles in the BAT by n-3 PUFA supplementation in obese models. More direct evidence is needed to support the roles of cardiolipins in BAT activation by n-3 PUFAs.

While a majority of studies reported that n-3 PUFAs protected against diet-induced obesity in rodents through UCP1-dependent thermogenesis by increasing energy expenditure via upregulating UCP1 mRNA and/or protein expression in iBAT [259, 292-296, 300-302], a few exceptions have been reported [298, 299]. Moreover, results from UCP1 deficient mice have indicated that n-3 PUFAs may enhance energy expenditure through UCP1-independent pathways [297, 298]. Fish oil or EPA-enriched fish oil administration protected both wild type and UCP1 knockout mice from high-fat diet-induced obesity with increased energy expenditure at both room temperature and thermoneutrality conditions [297, 298]. One possible UCP1-independent mechanism by which n-3 PUFAs enhance energy expenditure may be glycerol 3-phosphate dehydrogenase (GPD)-mediated thermogenic futile cycle [297]. It is a mechanism that regenerates reducing equivalent by substrates converting between glycerol 3-phosphate and dihydroxyacetone phosphate catalyzed through cytosolic/mitochondrial GDP [317]. mRNA expression of mitochondrial GPD (mGPD) was increased in the inguinal WAT

(iWAT) of UCP1 knockout mice treated with fish oil, indicating that n-3 PUFAs may enhance the mGDP-induced futile thermogenic metabolic cycle to increase energy expenditure [297]. In addition, EPA-enriched fish oil administration upregulated PGC-1 α protein and mRNA expression and increased mitochondrial DNA content in the iBAT of UCP1 knockout mice, suggesting that increased mitochondrial biogenesis may be, at least in part, responsible for the enhanced energy expenditure induced by n-3 PUFAs in UCP1 knockout mice [298].

C. Differential effects of DHA and EPA on BAT activity in obesity

Early evidence found that DHA or EPA supplementation for 4 weeks (11% energy intake from DHA or EPA) in rats protected against high-fat diet (40% energy intake from fat) induced abdominal WAT accumulation, possibly through activating BAT thermogenesis [292]. Whereas DHA significantly increased total DNA content and mitochondrial thermogenic activity in the iBAT compare to the control group, EPA significantly increased total mitochondrial and UCP content compared to the control group and DHA-treated group [292]. These results suggest that DHA or EPA administration may limit high-fat diet-induced white fat accumulation in rats, possibly by promoting BAT thermogenesis through different mechanisms. DHA may enhance BAT thermogenesis through hyperplasia effects, while EPA may induce mitochondrial biogenesis to enhance UCP1-dependent thermogenesis in rats fed with a high-fat diet.

In a mouse model, DHA or EPA administration (2% energy intake from DHA or EPA) for 5 weeks significantly decreased the lipid accumulation in the iBAT of mice fed a high-fructose diet [318]. However, the gene expression was differentially regulated by DHA and EPA. Gene expression of thermogenic markers, including UCP1 and PGC-1 α , in the iBAT were upregulated in both DHA- and EPA-treated groups, but UCP1 mRNA expression was more efficiently augmented in the EPA-treated mice compared to DHA-treated mice [318]. mRNA expression of genes involved in fatty acid utilization, such as LPL, were upregulated in the

DHA and EPA groups, while HSL mRNA expression was only increased in the EPA-treated group [318]. Both DHA and EPA treatment increased the PPAR α mRNA expression, whereas PPAR γ mRNA was only upregulated by the EPA treatment [318].

Although these studies suggest that DHA and EPA may promote BAT thermogenesis via different mechanisms to confer beneficial effects in rodents, many of the studies on DHA or EPA only showed upregulated thermogenic gene expression with no functional analysis of the thermogenesis. It is note that several other studies have reported that EPA increased the oxygen consumption in brown adipocytes [295, 300, 304, 319] and increased the energy expenditure in mice [295, 298, 300, 302, 303] by targeting thermogenetic genes in the iBAT to protect against obesity and associated metabolic disorders, which further confirms that the anti-obesity effects of EPA are at least in part originated from the improved BAT thermogenesis.

In addition, inconsistent results have been reported regarding the effects of DHA on fat mass and BAT thermogenic function. In one study, purified DHA supplementation (1% wt/wt) for 15 weeks in the obese mice fed a high-fat diet did not affect body weight but significantly decreased the iWAT mass and serum TG and TC levels in parallel with increased iBAT mass, ¹⁸FDG uptake in the iBAT, and whole-body oxygen consumption, possibly due to upregulated UCP1 expression in the iBAT [303]. These results suggest that DHA may limit WAT fat accumulation and improve hyperlipidemia, possibly through increased energy expenditure by activating BAT in mice. However, other studies failed to confirm the WAT-limiting and thermogenic effects of DHA in rodents [320-322]. DHA supplementation (1% wt/wt) for 16 weeks did not reduce body weight or WAT fat mass but improved the hypercholesterolemia in mice fed a high-fat, high-sucrose diet (45% energy intake and 14% energy intake from fat and sucrose, respectively) [323]. Another study reported that 1% DHA administration (1% wt/wt) for 12 weeks in insulin resistant mice prevented the high-fat diet (45% energy intake)-induced body weight gain and fat accumulation without improving the serum TG or TC levels, while

4% DHA treatment significantly decreased the serum TC and LDL cholesterol (LDL-c) levels without changing body weight gain or fat mass [322]. In addition, administration of purified DHA (1g/d/kg bw) for 4 weeks significantly decreased the plasma TG and TC levels but did not change the body weight, WAT fat mass, iBAT cellularity (DNA content), or the *ex vivo* oxygen consumption of the iBAT from rats [320]. Moreover, prolonged DHA administration for 16 weeks even significantly decreased the iBAT oxygen consumption in the basal state and NE-stimulated state [320]. In a most recent study, 12-month administration of a DHA-enriched high-fat diet (6.7% energy intake from DHA, 45% energy intake from fat) in the aged obese mice significantly improved the lipid profiles without affecting the body weight or adiposity [321]. Moreover, the activity of BAT in response to cold, measured by ¹⁸FDG uptake, was drastically decreased in the aged obese mice, which was not reversed by DHA supplementation even though a restored UCP1 protein expression and PRDM16 mRNA expression in BAT were observed [321]. Such discrepancy in the effects of DHA treatment may result from the different rodent species, study design, and the form and dose of DHA used in these studies.

In summary, current evidence for DHA supplementation is more profound and consistent in its lipids lowering effects in rodents compared to the fat-reducing effects, whereas EPA may be more protective against WAT accumulation in obesity. In addition, whether DHA and EPA have comparable effects on modulating BAT activity need further investigation. Moreover, the mechanisms by which DHA and EPA differentially regulate the thermogenic gene expression and the activity of BAT remain unclear. One possible speculation is that CYP-produced epoxides from DHA and EPA may partially contribute to their differential effects since these epoxides are bioactive metabolites participating in multiple physiological processes [276].

2.5 Effects of epoxy fatty acids from DHA and EPA in obesity

2.5.1 Overview: Production, Structure, and Metabolism

Inside the cell, the majority of n-3 PUFAs are incorporated into the cell membrane as phospholipids [264]. In addition, n-3 PUFAs are also incorporated into intracellular TG, mainly in adipose tissue [264]. Upon certain stimuli, n-3 PUFAs from TG or membrane phospholipids were released by the action of lipolytic enzymes, which then are converted to multiple bioactive metabolites through different enzymatic processes [264]. One of the major enzymes is CYP450 epoxygenase, which convert one of the double bonds in n-3 PUFAs to a monoepoxide group and generate epoxy fatty acids (EpFAs) [276]. Epoxydocosapentaenoic acids (EDPs) and epoxyeicosatetraenoic acids (EEQs) are EpFAs produced from DHA and EPA, respectively, with different regioisomers depending on the position of the monoepoxide group [276]. EDPs consist of 6 regioisomers, including 4,5-, 7,8-, 13,14-, 16-17, and 19,20-EDP, whereas EEQs consist of 5 regioisomers, including 5,6-, 8,9-, 11,12-, 14,15-, and 17,18-EEQ [276]. In rodents and humans, CYP 2C and 2J subfamilies are two major CYP epoxygenases efficiently metabolize n-3 PUFAs [276]. They metabolize DHA to 19,20-EDP and EPA to 17,18-EEQ in a predominate ratio [276].

N-3 PUFA-derived EpFAs (n-3 EpFAs) are reported to be involved in vasculature function, angiogenesis, inflammation, cell growth, and pain and are beneficial in cardiovascular diseases, pulmonary disease, neurodegenerative diseases, and cancer [276, 324-326]. However, these EpFAs are not stable and are quickly metabolized into far less active diols by sEH, an enzyme encoded by the *EPXH2* gene in humans [326]. Potent sEH inhibitors are developed and widely used to study the functional effects of EpFAs [326-328]. Many of the beneficial effects of EpFAs were potentiated by co-administration of an sEH inhibitor [326-328].

2.5.2 Effects of n-3 EpFA in obesity

It has been well-documented that n-3 PUFAs present cardioprotective and neuroprotective effects [329], which could be partially due to the production of n-3 EpFAs as they were reported to modulate a variety of physiological processes such as angiogenesis, vasodilation, inflammation, and cell growth/differentiation [276, 324-326].

Recent studies indicated that higher sEH activity was associated with obesity and metabolic diseases in rodents [330]. Moreover, sEH expression in the sWAT of obese adults was significantly higher than that of lean subjects [331]. Thereby, sEH has been targeted to treat or prevent obesity.

sEH knockout mice fed a high-fat diet have been reported to show decreased body weight [332] and improved glucose tolerance and insulin sensitivity [333, 334], accompanied by decreased inflammation in the colon or adipose tissue [332], suppressed gut bacterial leakage and translocation [332], and enhanced insulin signaling in the liver and adipose tissue [333]. Consistently, administration of sEH inhibitors 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS) [333], trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) [335, 336], or *t*-TUCB [337] have been demonstrated to improve fasting glucose [335], glucose tolerance [333, 336], insulin sensitivity [333], and plasma NEFA and LDL-c levels [336] in mice fed a high-fat diet, accompanied by enhanced insulin signaling in the adipose tissue and liver [333], improved hepatic steatosis [336], or decreased inflammation in the kidney, liver and adipose tissue [335-337]. In rats with diet-induced metabolic syndrome, *t*-AUCB also improved the glucose, insulin, and lipid profiles, decreased the systolic blood pressure, and restored the cardiovascular and hepatic structure and function [338].

Interestingly, sEH deletion in mice reduced body weight and visceral fat mass concurrently with significant reduced size of white adipocytes and increased mitochondrial and thermogenic

genes expression in the WAT, including mitofusin-1 (Mfn1), cytochrome c oxidase subunit 1 (COX1), and UCP1, suggesting browning of WAT induced by sEH ablation [339]. Moreover, sEH inhibitor 1-(1-acetypiperidin-4-yl)-3-adamantanylurea (AR9281 or APAU) was reported to promote weight loss and reduce visceral fat mass in obese mice fed a high-fat diet in parallel with increased oxygen consumption and UCP1 protein abundance in the iBAT, indicating a possible effect on activating BAT thermogenesis by sEH inhibitor [340].

Considering that bioactive EpFAs are metabolized by sEH, and many of the beneficial effects of EpFAs were potentiated by co-administration of an sEH inhibitor [326-328], sEH inhibition may improve obesity and its associated metabolic disorders due to, at least in part, stabilized endogenous EpFAs. However, the downstream EpFAs responsible for the anti-obesity effects of sEH inhibition have not been identified. Although the contribution of n-6 PUFA-derived EpFAs (n-6 EpFAs) cannot be ruled out, many studies have focused on the effects of n-3 EpFAs because of the well-recognized anti-obesity effects of n-3 PUFAs (discussed in section 2.4.3).

The transgenic mice fat-1 mice have provided a valuable model to study the effects of n-3 EpFAs in obesity and associated metabolic disorders [337]. Fat-1 mice expressed an n-3 desaturase that increases endogenous n-3 PUFAs in tissues [337]. It was found that insulin-sensitive tissues, including the liver and adipose tissue of fat-1 mice were enriched with n-3 EpFAs, such as 17,18-EEQ and 19,20-EDP [337]. When fed a high-fat diet, fat-1 mice gained significantly less body weight compared to the wild-type mice with a smaller adipocyte sizes and lower levels of inflammation and fibrosis in the epididymal WAT [337]. In addition, high-fat diet-induced hepatic inflammation and steatosis were significantly reversed in fat-1 mice, which were further reduced by the administration of sEH inhibitor *t*-TUCB [337]. Interestingly, the iBAT volume in fat-1 mice treated with *t*-TUCB was significantly increased compared to untreated controls, while *t*-TUCB treatment in wild-type mice did not affect the iBAT mass

[337]. But this study did not further investigate thermogenic genes expression in the iBAT or whole-body energy metabolism to qualify the BAT thermogenic activity.

Overall, these results suggest that sEH inhibition alone or combined with n-3 EpFAs-generating diet may be beneficial in obesity by targeting insulin-sensitive tissues, such as liver, WAT, and possibly BAT [337]. However, to our knowledge, no studies on the effects of specific n-3 EpFAs (EDPs/EEQs) on BAT in obesity treatment and prevention have been reported. Therefore, studies investigating the effects of EDPs/EEQs on BAT in obesity and the underlying mechanisms are warranted.

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Appendix

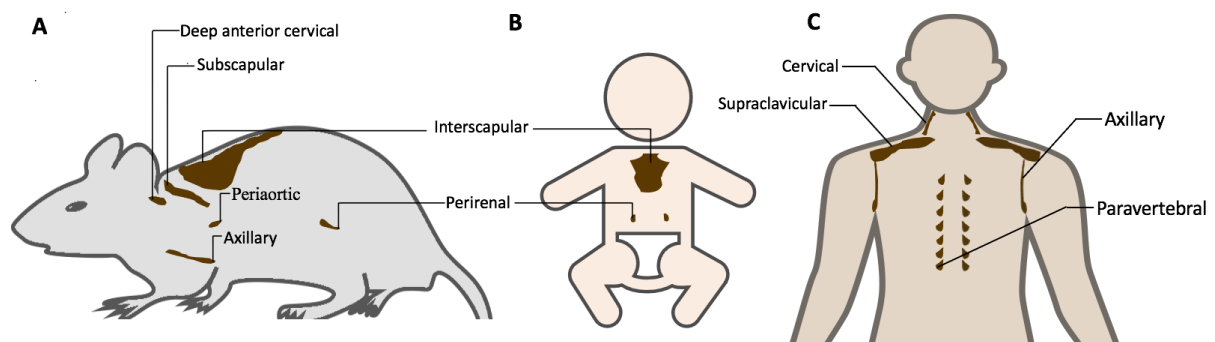


Figure 2.1. Major anatomic Locations of brown adipose tissue in mice and humans.

Brown adipose tissue (BAT) reside in the interscapular, axillary, deep anterior cervical, periaortic and perirenal area of mice. In infants, BAT major presents in interscapular and perirenal areas. In adult humans, it resides in the cervical, supraclavicular, axillary, and paravertebral regions. (Adapted from Kenji Ikeda and Pema Maretich et al. 2018 [341], additional reference [24-27, 34, 35])

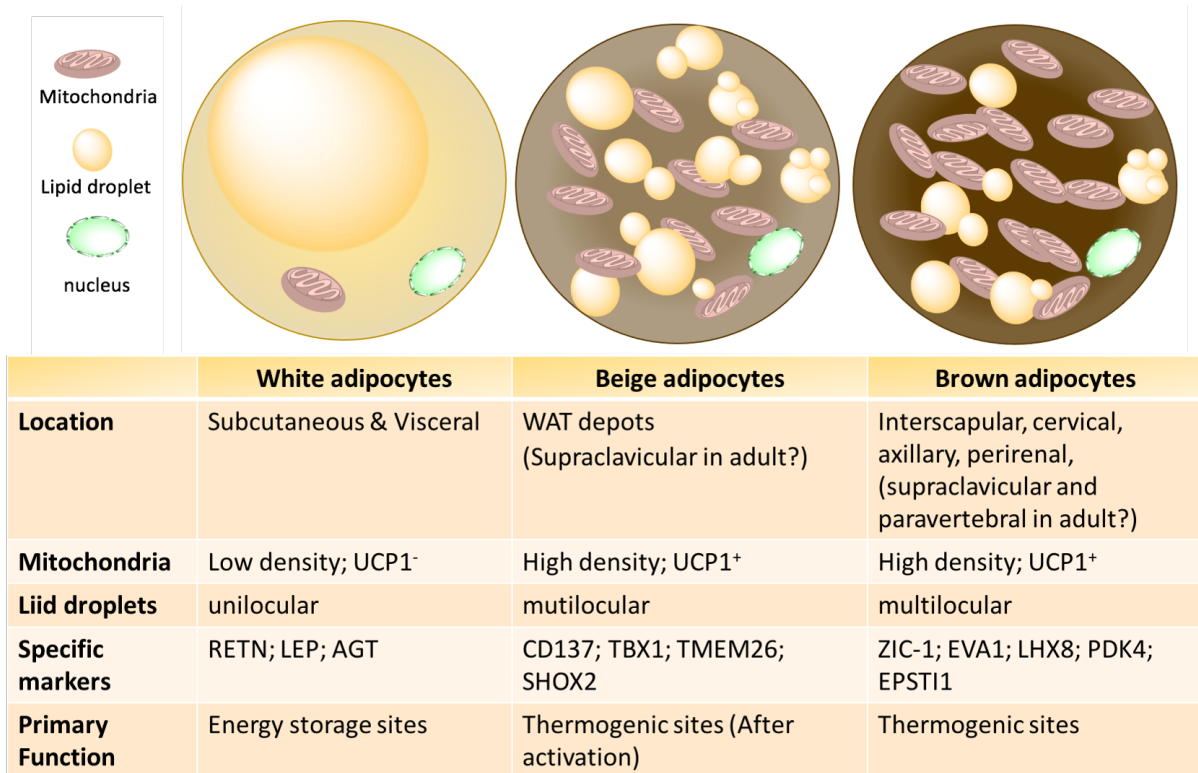


Figure 2.2. Schematic representation of three types of adipocytes. White, beige, and brown adipocytes are three types of adipocytes. Their major characters and markers are indicated below. (Adapted from Abhijit Babaji Shinde and Anying Song et al. 2021 [342]; additional reference[38-42].)

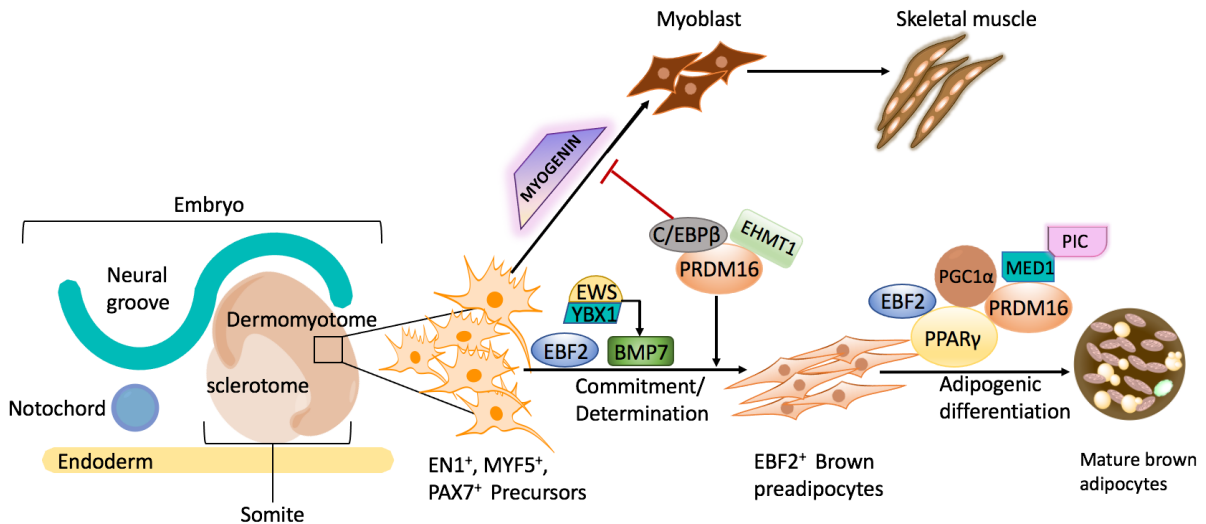


Figure 2.3. Developmental origins of brown adipocytes and the molecular regulators. EN1, MYF5, and PAX7 positive precursors, derived from the dermomyotome, are committed to brown preadipocytes. EBF2 or BMP7 regulates the commitment process from somatic precursors to brown preadipocytes. BMP7 is transcriptionally regulated by EWS/YBX1. PRDM16 complex (PRDM16/C/EBPβ/EHMT1) facilitates the brown adipose commitment while suppresses the myogenic process. Committed brown preadipocytes, marked by EBF2, differentiated to mature brown adipocytes regulated by multiple regulators including PPAR γ , EBF2, PGC-1 α , and PRDM16. EN1, engrailed 1; PAX7, paired box 7; MYF5, myogenic factor 5; EBF2, early B-cell factor 2; BMP7, bone morphogenetic protein 7; EWS, ewing sarcoma breakpoint region 1; YBX1, Y-box-binding protein 1; PRDM16, PR domain zinc finger protein 16; C/EBP β CCAAT/enhancer-binding protein-beta; EHMT1, euchromatic histone-lysine N-methyltransferase 1; PPAR γ , peroxisome proliferator activated receptor gamma; PGC-1 α , PPAR γ coactivator 1 alpha; PIC, preinitiation complex; MED1, mediator of RNA polymerase II transcription subunit 1. (Adapted from Wenshan Wang and Patrick Seale 2016[29])

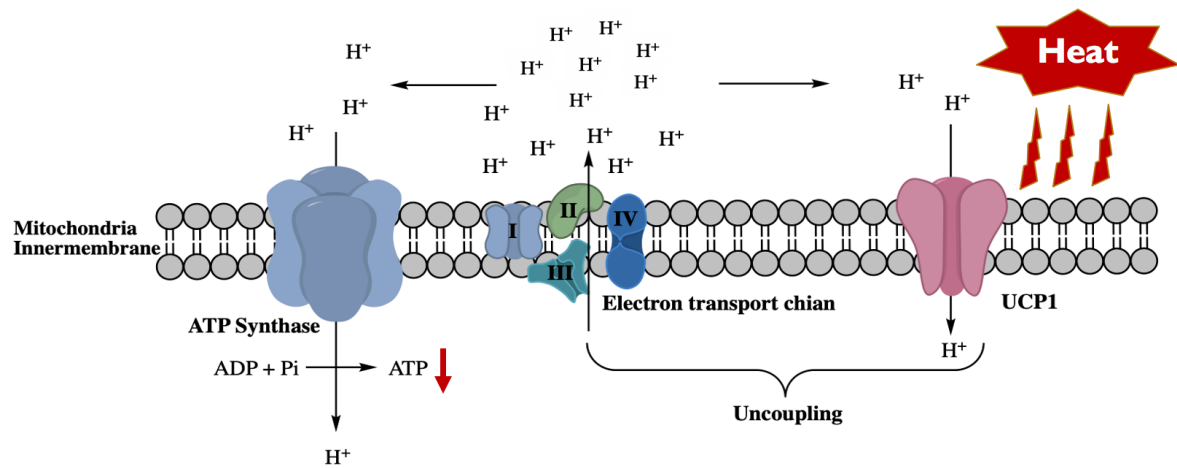


Figure 2.4. Uncoupling protein 1-mediated thermogenesis. Uncoupling protein 1(UCP1) presents in the inner membrane of mitochondria. It uncouples the proton gradient from ATP synthase to generate heat. (Adapted from Leticia de Almeida Brondani and Tais Silveira Assmann et al. 2012 [343])

**Chapter III Soluble Epoxide Hydrolase Inhibition by
t-TUCB Reduces Serum Triglycerides in Diet-Induced
Obese Mice**

A version of this chapter was originally published by Haley Overby, Yang Yang, Xinyun Xu, Katherine Graham, Kelsey Hildreth, Sue Choi, Debin Wan, Christophe Morisseau, Darryl C. Zeldin, Bruce D. Hammock, Shu Wang, Ahmed Bettaieb, and Ling Zhao:

Overby H*, Yang Y*, Xu X, Graham K, Hildreth K, Choi S, Wan D, Morisseau C, Zeldin DC, Hammock BD, Wang S, Bettaieb A, Zhao L. Soluble Epoxide Hydrolase Inhibition by *t*-TUCB Promotes Brown Adipogenesis and Reduces Serum Triglycerides in Diet-Induced Obesity. *Int J Mol Sci*. 2020 Sep 24;21(19):7039. * Denotes both authors contributed equally to this work and shared the first authorship.

This chapter has been revised from the published article in the following ways: *in vitro* results reported in Figure 1-8 in the article were removed and the abstract, introduction, result, and discussion sections were modified to fit with this dissertation content; font and figure numbers were altered to fit with this dissertation format. Some supplemental materials and methods and results were incorporated into the chapter.

Yang has contributed to experiments and data reported in this chapter (Figure 3.1-3.5) and the study designs, results, discussion, and other written portions of the manuscript regarding these data in the current chapter.

Abstract

BAT is an important target for obesity treatment and prevention. sEH converts bioactive EpFAs into less active diols. sEH inhibitors are beneficial in many chronic diseases by stabilizing EpFAs. Our lab previously found that sEH expression was increased in brown adipogenesis and in the BAT of diet-induced obese mice. In addition, sEH inhibitor *t*-TUCB promoted brown adipogenesis *in vitro*. However, the effects of sEH inhibitor in BAT activity in treating diet-induced obesity have not been reported. In this chapter, the effects of the sEH inhibitor *t*-TUCB were studied in obese mice via mini osmotic pump delivery. Although *t*-TUCB (3mg/kg/day) did not change body weight, fat pad weight, or glucose and insulin tolerance in the obese mice, it significantly decreased serum triglycerides level. In addition, the protein expression of PLIN, a lipid coating protein important for lipid metabolism, was significantly increased by *t*-TUCB treatment in the iBAT. Our results suggest that sEH inhibitor may be beneficial in improving BAT protein expression involved in lipid metabolism in high-fat diet-induced obese mice. Further studies using the sEH inhibitor combined with n-3 EpFAs substrates for obesity treatment and prevention are warranted.

3.1 Introduction

BAT has recently emerged as a novel target for obesity treatment/prevention [1-3]. In contrast to WAT, BAT is responsible for non-shivering thermogenesis through uncoupling ATP synthesis from respiration via UCP1, leading to dissipation of energy as heat [1]. It was long believed that BAT was transient within the first months of life and then drastically reduced in adulthood. Using PET/CT imaging technique, it was recently discovered that adult humans do have a significant amount of functional BAT [4-7]. These depots are either classical brown or beige fat [8-10]. Beige fat or brown-like fat is a type of fat that is formed in the WAT depot by a "browning" process in response to various stimuli [11, 12]. Current strategies to enhance functional BAT mass/activity include cold exposure and β -adrenergic stimulation; however, these strategies have issues of compliance and side effects. Novel effective strategies are needed for obesity treatment and prevention.

sEH (encoded by the *Ephx2* gene) is a predominant cytosolic enzyme that converts epoxy EpFAs into less active diols by adding a water molecule [13]. EpFAs are produced by CYP450 epoxygenase from n-6 PUFA, such as ARA, and n-3 PUFA, such as DHA and EPA. Many EpFAs are autocrine/paracrine lipid signaling molecules that play essential roles in pain, inflammation, vascular dilation, and cell growth/differentiation [13]; therefore, sEH, expressed in various tissues, including in white adipocytes and WAT [14], has become a pharmacological target. Potent small molecule sEH inhibitors have been developed to stabilize endogenous EpFAs and enhance their beneficial effects.

sEH inhibition and/or sEH deficiency have been shown to decrease ER stress [15] and inflammation [16] in the WAT and liver in diet-induced obesity and associated liver steatosis [16], cardiac remodeling [17], and endothelial dysfunction [18]. Interestingly, one study reported that sEH inhibitor-induced weight loss in high fat-high fructose-fed obese mice was associated with increased heat production and UCP1 protein expression in the iBAT [19]. In

another study, a different sEH inhibitor significantly increased the iBAT mass in the fat-1 mice [20], which had the transgenic expression of the n-3 desaturase, leading to enriched endogenous n-3 PUFA levels and higher n-3 EpFAs production [20]. However, whether an sEH inhibitor enhances iBAT activity and improves metabolic dysfunction in diet-induced obesity has not been investigated.

In the current chapter, the effects of sEH inhibition by *t*-TUCB were studied in obese C57BL/6J mice via osmotic pump delivery.

3.2 Materials and methods

3.2.1 Animal studies

All mice studies were approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville. Mice were singly caged at 22-23 °C with 12 hr light/dark shifts. Male C57BL/6J mice (n=10 per group) (The Jackson Laboratory) were purchased at 3 weeks old and were fed a high fat-diet diet (60% kcal from fat, D12492) (Research Diets) for 8 weeks to establish the obese condition. The mice were then surgically implanted with Alzet osmotic minipumps (model 2006) (DURECT Corporation, Cupertino, CA) filled *t*-TUCB into the subcutaneous compartment from an interscapular incision nearby the iBAT. *t*-TUCB was dissolved in a mixed solvent (25% DMSO in polyethylene glycol 400 (PEG400)) as described (7) and delivered at 3 mg/kg/day for 6 weeks. Insulin and glucose tolerance tests and indirect calorimetry were performed. Upon termination, four mice per group were randomly selected and subjected to cold tolerance tests. Immediately after the tests, whole blood was collected by cardiac puncture under anesthesia. After the mice were euthanized, various fat tissues, liver, and gastrocnemius muscle were collected and weighed. Some tissue was sampled for histological examination, and the rest was snap-frozen in liquid nitrogen then stored at -80 °C until analysis. The rest of the mice from each group were terminated the next day at room temperature (RT) following the same procedures.

3.2.2 Insulin and glucose tolerance test

For insulin tolerance tests, the mice were fasted for 6 hr before they were given insulin (Humulin) (Eli Lilly, Indianapolis, Indiana) at 0.5 U/kg of body weight through intraperitoneal injection. For glucose tolerance tests, the mice were fasted overnight (12-15 hr) before oral gavage with dextrose solution at 2 mg/g of body weight. Blood glucose levels were monitored and recorded at time 0, 15, 30, 60, 90, and 120 minutes after the administration of insulin or glucose with a handheld glucometer. These two tests were performed on all animals and one week apart.

3.2.3 Cold tolerance test

Mice were randomly selected (n=4 per group) and singly housed in their home cage without the filter top and bedding at 4 °C. Water and food were provided ad libitum. The mice body core temperatures were taken at time 0, 30, 60, 90, 120, 150, and 180 min, 4 hr, 5 hr, and 6 hr using RET 3 ISO rectal probe for mice and a portable temperature monitor (Physitemp Instruments, Clifton, NJ).

3.2.4 Lipid accumulation in the iBAT slides

Freshly isolated iBAT per mouse were sliced and fixed in 10% neutral buffered formalin before they were processed for Haematoxylin and Eosin staining at the University of Tennessee College of Veterinary Medicine Diagnostic Laboratory Service. 2-3 fields were taken per slide per mouse with a Nikon Eclipse E-600 microscopy. The pictures were analyzed by the Image J software.

3.2.5 Blood biochemical analysis

Plasma glucose was measured by Mouse Glucose Assay (Crystal Chem, Downers Grove, IL). Serum insulin was measured using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem). Serum triglycerides, NEFA, and cholesterol were measured using respective tests from Wako Diagnostics (FUJIFILM Wako Diagnostics, Mountain View, CA). Serum adiponectin

and leptin were measured using the Mouse Quantikine ELISA kit for the respective analyte (R&D Systems, Minneapolis, MN). All samples were analyzed according to the manufacturers' instructions.

3.2.6 Western blot analysis

Total cell lysates were prepared using 1X lysis buffer (Cell Signaling, Danvers, MA), and protein concentrations were determined using the BCA assay kit (Thermo Scientific, Waltham, MA). Proteins were separated on a 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked in TBST buffer (20 mM Tris Base, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4)) containing 5% nonfat milk. Membranes were then immunoblotted with the indicated primary antibodies against proteins of interest at 4 °C overnight, followed by 1 hr incubation with secondary antibodies conjugated with horseradish peroxidase. Bands of the protein of interest were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA). Densitometry of bands was quantified using Image Studio software (LI-COR Biosciences, Lincoln, NE) or ChemiDoc XRS+ system with Image Lab software (Bio-Rad, Hercules, CA).

3.2.7 Statistical analysis

The number of experiments and replicates are indicated in figure legends. Statistical analysis was performed using SigmaPlot 14.0 (Systat Software, San Jose, CA) or Prism 8 (GraphPad Software, San Diego, CA). Student's t-test was performed to determine the differences between treatment groups in body weight, food intake, tissue pad weight, core body temperature, cold tolerance test, oral glucose tolerance test (OGTT), and insulin tolerance test (ITT), respectively. Two-way ANOVA followed by the same multi group-wise comparison was performed to determine the differences between treatment groups and between the room temperature and cold condition. The level of significance was set at $p < 0.05$.

3.3 Results

3.3.1 *t*-TUCB delivered via mini osmotic pump did not decrease the body weight in diet-induced obese mice

To confirm whether the effects of sEH inhibition by *t*-TUCB on brown adipogenesis and function can be beneficial in diet-induced obesity in a mouse model, osmotic minipumps filled with *t*-TUCB to provide 3 mg/kg/day were implanted into the subcutaneous area on top of the iBAT of the diet-induced obese C57BL/6J male mice. The purpose of such delivery was to maximize the effects of *t*-TUCB on the iBAT while minimizing its systemic effects. After 6 weeks of treatment, *t*-TUCB did not change the body weight, food intake, fasting glucose level, or individual fat pad mass, including iBAT (Fig. 3.1). All figures are shown in the Appendix.

3.3.2 *t*-TUCB delivered via mini osmotic pump did not improve the cold tolerance in diet-induced obese mice

Since the important role of BAT in thermogenesis, we further investigated whether local delivery of *t*-TUCB can improve the cold tolerance in diet-induced obese mice. There was no difference in core body temperature at the baseline between two groups. In addition, *t*-TUCB did not improve the cold tolerance during 6 hr cold exposure at 4 °C compared to the controls (Fig. 3.2).

3.3.3 *t*-TUCB delivered via mini osmotic pump decreased serum triglycerides in diet-induced obese mice

To examine whether local delivery of *t*-TUCB can improve body metabolism, we performed an oral glucose tolerance test and insulin tolerance test (Fig. 3.3A-B). In addition, blood biochemical analysis was performed to analyze metabolic parameters (Fig. 3.3C-I). There were no improvements in glucose tolerance and insulin sensitivity by *t*-TUCB treatment compared to the groups (Fig. 3.3A-B). Although *t*-TUCB did not change blood levels of insulin, glucose, total cholesterol, NEFA, adiponectin, or leptin levels, serum TG levels at room

temperature (RT) were significantly decreased by *t*-TUCB compared to the controls ($p<0.05$ in RT) (Fig. 3.3C-I).

3.3.4 *t*-TUCB delivered via mini osmotic pump increased protein expression of genes involved in lipid metabolism in the iBAT of diet-induced obese mice

Histological analysis of the iBAT slides showed that *t*-TUCB treated mice tended to have less lipid accumulation caused by high-fat feeding than the control mice; however, the changes were not significant (Fig. 3.4A-B). Analysis of thermogenic protein expression in the iBAT revealed that *t*-TUCB treated mice had no significant increases in UCP1 and PGC1 α protein expression in the iBAT compared with the controls both in RT and the cold condition (Fig. 3.4C).

Since *t*-TUCB treated mice had significantly lower TG levels, we examined the protein expression of genes involved in fatty acid uptake from the circulation, including LPL and CD36, in the iBAT. *t*-TUCB treated mice had a higher LPL protein expression; however, the changes did not reach significance. There were no significant changes in CD36 expression in the iBAT in the *t*-TUCB treated mice compared to the controls (Fig. 3.5A-B).

We further analyzed the expression and phosphorylation of the proteins in the iBAT involved in lipolysis. The cold exposure significantly increased HSL protein abundance and phosphorylation of HSL at S565 and S660 ($p<0.05$) but did not affect the phosphorylation of HSL at S563. *t*-TUCB treated mice had a higher HSL protein abundance and higher phosphorylation of HSL at S563 and S660 at RT (Fig. 3.5C-D); however, the changes did not reach significance. Moreover, cold exposure decreased ATGL protein abundance ($p<0.01$); however, *t*-TUCB treatment did not affect ATGL protein expression (Fig. 3.5C-D). On the other hand, the cold exposure significantly decreased PLIN protein abundance ($p<0.01$) but increased phosphorylation of PLIN at S517 ($p<0.05$) (Fig. 3.5C-D). *t*-TUCB treated mice had significantly higher PLIN expression in the iBAT both in RT and the cold condition ($p<0.05$),

but no change in the phosphorylation of PLIN at S517 compared to the controls in either RT or the cold conditions (Fig. 3.5C-D).

3.4 Discussion

Although questions remain on the composition and origin of BAT in adult humans [21, 22], it is generally believed that BAT is a novel target to combat human obesity and associated metabolic disorders.

Our lab previously found that sEH expression was increased during both murine and human brown adipocyte differentiation [23], which are in line with sEH expression patterns in 3T3-L1 white adipocyte differentiation [14]. In addition, some diol/epoxide ratios were significantly higher in the media from differentiated brown adipocytes than that from the preadipocytes, which indicates higher sEH activities in the differentiated brown adipocytes [23]. Moreover, the sEH inhibitor *t*-TUCB dose-dependently promoted brown adipogenesis and mitochondrial uncoupling *in vitro* [23].

Based on these brown cell culture results, we have focused on the effects of sEH inhibition on the iBAT in the animal studies in this chapter. To explore the mechanisms by which *t*-TUCB improved serum TG levels in diet-induced obese mice, we analyzed protein expression involved in TG uptake and breakdown in the iBAT. It has been reported that LPL and CD36, two genes primarily responsible for the hydrolysis of circulating TRLs and taking up fatty acids, respectively, were both upregulated in the BAT and required for increased TG clearance in response to the cold [24]. In addition, LPL expression was also increased by cold-induced lipokine 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DiHOME) in the BAT [25]. Injection of 12,13-DiHOME into obese B6 mice was shown to decrease serum TG without affecting body weight and glucose tolerance by increasing fatty acid uptake into the BAT [25]. Increased fatty acid uptake was attributed to increased translocation of fatty acid transporters, such as CD36, in brown adipocytes by the lipokine [25]. Due to limited tissue samples, we could not

investigate the effects of *t*-TUCB on CD36 translocation. Future studies are needed to investigate the effects of *t*-TUCB on fatty acid uptake in brown adipocytes. It is worth noting that we previously found that *t*-TUCB dose-dependently increased both mRNA and protein expression of CD36 and protein (but not mRNA) expression of LPL *in vitro* [23]. Therefore, decreased serum TG levels by *t*-TUCB local delivery seems to be consistent with the published reports on BAT activation by the cold and the lipokine [24, 25] and suggests that *t*-TUCB may enhance BAT activity, which in turn can promote TG clearance from circulation by increasing hydrolysis of TG and possibly fatty acid uptake into the brown adipocytes.

In adaptive thermogenesis, BAT responds to the β -adrenergic stimulation by increasing lipolysis to provide fatty acids as substrates for heat production via uncoupling. As the most abundant lipid-coating protein on mature lipid droplets (LD), Plin1 is critical for NE-induced lipolysis in the BAT and thermal response to NE *in vivo* [26]. Moreover, transgenic mice with adipose-specific aP2 promoter/enhancer-driven murine Plin1 overexpression led to significant increases of mRNA expression of fatty acid oxidative genes in the BAT, but not in the WAT [27, 28]. Furthermore, phosphorylation of murine PLIN sequence on serine 517 (equivalent to human PLIN serine 522) by activated PKA in response to cold exposure is essential for the ATGL activity [29]. ATGL catalyzes the removal of sn-1 fatty acid from the stored TGs to produce diacylglycerol DAG [30]. DAG can be further hydrolyzed by HSL producing monoacylglycerol, which is further hydrolyzed by the monoacylglycerol lipase [31]. Phosphorylation of HSL on Ser 563, Ser 565, and Ser 660 have been shown to affect HSL-mediated lipolysis [32-34]. It is worth noting that our lab previously found that *t*-TUCB dose-dependently increased mRNA and protein expression of *Plin1* and mRNA expression of *Atgl* and *Hsl* in murine brown adipocytes *in vitro* [23]. Increases in lipolytic gene expression, together with increases in thermogenic UCP1 and PGC-1 α expression, are consistent with increases in ISO-stimulated oxygen consumption and mitochondrial uncoupling in *t*-TUCB

treated brown adipocytes *in vitro* [23]. In this study, we demonstrated that *t*-TUCB increased the PLIN protein expression in iBAT of obese mice. Combined with previous *in vitro* data, results from the current animal study suggest that *t*-TUCB may promote BAT activity by increasing protein expression involved in lipolysis, leading to less lipid accumulation in the BAT and significantly lower serum TG levels in diet-induced obesity.

It should be noted that our studies cannot rule out the effects of *t*-TUCB on other tissues, such as subcutaneous white fat and liver, to improve lipid metabolism. Previous studies have shown sEH inhibition reduced inflammation [16], reduced ER stress [15], and restored autophagy [20] in the white adipose and liver, and improved the overall metabolic health in DIO of mice. In addition, it is worth noting that the BAT is partially activated at 22-23 °C (RT) and further activated at 4 °C (Cold) in our studies; however, there was minimal browning of iWAT and eWAT in the mice either at RT or at 4 °C for only 6 hr (data not shown). Therefore, the observed decreases in TG levels in *t*-TUCB treated mice at RT are more likely due to the enhanced BAT activities rather than enhanced browning of white fat. Previous studies by others have also investigated this topic. It was reported that sEH genetic deficiency led to the reprogramming of white fat with increases in mitochondrial and thermogenic *Ucp1* mRNA expression [35]. Similarly, an EET analog was shown to induce UCP1 protein expression in 3T3-L1 adipocytes [36]. Therefore, it is possible that sEH inhibition could affect browning; however, our current study designs do not support the study of browning of the WAT. Future studies are needed to study the effects of *t*-TUCB on the browning of WAT.

In a preventive study, *t*-TUCB was systemically administered at ~1.67 mg/kg/day for 16 weeks starting at the same time with a high-fat diet; it increased BAT volume of fat-1 mice, but not the WT controls [20]. Due to overexpression of an n-3 desaturase, fat-1 mice had increased endogenous levels of n-3 PUFA and n-3 PUFA-derived EpFAs. The *t*-TUCB administration further increased n-3 PUFA derived EpFAs in the fat-1-mice. In contrast, *t*-

TUCB did not seem to affect the weight gain or total (white) fat volume in either fat-1 or WT mice on the high-fat diet [20]. Our studies further add to the current understanding by demonstrating the effects of *t*-TUCB on improving TG levels in high fat diet-induced obese mice. The *t*-TUCB dose at 3 mg/kg/day we used has been demonstrated by other studies to achieve beneficial effects with minimal toxicity in mice [20, 37]. On the other hand, our findings that *t*-TUCB at this dose for 6-weeks improved TG levels but did not alter body weight in the obese mice seem to support the notion that enhanced BAT mass/activity may improve metabolisms in a relatively short period but may require a longer duration of intervention to decrease body weights. For example, BAT transplantation improved high fat-induced glucose intolerance after 8 weeks of transplantation and only partially attenuated the body weight after 12 weeks of transplantation [38]. Together, these results suggest that sEH inhibition by *t*-TUCB may be a novel strategy for diet-induced obesity by promoting BAT activities; however, a long duration of treatment or combination with EpFAs-generating diets may be necessary to reduce the body weight gain and other aspects of metabolism in obesity.

The mechanisms by which the sEH inhibitors improved BAT protein expression in obese mice remain to be determined. Our lab previously found that *t*-TUCB activated both PPAR γ and PPAR α in brown preadipocytes at the tested concentrations *in vitro* [23]. PPAR α and PPAR γ are known to be expressed in the BAT [39, 40]. Both PPARs modulate gene expression by binding to the PPAR response element(s) present in the target genes' promoter in heterodimers with the RXR. PPARs target genes include *Ucp1* [12, 39], *Lpl* [39], and *Plin1* [40], which were found to be induced by *t*-TUCB in the current *in vivo* study and our previous *in vitro* studies[23]. Herein, *t*-TUCB most likely functions either through the stabilization of EpFAs or by acting as a PPAR agonist. EETs, among the main EpFAs generated by CYPs from AA, were shown to bind and activate PPARs and induce target gene transcription [41]. Some of the biological effects of EETs and sEH inhibitors have been shown to be mediated through

PPAR γ in various disease models [41-45]. However, it is also possible that *t*-TUCB may mimic the effects of EpFAs by binding to PPARs directly as an agonist, as the structural component of urea present in *t*-TUCB has been used to mimic epoxides [46]. Future studies are needed to explore the mechanisms by which *t*-TUCB activates PPARs to modulate gene expression in brown adipocytes and BAT.

3.5 Conclusion

In conclusion, although the exact molecular mechanisms are yet to be determined, the results presented herein show that sEH inhibition may be beneficial in improving BAT protein expression involved in lipid metabolism in diet-induced obesity. Further studies using the sEH inhibitors combined with EpFAs substrates for obesity treatment and prevention are warranted.

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Appendix

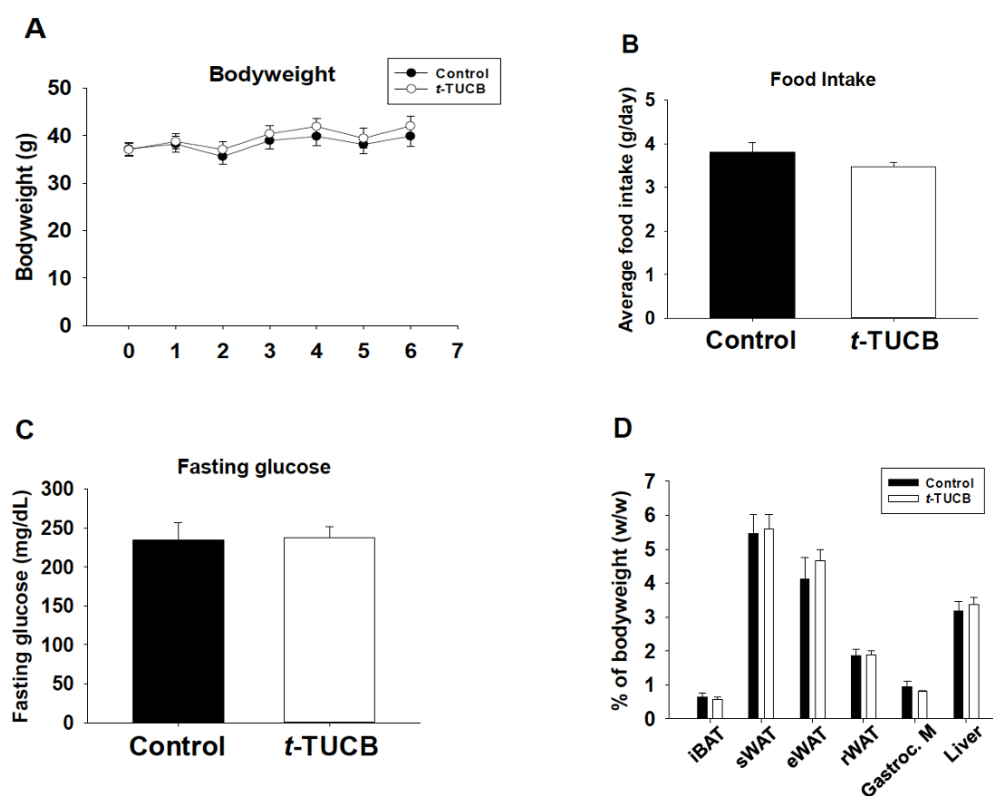


Figure 3.1. *t*-TUCB delivered via mini osmotic pump did not decrease the body weight in diet-induced obese mice. C57BL/6J mice were fed a high-fat diet for 8 weeks, followed by *t*-TUCB (3 mg/kg/day) (open bar or circle) or the vehicle (solid bar or circle) containing osmotic minipump implantation for 6 weeks. Body weight, food intakes, fasting glucose, and various tissue pad weights were recorded (A) Changes in body weight after the mini pump implantation; (B) Average food intake per day after the mini pump implantation; (C) Fasting glucose levels after 6 weeks of mini pump implantation; (D) Fat pad weights at termination. No significant differences were detected between the treated and the control group (Student's t-test).

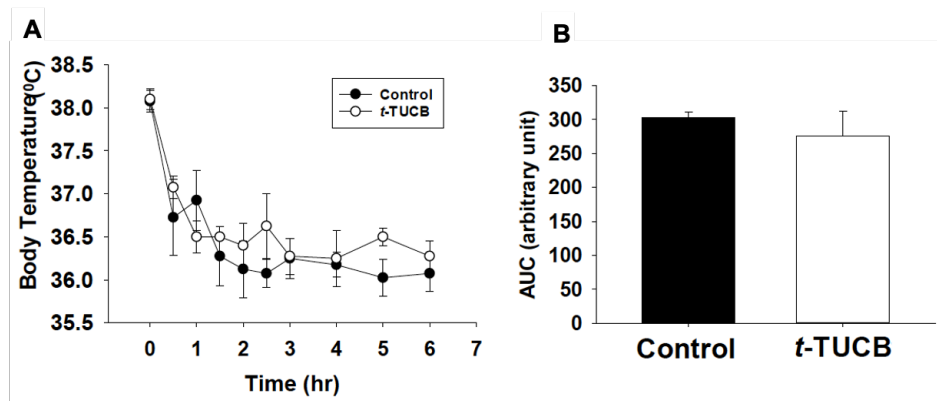


Figure 3.2. *t*-TUCB delivered via mini osmotic pump did not improve the cold tolerance in diet-induced obese mice. C57BL/6J mice were fed a high-fat diet for 8 weeks, followed by *t*-TUCB (3 mg/kg/day) (open bar or circle) or the vehicle (solid bar or circle) containing osmotic minipump implantation for 6 weeks. Core body temperature (A) and cold tolerance tests (B) were performed as described. No significant differences were detected between the treated and the control group (Student's t-test).

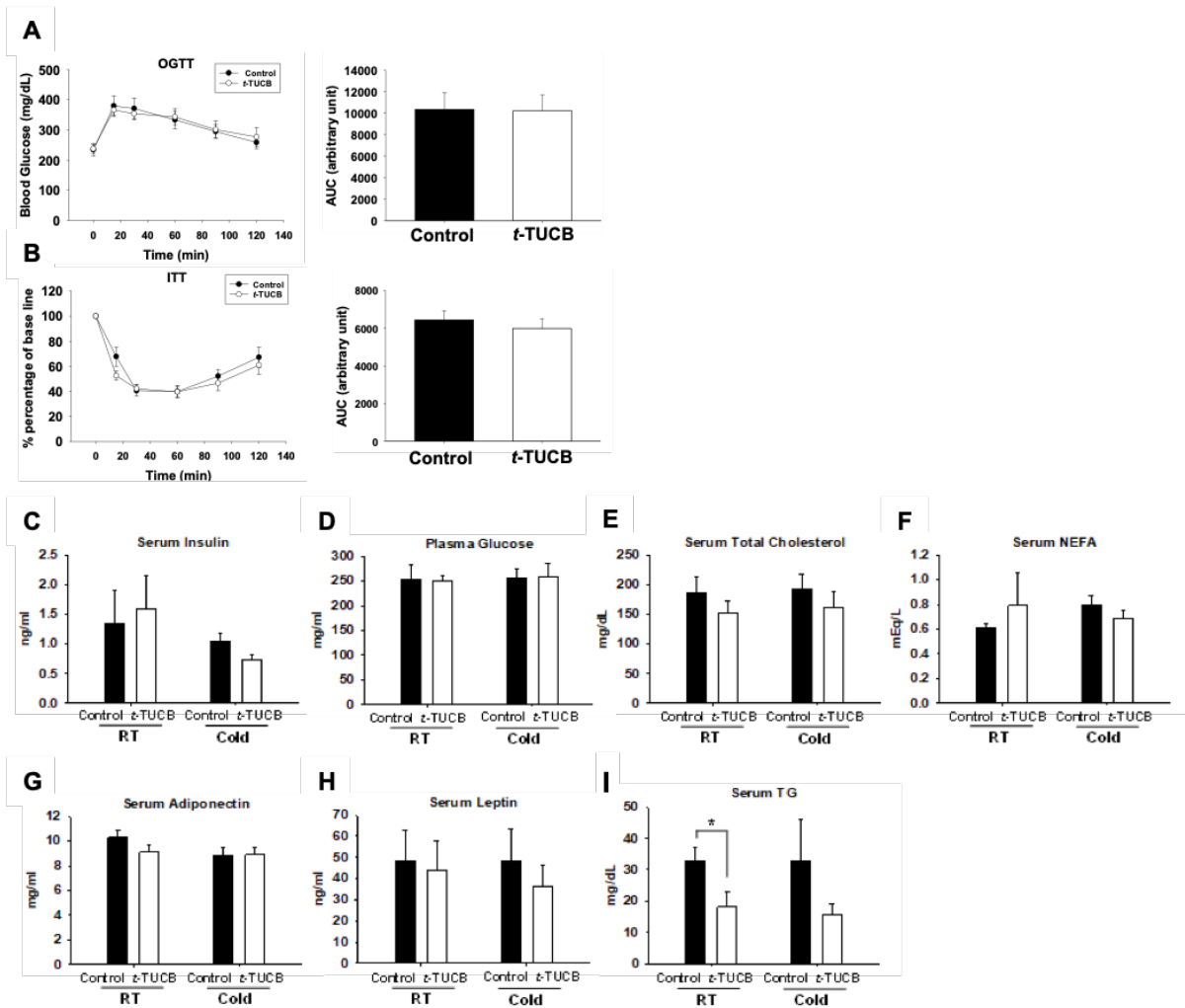


Figure 3.3. *t*-TUCB delivered via mini osmotic pump decreased serum triglycerides in diet-induced obese mice. C57BL/6J mice were fed a high-fat diet for 8 weeks, followed by *t*-TUCB (3 mg/kg/day) or the vehicle containing osmotic minipump implantation for 6 weeks as described. Oral glucose test (A) and insulin tolerance test (B) were performed as described. Serum insulin (C), plasma glucose (D), serum total cholesterol (E), NEFA (F), adiponectin (G), leptin (H), and TG (I) levels in the control and *t*-TUCB treated mice in the RT and cold condition. Data=Mean+SEM (n=4-5). *, **, ***, $p<0.05$, $p<0.01$, and $p<0.001$ compared to the controls, respectively. No significant differences were detected between the treated and the control group in the glucose tolerance test and insulin tolerance test (Student's *t*-test).

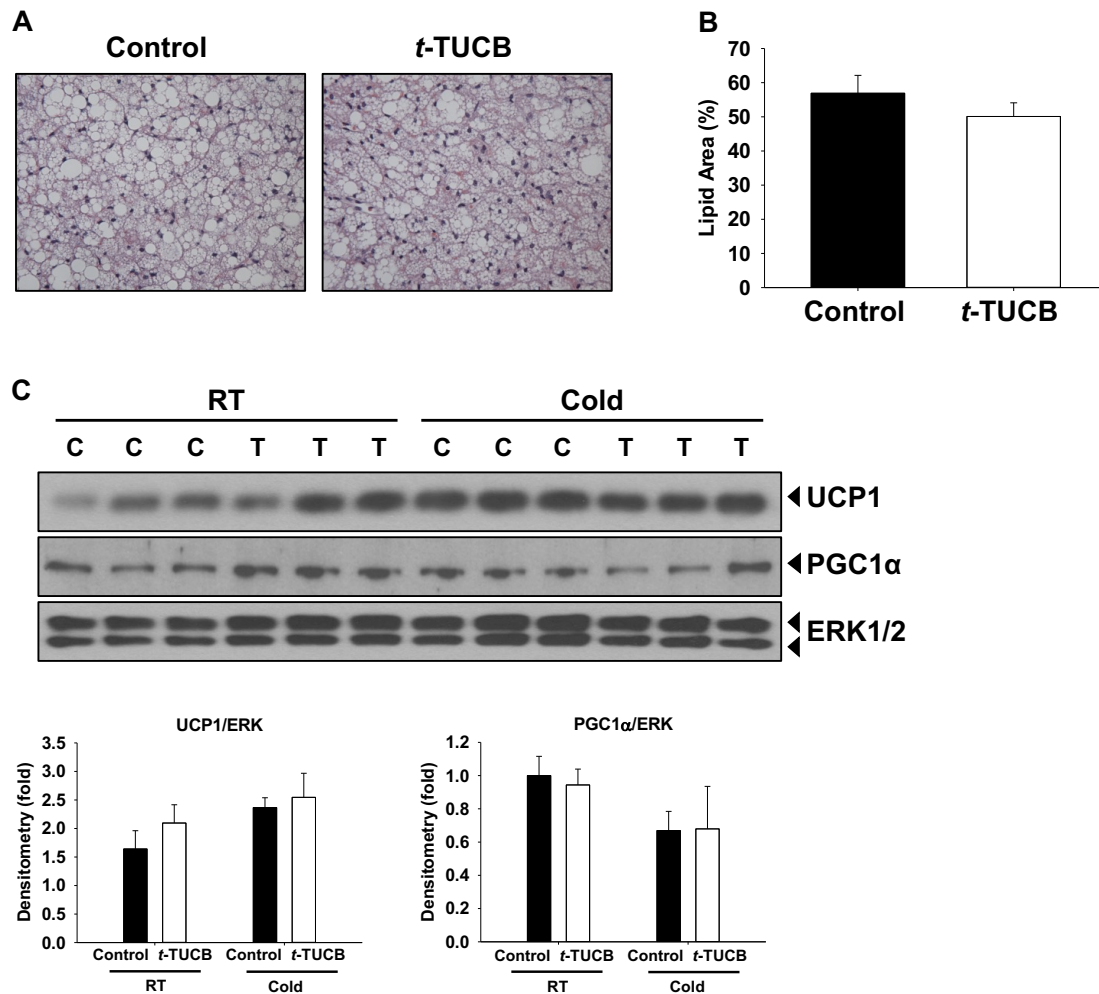


Figure 3.4. The effects of *t*-TUCB minipump delivery on lipid accumulation and UCP1 and PGC1α protein expression in the iBAT of obese C57BL/6J mice. After termination, the iBAT tissue slides from mice were stained with Hematoxylin and Eosin (H&E) (A), and % area occupied by lipid from 2-3 fields per slide per mouse (n=9-10) were measured using Image J software as described and graphed in (B). (C) Protein expression of UCP1 and PGC1α in the BAT of *t*-TUCB treated or the control mice. Bar graphs show normalized densitometry for UCP1/ERK and PGC1α/ERK. Data=Mean+SEM (n=3). No significant differences were detected between the groups.

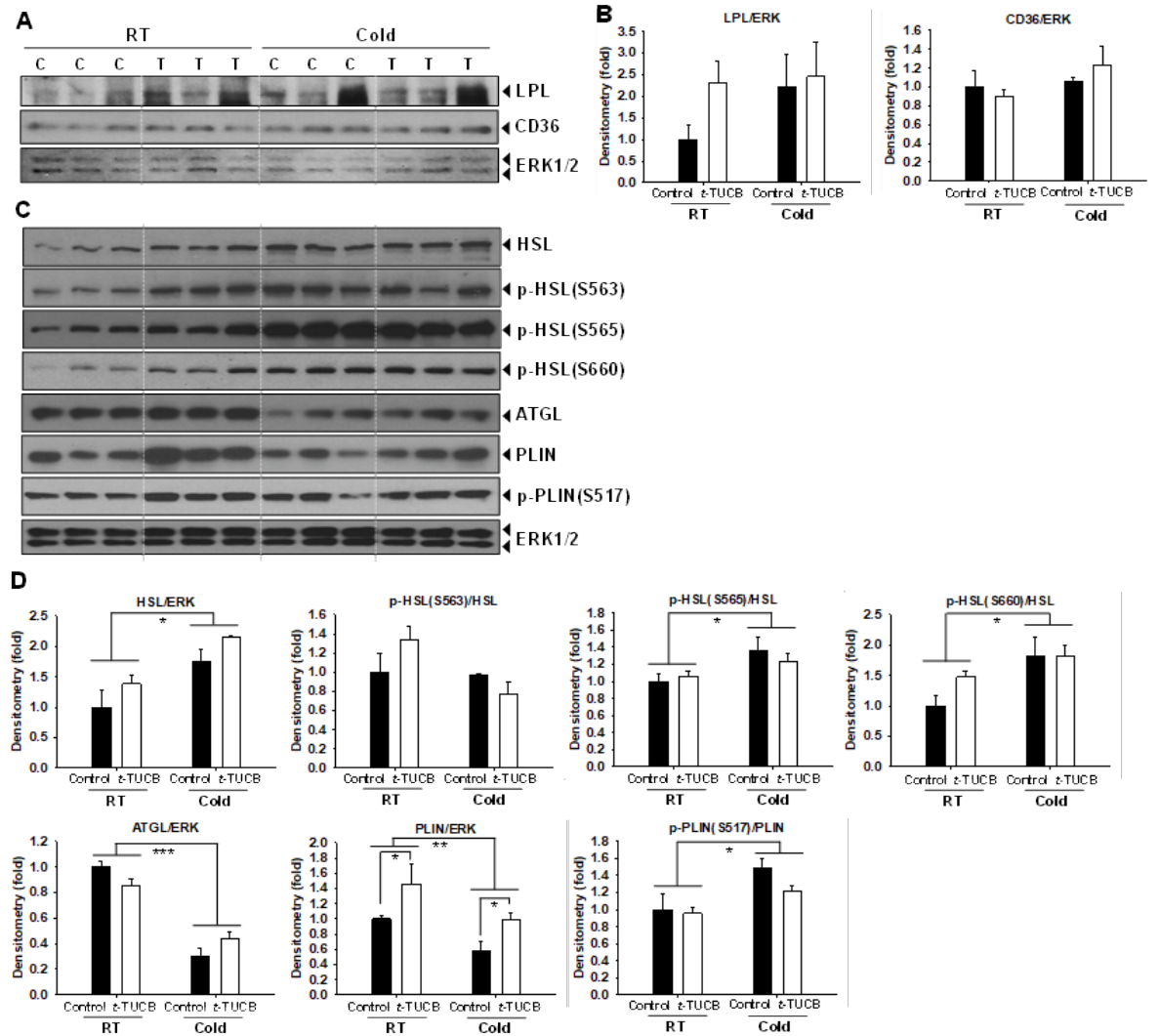


Figure 3.5. *t*-TUCB delivered via mini osmotic pump increased protein expression of genes involved in lipid metabolism in the iBAT of diet-induced obese mice. (A-B) Protein expression of LPL, CD36, and the loading control ERK in the iBAT of *t*-TUCB treated and the control mice, and their densitometry. (C-D) Protein expression of HSL, ATGL, PLIN, the phosphorylation of HSL and PLIN, and the loading control ERK in the iBAT of *t*-TUCB treated or the control mice and their densitometry. Bar graphs show normalized densitometry for LPL/ERK1/2 and CD36/ERK1/2 in (B) HSL/ERK1/2, ATGL/ERK1/2, PLIN/ERK1/2, and p-HSL(S563)/HSL, p-HSL(S565)/HSL, p-HSL(S660)/HSL and p-PLIN(S517)/PLIN in (D). Data=Mean+SEM (n=3). *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared to the controls, respectively.

**Chapter IV Effects of 19,20-EDP Combined with
Soluble Epoxide Hydrolase Inhibition by *t*-TUCB in
Diet-Induced Obese Mice**

Abstract

Obesity is a worldwide epidemic associated with various comorbidities. BAT becomes a promising target for obesity treatment and prevention due to its role in energy homeostasis. 19,20-EDP is a bioactive EpFAs derived from an n-3 PUFA DHA by CYP450 epoxygenase. 19,20-EDP has been reported to be anti-inflammatory and cardioprotective recently. However, endogenous EpFAs are not stable and are metabolized into less active diols *in vivo* by a cytosolic enzyme, sEH. sEH inhibitors are, therefore, beneficial in many chronic diseases by stabilizing endogenous EpFAs, including 19,20-EDP. We have found that *t*-TUCB, a potent sEH inhibitor, promoted brown adipogenesis *in vitro*. In addition, minipump delivery of *t*-TUCB (3mg/kg/day) to the iBAT decreased serum triglycerides and increased protein expression of genes important for lipid metabolism in the BAT of diet-induced obese mice (Chapter III). However, whether the addition of 19,20-EDP can enhance the beneficial effects of *t*-TUCB is unknown. Therefore, in this chapter, we investigate the potential beneficial effects of 19,20-EDP alone or combined with *t*-TUCB via osmotic minipump in diet-induced obese mice. We found that 19,20-EDP alone or combined with *t*-TUCB improved the cold tolerance test in the obese mice. However, body weight, fat pad mass, glucose tolerance, insulin tolerance, or blood metabolic parameters were not changed by 19,20-EDP alone or combined with *t*-TUCB. Future studies on the mechanisms by which 19,20-EDP combined *t*-TUCB improves cold tolerance and on the effective treatment time and dose are warranted.

4.1 Introduction

BAT has become a novel target for obesity treatment or prevention due to its thermogenic function [1-3]. Current strategies to enhance functional BAT mass/activity to combat obesity, such as cold exposure and β -adrenergic agonists, have been challenged by incomppliance and side effects [4]. Novel effective strategies targeting BAT to fight against obesity and associated metabolic disorders are needed.

Dietary ingredients, such as fish oil with enriched DHA and EPA, have been reported to promote BAT activity [5, 6]. It has been reported that fish oil, commonly enriched with EPA and DHA, increased the UCP1 mRNA expression and protein expression in the iBAT of rats fed with a high-fat diet, which was accompanied by decreased WAT pad weight [7, 8]. Similarly, fish oil intake increased the core body temperature and heat production by upregulating UCP1 and β_3 -AR of the iBAT in mice fed a chow diet [9]. Moreover, fish oil prevented the high-fat diet-induced body weight gain and hypertriglyceridemia and improved the glucose tolerance in part by enhanced BAT thermogenesis, as evidenced by upregulated expression of UCP1, PGC1 α , and β_3 -AR in the iBAT [10]. The mechanisms by which n-3 PUFA promotes BAT activity to combat obesity may partially be attributed to the production of n-3 PUFA-derived metabolites, but the specific metabolites are not fully determined [11].

N-3 EpFAs are bioactive metabolites generated by CYP450 epoxygenases from n-3 PUFAs [12, 13]. These EpFAs are known to modulate angiogenesis, vascular dilation, inflammation, and cell growth and differentiation and are implicated in tumor growth and metastasis, cardiovascular disease, type 2 diabetes, metabolic syndromes, and pain [14-16]. However, these EpFAs are not stable and are metabolized into less active diols quickly *in vivo* by soluble sEH, encoded by the *Ephx2* gene [17]. Therefore, many of the beneficial effects of these EpFAs were potentiated by co-administration of an sEH inhibitor [13, 15, 16]. Interestingly, it has been reported that an sEH inhibitor significantly increased the iBAT mass

in the fat-1 mice [18], which had the transgenic expression of the n-3 desaturase, leading to the accumulation of endogenous n-3 PUFA and its derived EpFA [18]. Moreover, sEH inhibitor alone did not show strong beneficial effects in some disease models unless it was combined with an EpFAs [19] or enriched tissue levels of n-3 PUFAs [18]. These results led us to hypothesize that n-3 EpFAs may be responsible for the anti-obesity effects of n-3 PUFAs by regulating BAT activity.

We have previously demonstrated that *t*-TUCB promoted brown adipogenesis *in vitro* [20], decreased serum TG levels, and increased protein expression of lipid metabolic genes in the iBAT of diet-induced obese mice (Chapter III). However, the effects of n-3 EpFAs in obesity are unknown. 19,20-EDP is the predominant EpFA derived from DHA, which is enriched in fish oil. Therefore, here we investigate the effects of 19,20-EDP alone or combined with *t*-TUCB on BAT activation in obese C57BL/6J mice via osmotic pump delivery.

4.2 Materials and methods

4.2.1 Animal studies

All mice studies were approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville. Male C57BL/6J mice (n=15 per group) (The Jackson Laboratory) were purchased at 3 weeks old and were singly housed at 22-23°C with 12 hr light/dark shifts. After one week of acclimation, the mice were fed a high fat-diet diet (60% kcal from fat, D12492) (Research Diets) for 8 weeks to establish diet-induced obesity. The mice were then surgically implanted with Alzet osmotic minipumps (model 2006) (DURECT Corporation, Cupertino, CA) filled with the following: mixed solvent (Control), 19,20-EDP alone (0.05 mg/kg/d) (EDP) or 19,20-EDP (0.05 mg/kg/d) combined with *t*-TUCB (3 mg/kg/d) (E+T). All pumps were implanted into the subcutaneous compartment from an interscapular incision nearby the iBAT. *t*-TUCB, 19,20-EDP were dissolved in a mixed solvent (25% DMSO in polyethylene glycol 400 (PEG 400) as previously described [21]. Insulin and glucose

tolerance tests and indirect calorimetry were performed. Upon termination, seven to eight mice per group were randomly selected and subjected to cold tolerance tests (6 hours cold exposure from 10 am to 4 pm) and remained in the cold environment until 24 hr. After 24hr-cold exposure, whole blood was collected by cardiac puncture under anesthesia. After the mice were euthanized, various fat tissue, liver, and gastrocnemius muscle were collected and weighed. Some tissues were sampled for histological examination, and the rest was snap-frozen in liquid nitrogen then stored at -80°C until analysis. The rest of the mice from each group were terminated the next day at room temperature following the same procedures.

4.2.2 Insulin and glucose tolerance test

For insulin tolerance tests, mice were randomly selected (n=7 per group) and were fasted for 6 hr before giving the insulin (Humulin) (Eli Lilly, Indianapolis, Indiana) at 0.5 U/kg of body weight through intraperitoneal injection. For glucose tolerance tests, 9-10 mice per group were randomly selected and were fasted overnight (12-15 hr) before oral gavage with dextrose solution at 1.5 g/kg of body weight. Blood glucose levels were monitored and recorded at time 0, 15, 30, 60, 90, and 120 min after the administration of insulin or glucose with a handheld glucometer. These two tests were performed 5 days apart.

4.2.3 Cold tolerance test

Mice were randomly selected (n=7-8 per group) and subjected to three-day cold acclimation before the cold tolerance test. Briefly, from day one to day three, mice were singly housed in their home cage without the filter top and bedding at 4°C for 6 hours from 10 am-4 pm, and then moved back to the single cage with filter top and bedding at room temperature 22-23°C for the rest of time. Water and food were provided ad libitum. On day 4, the mice were housed at 4 °C, and body core temperatures were taken at time 0, 15, 30, 60, 90, 120, 150, and 180 min, 4 hr, 5 hr, and 6 hr using RET 3 ISO rectal probe for mice connected with a portable temperature monitor (Physitemp Instruments, Clifton, NJ).

4.2.4 Blood biochemical analysis

The Mouse Glucose Assay kit (Crystal Chem, Downers Grove, IL) is used to measure plasma glucose. The Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem) was used to quantify the serum insulin level. Wako Diagnostics (FUJIFILM Wako Diagnostics, Mountain View, CA) was utilized to analyze serum triglycerides, NEFA, and cholesterol levels. All samples were analyzed according to the manufacturers' instructions.

4.2.5 Statistical analysis

The number of experiments and replicates are indicated in figure legends. Prism 8 (GraphPad Software, San Diego, CA) was used in statistical analysis. The differences between treatment groups (in body weight, food intake, tissue pad weight, core body temperature, cold tolerance test, OGTT, and ITT, respectively) were determined by Student's *t*-tests. The differences between treatment groups and between the room temperature and cold condition were analyzed by Two-way ANOVA followed by a multi-group-wise comparison. The level of significance was set at $p < 0.05$.

4.3 Results

4.3.1 19,20-EDP alone or combined with *t*-TUCB delivered via mini osmotic pump did not decrease the body weight, food intakes, and fasting glucose in diet-induced obese mice

To investigate whether the effects of 19,20-EDP alone or combined with sEH inhibitor *t*-TUCB on BAT function in the diet-induced obese mouse model, osmotic minipumps filled with 19,20-EDP or 19,20-EDP combined with *t*-TUCB were implanted into the subcutaneous area on top of the iBAT of the diet-induced obese C57BL/6J male mice, which had been fed with a high-fat diet for 8 weeks. After 6 weeks of treatment, 19,20-EDP alone or combined with *t*-TUCB did not change the body weight, food intake, fasting glucose level, or individual fat pad mass, including iBAT (Fig. 4.1). All figures are shown in the Appendix.

4.3.2 19,20-EDP alone or combined with *t*-TUCB via mini osmotic pump improved the cold tolerance in diet-induced obese mice

Due to the important role of BAT in thermogenesis, we further investigated whether local delivery of 19,20-EDP alone or combined with *t*-TUCB can improve the cold tolerance in diet-induced obese mice. Although there was no difference in core body temperature at the baseline in the two treated groups (the EDP and E+T group) compared to the controls, both 19,20-EDP alone or combined with *t*-TUCB significantly improved the cold tolerance during 6 hr cold exposure at 4°C compared to the controls ($p < 0.01$ for the EDP group and $p < 0.001$ for E+T group) (Fig. 4.2).

4.3.3 19,20-EDP or combined with *t*-TUCB via mini osmotic pump did not improve systemic metabolism in diet-induced obese mice

To examine whether local delivery of 19,20-EDP or 19,20-EDP combined with *t*-TUCB can improve metabolism, we performed the oral glucose tolerance test and insulin tolerance test (Fig. 4.3 A-B). In addition, blood biochemical analysis was performed to analyze metabolic parameters (Fig. 4.3 C-G). There were no improvements in glucose tolerance and insulin sensitivity by either 19,20-EDP alone or 19,20-EDP combined with *t*-TUCB treatment compared to the controls (Fig. 4.3 A-B). Cold exposure significantly decreased the serum insulin and TG levels while significantly increased the serum NEFA levels in all three groups (Fig. 4.3D-F). However, 19,20-EDP alone or combined with *t*-TUCB did not change plasma glucose, serum insulin, TG, NEFA, or total cholesterol (TC) levels compared to the controls (Fig. 4.3 C-G).

4.4 Discussion

In the current study, we tested the effects of 19,20-EDP, a prominent epoxy metabolite of DHA, alone or combined with *t*-TUCB in reducing the body weight and improving metabolic health in diet-induced obese mice. Under the experimental conditions (e.g., with doses and

treatment time), 19,20-EDP alone or combined with *t*-TUCB did not decrease the body weight nor improved the metabolic health in the obese mice. However, both 19,20-EDP alone and 19,20-EDP combined with *t*-TUCB significantly improved cold tolerance in the obese mice.

A few studies have tested whether enhanced BAT mass or activities can be of therapeutic value for obesity. For example, when small pieces of BAT were transplanted into high-fat diet-induced obese mice, high-fat diet-induced glucose intolerance was improved after 8 weeks' transplantation, and the body weight gains were only partially attenuated after 12 weeks of transplantation [22]. However, in another study, the body weights of diet-induced obese mice were significantly decreased as early as 4 weeks after BAT transplantation [23]. This discrepancy may be due to the different study designs between these two studies. In the first study [22], the brown fat pads (0.1 g per mouse) were taken from 12-weeks old donors and were transplanted into the visceral cavity of the recipient mice. In contrast, the BAT (0.15 g), taken from 6-weeks old donors, was transplanted into the subcutaneous interscapular area in the second study [23]. It is worth noting, the initial average body weight of mice before BAT transplantation was around 32-35 grams after 6-8 weeks of high-fat diet feeding (60% kcal from fat) in these two studies [22, 23]. The average body weight of non-transplanted mice reached to 45-50 grams with additional 10-12 weeks of high-fat feeding at the end of the study [22, 23]. However, the initial average body weight of mice before osmotic pump implantation, was already reached 42 grams in our study. Therefore, the unchanged body weight and systemic metabolisms by 19,20-EDP alone or combined with *t*-TUCB in our study may be partially due to the severe obese condition and the relatively short treatment duration (6 weeks).

The current dose of 19,20-EDP (0.05 mg/kg/day) used in our study was based on the serum levels achieved in mice fed an n-3 PUFAs enriched diet [24], which were reported to show beneficial effects in several disease models [21, 24, 25]. However, it may not be sufficient to reverse existing severe obesity in our current study. A higher dose of 19,20-EDP have been

used in other studies. 19,20-EDP treatment by osmotic pump delivery at the dose of 1 mg/kg/day for 10-14 days was shown to protect kidney function in renal fibrosis and hypertensive mouse models [26, 27]. Therefore, further studies are needed to determine the optimal dose of 19,20-EDP, possibly a range from 0.05 mg/kg/day to 1 mg/kg/day, to improve BAT activity to treat obesity.

Interestingly, although the core body temperature was not affected by 19,20-EDP alone or combined with *t*-TUCB at room temperature, mice treated with 19,20-EDP alone or combined with *t*-TUCB maintained higher core body temperature under 6-hours cold exposure at 4 °C. These results indicate that 19,20-EDP may facilitate BAT thermogenic function under certain stress conditions, such as cold exposure. The molecular mechanisms by which 19,20-EDP improves BAT thermogenesis after stimulation need further investigation. For example, we could investigate whether the beneficial effects of 19,20-EDP in cold tolerance observed here are associated with improved brown adipogenesis and enhanced thermogenic capacity at cell level using *in vitro* cell models. Brown adipogenesis can be studied by oil red O staining and brown marker gene expression, whereas the thermogenic capacity can be investigated by the mitochondrial stress test, which provides multiple parameters to analyze mitochondrial function, including basal and maximal respiration, ATP-production, and proton leak (i.e., uncoupling) [20]. In addition, stimulation with β -AR agonist, such as isoproterenol (ISO), can be used to mimic the cold exposure in mice [28]. Therefore, we can further investigate whether 19,20-EDP treatment can enhance the thermogenic capacity of murine brown cells with or without the ISO stimulation.

In addition, the EPA, another major n-3 PUFA enriched in fish oil, has been reported to improve BAT activity to combat obesity in mice [29], which may have targeted BAT and contributed to anti-obesity effects observed with fish oil. Moreover, in *fat-1* mice with a transgenic expression of the n-3 desaturase that increases endogenous n-3 PUFAs in tissues,

both 17,18-EEQ and 19,20-EDP were enriched in insulin-sensitive tissues, including liver and adipose tissue [18]. 17,18-EEQ is the major n-3 EpFA derived from EPA [30]. Furthermore, differential effects of DHA and EPA were reported in various disease models [31]. Therefore, the effects of 17,18-EEQ on BAT activation in reversing obesity and whether the effects of 17,18-EEQ are different from 19,20-EDP warrant further investigation in the future.

4.5 Conclusion

We report that 19,20-EDP alone or combined with *t*-TUCB improved cold tolerance but did not affect body weight and metabolic health in diet-induced obese mice under our study conditions. Future studies on the mechanisms by which 19,20-EDP combined with *t*-TUCB improve cold tolerance and on determining the effective treatment time and dose are warranted.

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Appendix

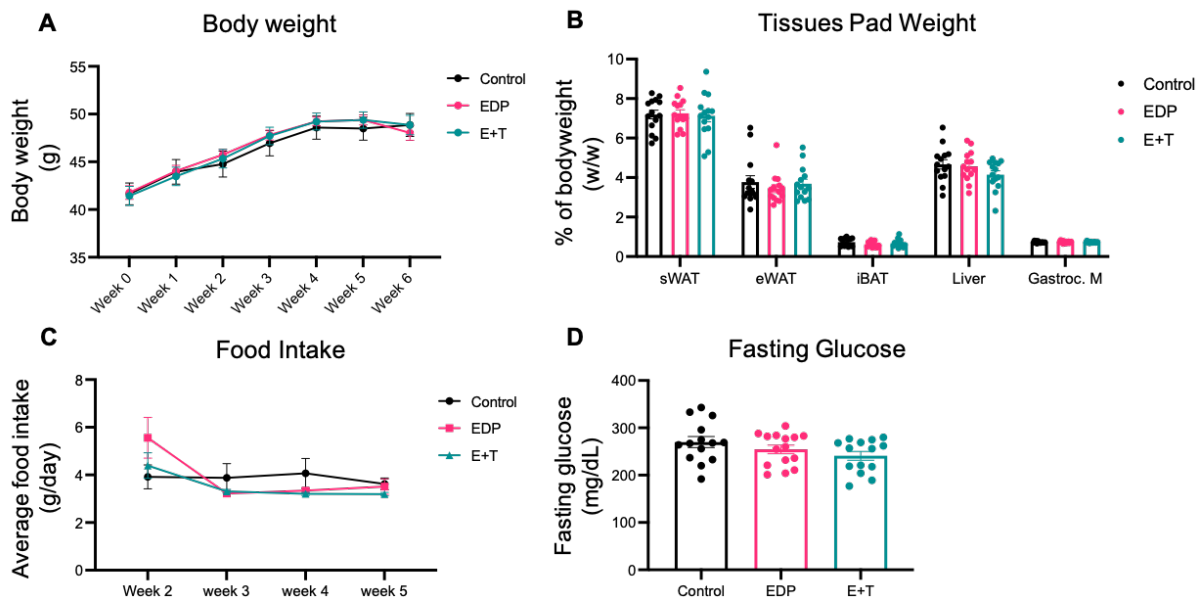


Figure 4.1. 19,20-EDP along or combined with *t*-TUCB delivered via mini osmotic pump did not decrease the body weight, food intake, and fasting glucose in diet-induced obese mice. C57BL/6J mice were fed the high-fat diet for 8 weeks, followed by implantation of osmotic minipumps filled with 19,20-EDP (0.05 mg/kg/d) (magenta color) or 19,20-EDP combined with *t*-TUCB (3 mg/kg/day) (turquoise color) or the vehicle control (black color) for 6 weeks. Body weight, food intakes, fasting glucose, and various tissue pad weights were recorded. (A) Changes in body weight after the mini pump implantation; (B) Fat pad weights at termination; (C) Average food intake per day after the mini pump implantation; (D) Fasting glucose levels after 6 weeks of mini pump implantation; Data=Mean±SEM. No significant differences were detected between the treated and the control group (Student's t-test).

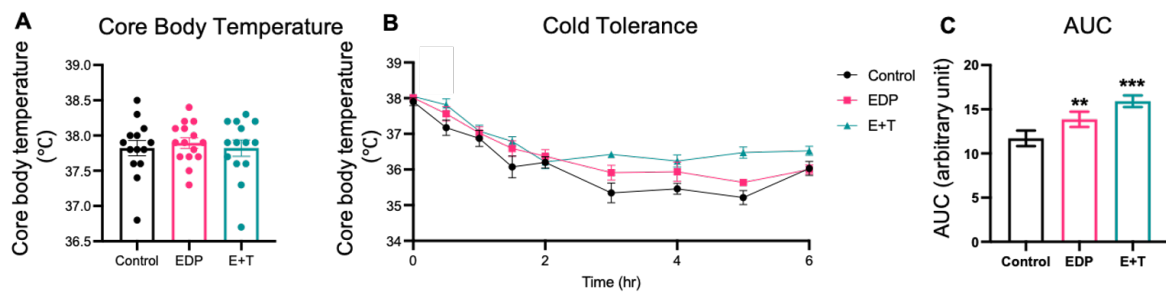


Figure 4.2. 19,20-EDP alone or combined with *t*-TUCB delivered via mini osmotic pump improved cold tolerance in diet-induced obese mice. C57BL/6J mice were fed the high-fat diet for 8 weeks, followed by implantation of osmotic minipumps filled with 19,20-EDP (0.05 mg/kg/day) (magenta color) or 19,20-EDP combined with *t*-TUCB (3 mg/kg/day) (turquoise color) or the vehicle control (black color) for 6 weeks. Core body temperature (n=14-15) (A) and cold tolerance tests (n=7-8) (B) were performed as described. The area under the curve (AUC) was calculated (C). Data=Mean±SEM. *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the controls, respectively (Student's t-test).

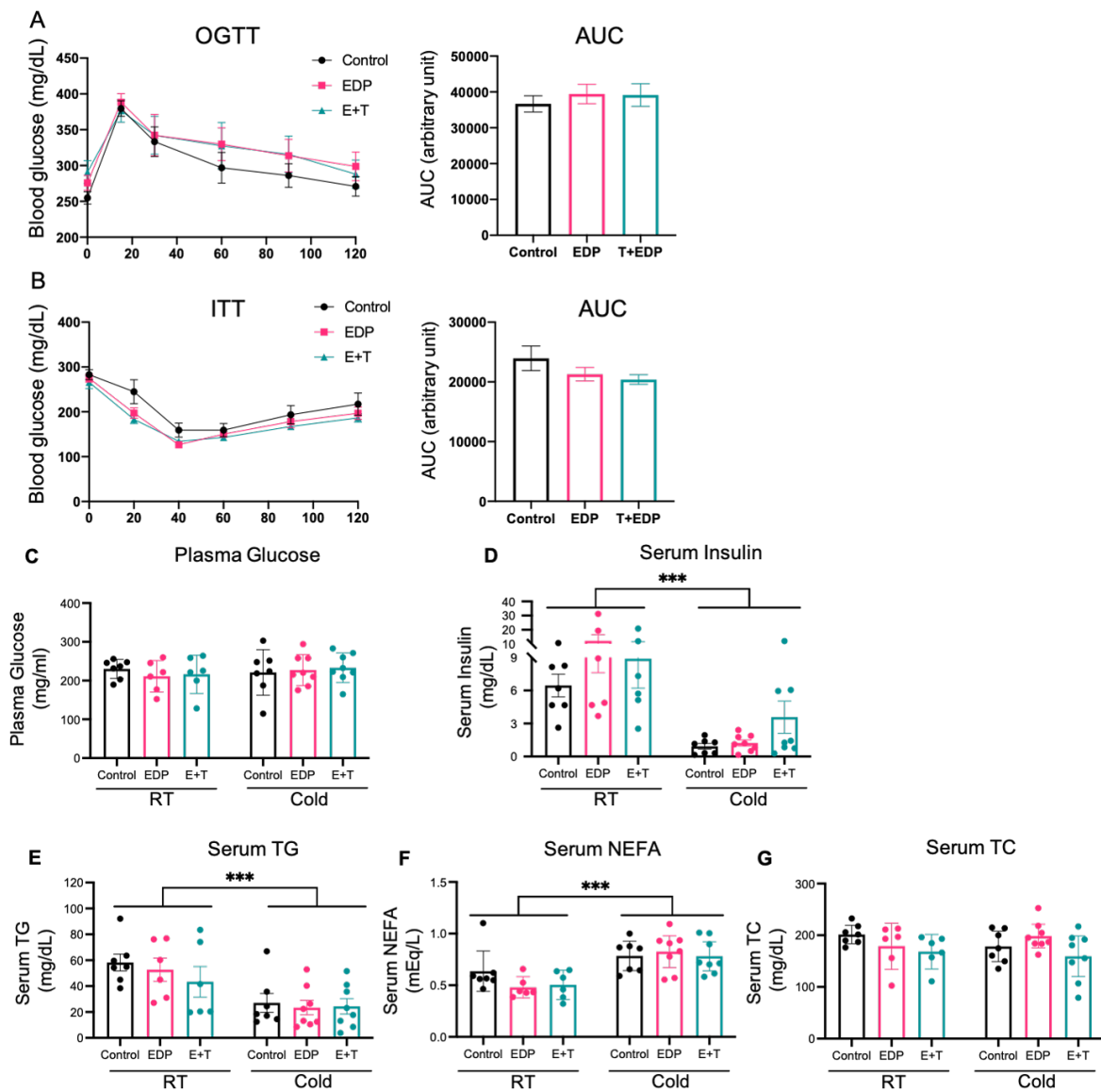


Figure 4.3. 19,20-EDP alone or combined with *t*-TUCB delivered via mini osmotic pump did not improve body metabolism in diet-induced obese mice. C57BL/6J mice were fed a high-fat diet for 8 weeks, followed by *t*-TUCB (3 mg/kg/day) or the vehicle containing osmotic minipump implantation for 6 weeks as described. Oral glucose test (A) and insulin tolerance test (B) were performed as described. Plasma glucose (C), Serum insulin (D), serum TG (E), NEFA (F), and TC (G) levels in the control, EDP, or E+T group in the RT and cold condition are shown. Data=Mean±SEM (n=7-8). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the room temperature, respectively. No significant differences were detected between the treated and the control groups in the glucose tolerance test and insulin tolerance test (Student's *t*-test).

**Chapter V Differential Effects of 17,18-EEQ and
19,20-EDP Combined with Soluble Epoxide
Hydrolase Inhibitor *t*-TUCB on Diet-induced Obesity
in Mice**

A version of this chapter has been published:

Yang Yang, Xinyun Xu, Haoying Wu, Jun Yang, Jiangang Chen, Christophe Morisseau, Bruce D. Hammock, Ahmed Bettaieb, and Ling Zhao. Differential Effects of 17,18-EEQ and 19,20-EDP Combined with Soluble Epoxide Hydrolase Inhibitor *t*-TUCB on Diet-induced Obesity in Mice. *Int. J. Mol. Sci.* 2021, 22(15), 8267; <https://doi.org/10.3390/ijms22158267>

This chapter has been revised from the article in the following ways: Introduction and results sections were modified to fit with this dissertation content; font and figure numbers were altered to fit with this dissertation format. Some supplemental materials were incorporated into the chapter and the caption was modified to fit with this dissertation content.

Yang has contributed to experiments and data reported in this chapter and the study designs, results, discussion, and other written portions of the manuscript regarding these data in the current chapter.

Abstract

17,18-EEQ and 19,20-EDP are bioactive epoxides produced from EPA and DHA, respectively, which are quickly metabolized to less active diols by cytosolic enzyme, sEH. We have previously demonstrated that an sEH inhibitor *t*-TUCB decreased serum TG and increased lipid metabolic protein expression in the iBAT of diet-induced obese mice. This study investigated the preventive effects of *t*-TUCB (T) alone or combined with 19,20-EDP (T+EDP) or 17,18-EEQ (T+EEQ) on BAT activation in the development of diet-induced obesity and metabolic disorders via osmotic minipump delivery in mice. Both T+EDP and T+EEQ groups showed significant improvement in fasting glucose, serum triglycerides, and higher core body temperature, whereas heat production was only significantly increased in the T+EEQ group. Moreover, both T+EDP and T+EEQ groups showed less lipid accumulation in the BAT. Although UCP1 expression was not changed, PGC1 α expression was increased in all three treated groups. In contrast, the expression of CPT1A and CPT1B, which are responsible for the rate-limiting step for fatty acid oxidation, was only increased in the T+EDP and T+EEQ groups. Interestingly, as a fatty acid transporter, CD36 expression was only increased in the T+EEQ group. Furthermore, both T+EDP and T+EEQ groups showed decreased inflammatory NF κ B signaling in the BAT. Our results suggest that 17,18-EEQ or 19,20-EDP combined with *t*-TUCB may prevent high-fat diet-induced metabolic disorders, in part through increasing thermogenesis, upregulating lipid metabolic protein expression, and decreasing inflammation in the iBAT.

5.1 Introduction

Obesity remains one of the biggest public health issues worldwide [1], which is associated with various diseases, including dyslipidemia, cardiovascular diseases, and certain types of cancers, leading to a shorter lifespan and higher medical costs [2, 3]. Recent studies have indicated that obesity is also associated with the high prevalence and severity of COVID-19, an infectious disease caused by coronavirus SARS-CoV-2 [4, 5]. A surge of studies targeting BAT to treat or prevent obesity due to its ability to increase energy expenditure through non-shivering thermogenesis [6-9]. Strategies that enhance BAT metabolic activity and improve BAT thermogenic function may confer beneficial diet-induced obesity and associated metabolic comorbidities.

17,18-EEQ and 19,20-EDP are the major EpFAs derived from EPA and DHA, respectively [10, 11]. These EpFAs modulate angiogenesis, vascular dilation, inflammation, and cell growth and differentiation and are implicated in tumor growth and metastasis, cardiovascular disease, diabetes, metabolic syndromes, and pain [12-14]. Because these epoxides are unstable and quickly metabolized to less active diols by a cytosolic enzyme, sEH (encoded by the *Ephx2* gene) [11], many of the beneficial effects of these EpFAs were enhanced by simultaneously providing an sEH inhibitor [11, 13, 14].

We have previously demonstrated that *t*-TUCB promoted brown adipogenesis *in vitro* [15], decreased serum TG levels, and increased protein expression of lipid metabolic genes in the BAT of diet-induced obese mice (Chapter III). In addition, 19,20-EDP alone or combined with *t*-TUCB significantly improved the cold tolerance in diet-induced obese mice (Chapter IV). In this study, we further investigated the effects of *t*-TUCB (T) alone or combined with 19,20-EDP (T+EDP) or 17,18-EEQ (T+EEQ) on BAT activation in preventing the development of diet-induced obesity and associated metabolic disorders in mice by mini pump delivery.

5.2 Materials and methods

5.2.1 Reagents

Antibodies used in the study are shown in Table 5.1. All tables and figures are shown in the Appendix.

5.2.2 Animal study

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee, Knoxville. Mice were housed individually at 22-23 °C with a 12-hour light/dark cycle.

3 weeks old male C57BL/6J mice were purchased from the Jackson Laboratory and were fed a regular chow diet (14% kcal from fat, 8604) (Teklad Rodent Diet, ENVIGO) for 5 weeks. At 8 weeks old, 10 mice were randomly selected as the non-transplanted controls. They were fed a low-fat diet (10% kcal from fat, D12450H) or a high-fat diet (45% kcal from fat, D12451) (Research Diets) for 6 weeks. At the same time, the rest mice were randomly divided into 4 groups (n=5-6 per group) and were fed the same high-fat diet (45% kcal from fat, D12451) (Research Diets). In addition, these 4 groups of mice were surgically implanted with Alzet osmotic minipumps (model 2006) (DURECT Corporation, Cupertino, CA) filled with the following: mixed solvent (the vehicle control), *t*-TUCB alone (T), or *t*-TUCB combined with 19,20-EDP (T+EDP) or 17,18-EEQ (T+EEQ). All pumps were implanted into the subcutaneous compartment from an interscapular incision nearby the iBAT. *t*-TUCB, 19,20-EDP, and 17,18-EEQ were dissolved in a mixed solvent (25% DMSO in polyethylene glycol 400 (PEG 400)) as previously described [16]. The deliver rate was 3 mg/kg/day for *t*-TUCB, and 0.05 mg/kg/day for 19,20-EDP and 17,18-EEQ, respectively. The maximal delivery duration was 6 weeks by design for the model. Body weight and food intake were measured every week. Insulin and glucose tolerance tests, indirect calorimetry, and cold tolerance tests were performed as previously described [15]. The animals were then terminated at room

temperature. Whole blood was collected by cardiac puncture under anesthesia. Subcutaneous WAT, epididymal WAT, interscapular BAT, liver, and gastrocnemius muscle were collected and weighed. A small piece of tissue samples was fixed for histopathological analysis, and the rest of tissue samples were snap-frozen in liquid nitrogen and then stored at -80 °C until analysis.

5.2.3 Blood biochemical analysis

The Mouse Glucose Assay kit (Crystal Chem, Downers Grove, IL) is used to measure plasma glucose. The Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem) was used to quantify the serum insulin level. Wako Diagnostics (FUJIFILM Wako Diagnostics, Mountain View, CA) was utilized to analyze serum triglycerides, NEFA, and cholesterol levels. All samples were analyzed according to the manufacturers' instructions.

5.2.4. Western blot analysis

Tissue samples were homogenized by an electric homogenizer in 1xRIPA lysis buffer (Cell Signaling, Danvers, MA) and then centrifuge for 15 minutes at 12000 rpm at 4 °C to obtain protein samples. BCA assay kit (Thermo Scientific, Waltham, MA) was used to determine protein concentrations. Protein samples (1-45 µg per lane) were separated on 10% or 12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes purchased from Bio-Rad (Hercules, CA) in the transfer buffer (25 mM Tris base, 190mM glycine, 20% methanol) at 25 volts for 20 hours. Then the membranes were blocked in TBST buffer (20 mM Tris Base, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4)) with 5% nonfat milk for 1 hour at room temperature. Next, membranes were immunoblotted with the indicated primary antibodies (Table 1) against proteins of interest at 4 °C overnight at 1:1000 dilution (at 1:2000 dilution for UCP1 antibody, 1:500 dilution for CD36 and HSL antibodies). After 10 minutes of wash by TBST buffer 3 times, membranes were incubated with secondary antibodies (horseradish peroxidase conjugated) at 1:4000 dilution for 1 hour. Bands of the

protein of interest were visualized by Pierce ECL Western Blotting Substrate or SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA). To facilitate the detection of some proteins of interest, two gels were run and detected at the same time to accommodate all samples and/or large sample volumes. Densitometry of bands was quantified using ChemiDoc XRS+ system with Image Lab software (Bio-Rad, Hercules, CA) or Image J software (an open source from NIH).

5.2.5 BAT lipid accumulation and sWAT adipocyte area determination

The iBAT and sWAT from each mouse were isolated. A small piece was fixed in 10% neutral buffered formalin and then processed for hematoxylin and eosin staining at the University of Tennessee College of Veterinary Medicine Diagnostic Laboratory Service. Nikon Eclipse E-600 microscopy was used to take pictures from 2-3 fields of each slide of iBAT or sWAT from each mouse. The % area occupied by lipid or adipocyte areas from 2-3 fields per slide per mouse was then quantified by the Image J software.

5.2.6. Statistical analysis

Prism 8 (GraphPad Software, San Diego, CA) was used to conduct statistical analysis. The two-tailed Student's t-test was used to analyze the differences in group means among the treatment groups and the control group. For the OGTT, ITT, cold tolerance tests, and heat production, areas under the curve were calculated using Prism 8, and a two-tailed Student's t-test was used to compare differences between the treatment and the control group. The level of significance was set at $p < 0.05$.

5.3 Results

5.3.1 17,18-EEQ or 19,20-EDP combined with *t*-TUCB differentially affected body weight in diet-induced obesity

To investigate whether treatments of *t*-TUCB alone or combined either with 17,18-EEQ or 19,20-EDP are beneficial in preventing the development of diet-induced obesity, osmotic

minipumps filled with vehicle control, *t*-TUCB (T) alone or combined with 19,20-EDP (T+EDP) or 17,18-EEQ (T+EEQ) were implanted into the subcutaneous compartment on top of the interscapular BAT (iBAT) of C57BL/6J male mice fed a high-fat diet (45% kcal from fat). In addition, we set up another two groups (n=5 per group) of mice fed either a low-fat diet (10% kcal from fat) or a high-fat diet (45% kcal from fat) for 6 weeks without osmotic minipumps implantation as controls.

6-weeks administration of the high-fat diet did not affect the food intake, body weight, brown fat weight but induced a significant increase in subcutaneous and epididymal WAT mass (Fig. 4.1A-E). Although the fasting glucose level was not affected, the glucose tolerance was impaired in mice fed the high-fat diet (Fig. 5.1F-G). In addition, the high-fat diet induced a significant increase in serum TG level without affecting serum NEFA and TC levels (Fig. 5.1H-J). Moreover, core body temperature and heat production were not different between low-fat diet-fed mice and high-fat diet-fed mice (Fig. 5.1K-L).

After 6 weeks of treatment, *t*-TUCB alone or combined with either 19,20-EDP or 17,18-EEQ did not change the food intakes and individual fat pad mass, including sWAT, epididymal WAT (eWAT), and iBAT (Fig. 5.2A, C-E). However, there were small but significant increases in body weight in both T and T+EDP groups compared with the vehicle control group (Fig. 5.2B). Although not significantly different from the control group, the T+EEQ group had a lower body weight than the T and T+EDP groups ($p<0.01$ and $p<0.001$, respectively) (Fig. 5.2B).

5.3.2 17,18-EEQ or 19,20-EDP combined with *t*-TUCB improved fasting glucose and serum TG levels in diet-induced obesity

To investigate the effects of sEH inhibition combined with 17,18-EEQ or 19,20-EDP on glucose metabolism, we performed ITT and OGTT. Although there were no differences in ITT among groups (Fig. 5.3A-B), the T+EEQ group showed a trend of improvement in glucose

tolerance tests ($p=0.079$) (Fig. 5.3C-D). Additionally, while there were no significant differences in serum insulin levels among groups (Fig. 5.3F), both T+EEQ and T+EDP groups showed significant improvement in fasting glucose compared to the controls (208.8 ± 36.4 mg/d vs. 214 ± 10.8 mg/dL vs. 262.8 ± 8.9 mg/dL) ($p<0.05$ and $p<0.01$, respectively) (Fig. 5.3E).

We have previously shown that *t*-TUCB decreased the serum TG levels in diet-induced obese mice (Chapter III). To determine whether *t*-TUCB combined with 17,18-EEQ or 19,20-EDP can improve lipid metabolism in diet-induced obesity, we investigated blood lipid parameters (Fig. 5.3G-I). While the serum TC and NEFA levels were unchanged in response to treatment with *t*-TUCB alone or combined with 17,18-EEQ or 19,20-EDP (Fig. 5.3G-H), serum TG levels were significantly decreased in the T+EDP (45.8 ± 2.2 mg/dL; $p<0.01$) and T+EEQ (38.3 ± 6.7 mg/dL; $p<0.05$) groups compared to the controls (69.7 ± 7.2 mg/dL) (Fig. 5.3I).

5.3.3 17,18-EEQ or 19,20-EDP combined with *t*-TUCB increased thermogenesis in diet-induced obesity

In Chapter IV, we found that 19,20-EDP alone or combined with *t*-TUCB improved the cold tolerance in diet-induced obese mice. Here, to investigate whether the beneficial effects of 17,18-EEQ or 19,20-EDP combined with *t*-TUCB on metabolism are correlated with improved thermogenesis, we measured the core body temperature and performed cold tolerance test and indirect calorimetry (Fig. 5.4). These tests were performed at room temperature. Core body temperature was not changed by *t*-TUCB (37.3 ± 0.08 °C) but was significantly increased in the T+EDP (37.9 ± 0.06 °C; $p<0.01$) and T+EEQ (38.1 ± 0.09 °C; $p<0.001$) groups compared to the controls (37.3 ± 0.14 °C) (Fig.5.4A). In addition, both T+EDP and T+EEQ groups showed significantly higher core body temperature than the T group ($p<0.01$ and $p<0.001$, respectively). Consistently, both T+EDP and T+EEQ groups

showed significantly improved cold tolerance compared to the control ($p<0.05$ and $p<0.001$, respectively) (Fig. 5.4B-C). Also, the T+EEQ group showed better cold tolerance than the T group ($p<0.05$). Furthermore, heat production, measured by indirect calorimetry, was significantly higher in the T+EEQ group ($p<0.001$) but not in the T and T+EDP groups compared to the control group (Fig. 5.4D-E).

5.3.4 17,18-EEQ or 19,20-EDP combined with *t*-TUCB decreased lipid accumulation and regulated protein expression of thermogenic genes in the iBAT of diet-induced obesity

Since 17,18-EEQ or 19,20-EDP combined with *t*-TUCB alleviated some of the obesity-induced metabolic dysregulations and increased thermogenesis, we further investigated whether 17,18-EEQ or 19,20-EDP combined with *t*-TUCB modulated BAT activation, leading to the beneficial effects.

First, we examined changes in lipid accumulation in the iBAT by measuring the % area occupied by lipid from hematoxylin-eosin (H&E) stained iBAT slides of each mouse (Fig. 5.5A-B). *t*-TUCB alone did not change the lipid accumulation compared to the controls, but the lipid accumulation in the iBAT of the T+EDP and T+EEQ groups was significantly lower than the controls ($p<0.05$ and $p<0.01$, respectively).

Next, we analyzed the protein expression of thermogenic genes in the iBAT (Fig. 5.5C-D). We found that UCP1 protein expression in the iBAT was not changed in the T and T+EDP groups, but the T+EEQ group showed a trend of increase in UCP1 protein expression compared to the controls ($p=0.07$). On the other hand, PGC1 α protein expression in the T, T+EDP, and T+EEQ groups was significantly increased compared to the control group ($p<0.05$, $p<0.001$, and $p<0.05$, respectively).

5.3.5 17,18-EEQ or 19,20-EDP combined with *t*-TUCB regulated protein expression of genes involved in lipid metabolism in the iBAT of diet-induced obesity

We have previously demonstrated that *t*-TUCB decreased serum TG levels accompanied by increased protein expression of PLIN, a gene involved in lipid lipolysis, in the iBAT of diet-induced obese mice (Chapter III). Consistently, we show that the T+EDP and T+EEQ groups had significantly lower serum TG levels in this study. Moreover, the lipid accumulation in the iBAT was significantly decreased by 19,20-EDP or 17,18-EEQ combined with *t*-TUCB. Therefore, we further investigated the protein expression of genes involved in lipid metabolisms in the iBAT of diet-induced obesity (Fig. 5.6-5.7).

First, protein expression of LPL, CD36, FABP4, CPT1A, and CPT1B, which are involved in fatty acids uptake, transport, binding, and oxidation in the iBAT, were analyzed by western blot analysis (Fig. 5.6). There were no significant changes in LPL expression in the iBAT among groups. In contrast, CD36 expression was not changed in both T and T+EDP groups but was significantly increased in the T+EEQ group compared to the control, T, or T+EDP group ($p<0.01$) (Fig. 5.6). Moreover, CPT1A and CPT1B protein expression were significantly increased in both T+EDP ($p<0.05$ for CPT1A and $p<0.01$ for CPT1B) and T+EEQ groups ($p<0.01$ for CPT1A and $p<0.001$ for CPT1B), but not in the T group (Fig. 5.6). In addition, FABP4 protein expression was significantly higher in both T and T+EDP groups ($p<0.01$ and $p<0.001$, respectively) (Fig. 5.6), consistent with their increased body weights.

Next, we examined the protein expression and phosphorylation of genes involved in lipolysis (Fig. 5.7). Consistent with previous results, PLIN expression was significantly increased in the iBAT of mice treated with *t*-TUCB ($p<0.05$) alone or combined with 19,20-EDP ($p<0.001$) or 17,18-EEQ ($p<0.05$). In addition, phosphorylation of PLIN at S517 was significantly higher in the T, T+EDP, and T+EEQ groups compared to the controls ($p<0.05$, $p<0.05$, and $p<0.01$, respectively). Moreover, HSL protein expression and phosphorylation

were differentially regulated in the T+EEQ group compared to the other groups (Fig. 5.7). There were no significant changes in ATGL protein expression in the iBAT among all groups (Fig. 5.7).

5.3.6. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB decreased inflammatory response in the iBAT of diet-induced obesity

It has been reported that BAT content and activity declined in the obese animals partly because of the inflammatory microenvironment of BAT caused by low-grade inflammation in obesity [17, 18]. Targeting systematic and local inflammation could be a possible way to restore BAT function, thereby improving metabolic health [19]. As the parental PUFAs of 17,18-EEQ and 19,20-EDP, EPA and DHA have long been reported to regulate inflammatory processes in both rodents and humans [20, 21]. Moreover, the anti-inflammatory roles of 17,18-EEQ or 19,20-EDP have been revealed in multiple disease models [22]. Therefore, we analyzed whether 17,18-EEQ or 19,20-EDP combined with *t*-TUCB can suppress inflammatory pathways in the iBAT of diet-induced obese mice, thereby improving the iBAT activity (Fig. 5.8).

As the hallmarks of NF κ B activation, the phosphorylation of NF κ B inhibitor alpha (I κ B α) at S32 was significantly lower, and the I κ B α abundance was significantly higher in both T+EDP and T+EEQ groups than in the control group ($p < 0.05$) (Fig. 4.9). However, the phosphorylation of c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), the hallmarks of activation of inflammatory MAPK pathways, were only decreased in the T+EDP group compared to the control group ($p < 0.05$) (Fig. 5.8).

5.4 Discussion

BAT has emerged as a promising target to combat obesity and its associated metabolic disorders due to its role in non-shivering thermogenesis [23-25]. In the current study, we have demonstrated that fatty acid epoxide 17,18-EEQ or 19,20-EDP combined with *t*-TUCB via

mini osmotic pump delivery significantly decreased fasting glucose and serum TG levels while increased the core body temperature, improved cold tolerance, and thermogenesis in diet-induced obesity. In addition, 17,18-EEQ or 19,20-EDP combined with *t*-TUCB reduced the lipid accumulation in the iBAT, which was accompanied by enhanced protein expression of genes involved in thermogenesis and lipid metabolism and reduced inflammatory response in the iBAT.

5.4.1 17,18-EEQ and 19,20-EDP contribute to the beneficial effects of EPA and fish oil

As the precursors of EEQs and EDPs, EPA and fish oil (known to be enriched with both DHA and EPA) have long been reported to improve obesity and its associated metabolic disorders at least in part by regulating energy expenditure and/or thermogenesis in rodents [26-29]. Many of the animal studies with fish oil [30-32] showed increased energy expenditure and/or thermogenesis and improvement in glucose and lipid metabolism with concurrent increases of thermogenic *Ucp1* and/or *Pgc1 α* expression in the BAT. However, increased energy expenditure and improvement in metabolism without significant increases in *Ucp1* and/or *Pgc-1 α* expression have also been reported [33], possibly due to the differences in study designs, fish oil source and composition, and the dose and duration of the treatments. Mice studies with pure EPA showed dose-dependent increases in energy expenditure [34] and increased UCP1 protein expression at the highest dose tested [35]. However, whether the downstream epoxidized metabolites of EPA and DHA are at least partially responsible for the beneficial effects remains elusive. Our study has shed new light on the mechanisms underlying the anti-obesity effects of n-3 PUFAs by demonstrating that their downstream epoxide metabolites 17,18-EEQ and 19,20-EDP, when combined with *t*-TUCB, had similar regulatory effects on energy expenditure, thermogenesis, and glucose and lipid metabolism accompanied by changes in gene expression of *Pgc-1 α* and *Ucp1*. Our results suggest that 17,18-EEQ and

19,20-EDP may at least in part contribute to the beneficial effects reported for EPA and fish oil.

5.4.2 17,18-EEQ is more potent in increasing thermogenesis and improving metabolism in diet-induced obesity

Interestingly, differential effects among three treatment groups are noted in our study. Even though both T+EEQ and T+EDP groups showed beneficial effects in thermogenesis and metabolic biomarkers (i.e., increased core body temperature and cold tolerance and improved fasting glucose and serum TG levels, and less lipid accumulation in the BAT), these beneficial effects were not observed in the T group. Moreover, significant increases in heat production were only found in the T+EEQ group, suggesting that T+EEQ has more potent effects than T+EDP. Limited studies comparing the effects of EPA and DHA have suggested that EPA seemed to be more potent than DHA as a thermogenic stimulus [26]. To explore the molecular mechanisms by which the 17,18-EEQ or 19,20-EDP combined with sEH inhibitor showed differential effects, we further analyzed the protein expression of genes involved in lipid metabolism and inflammatory pathways in the BAT.

Upon cold stimulation, BAT consumes free fatty acids primarily derived from the lipolysis of intracellular lipid droplets [36, 37], which need to be replenished by the uptake of circulating TG-enriched lipoproteins [9, 38]. In addition, fatty acid oxidation (FAO) in mitochondria is enhanced to meet the high energetic demands [39, 40]. Therefore, activated BAT shows increases in FAO and enhances TG clearance from circulation [9]. We found that serum TG levels and lipid accumulation in the iBAT were decreased by 17,18-EEQ or 19,20-EDP combined with *t*-TUCB. Therefore, we further investigated the protein expression of genes responsible for lipolysis, FAO, and fatty acids uptake in the BAT.

In adaptive thermogenesis, lipolysis in brown adipocytes was mediated by PLIN, a lipid-coating protein whose phosphorylation promotes lipase activity in response to β -adrenergic

stimulation [41]. We found that in all three treatment groups, PLIN protein abundance and phosphorylation of PLIN at S517 were significantly increased, indicating that enhanced intracellular lipolysis for all three treatment groups, consistent with previous results (Chapter III). In addition, we observed that HSL protein expression and phosphorylation were differentially regulated in the T+EEQ group compared to the other groups. HSL hydrolyzes diacylglycerol and produces monoacylglycerol, which is further hydrolyzed by the monoacylglycerol lipase [42]. Phosphorylation of HSL has been shown to affect HSL-mediated lipolysis [43-45]. The effects of 17,18-EEQ on lipolysis need to be determined in future studies.

As the rate-limiting enzyme in FAO, the CPT1 family has three isoforms, including CPT1A, B, and C, distributed differently among tissues [46-48]. Although both CPT1A and CPT1B are expressed in the BAT of mice and rats, CPT1B has a much higher expression than CPT1A [46, 48]. Specific CPT1 inhibition by 2-tetradecylglycidic acid (McN-3802) decreased palmitic acid-induced mitochondrial respiration in murine primary brown adipocytes [49]. While *Cpt1b*^{-/-} mice were embryonically lethal, more than half of the *Cpt1*^{+/-} mice developed fatal hypothermia after a prolonged cold exposure compared to 21% of *Cpt1b*^{+/+} mice [50]. Moreover, overexpressing an active mutant form of CPT1A (insensitive to malonyl-CoA inhibition) in fully matured rat brown adipocytes significantly enhanced FAO, lipolysis, and mitochondria activity, which in turn inhibited the palmitate-induced triglyceride accumulation [51]. Our results show that both T+EEQ and T+EDP groups had significantly increased CPT1A and CPT1B protein expression in the BAT, suggesting that these epoxides combined with *t*-TUCB, but not the *t*-TUCB alone, may enhance FAO, thereby promoting BAT activity in diet-induced obesity.

LPL is a critical lipase that is involved in TG-enriched lipoprotein hydrolysis for the uptake of free fatty acids into the cells [52]. Short-term cold exposure [53] and cold-induced lipokine [54] increase free fatty acids uptake in the BAT by regulating the LPL activity and expression.

However, we did not find changes in LPL protein expression among the different treatment groups, which were housed under room temperature. CD36 is a cell surface protein responsible for fatty acid uptake from circulation and was also upregulated in the BAT by the cold temperature and contributed to cold-induced TG clearance [53]. *Cd36*^{-/-} mice showed decreases in fatty acid uptake and thermogenic gene expression in the BAT after cold exposure, leading to impaired cold tolerance [55]. In addition, CD36 is indispensable for the BAT thermogenic function by mediating coenzyme Q uptake from circulation for mitochondria respiration [56]. Interestingly, we found that CD36 protein expression was only upregulated in the T+EEQ group compared to other treated groups, consistent with the increased heat production in the T+EEQ group. Our results suggest that CD36 may be specifically regulated by 17,18-EEQ but not by 19,20-EDP, which partially contributes to more potent thermogenic effects observed in the T+EEQ group. The molecular mechanisms by which 17,18-EEQ upregulates CD36 need further investigation.

5.4.3 Both 17,18-EEQ and 19,20-EDP suppress NFκB activation in the BAT of diet-induced obesity

Obesity is associated with low-grade chronic inflammation, leading to impaired BAT activity, which contributes to the development of insulin resistance and type 2 diabetes [17, 18]. Therefore, targeting the inflammatory pathways may reverse the BAT dysfunction and prevent the development of metabolic disorders [19]. We found that the fasting glucose levels were significantly decreased by both 17,18-EEQ and 19,20-EDP combined with *t*-TUCB, and there was a trend of improvement in glucose tolerance in the T+EEQ group. Therefore, we further investigated the hallmarks of activation for inflammatory pathways in brown adipocytes [57]. We found that while activation of JNK and ERK pathways were suppressed by T+EDP, NFκB activation was significantly suppressed by both epoxides combined with *t*-TUCB. In contrast, *t*-TUCB alone did not suppress any of these inflammatory pathways under our study

conditions. These results suggest anti-inflammatory effects of 17,18-EEQ and 19,20-EDP, which may contribute to the improvement of glucose metabolism and thermogenesis in diet-induced obesity.

Limited studies have been reported on the anti-inflammatory effects of 17,18-EEQ and 19,20-EDP on adipose tissue in diet-induced obesity [58] [59]. In fat-1 mice with enriched endogenous n-3 PUFA by transgenic expression of n-3 desaturase, 17,18-EEQ and 19,20-EDP were significantly increased in the liver and eWAT, accompanied by decreased macrophage infiltration and reduced pro-inflammatory markers in the eWAT compared to the wild type controls [58]. Moreover, injection of 17,18-EEQ combined with two metabolites of EPA from lipoxygenase (5-HEPE and 9-HEPE) for 4 days significantly reduced the inflammatory response in the adipose tissue of high-fat induced obese mice [59]. Our results further suggest anti-inflammatory effects of 17,18-EEQ and 19,20-EDP on the BAT in diet-induced obesity. However, due to the limited amount of tissue samples, we were not able to analyze further the downstream mechanisms involved in glucose metabolisms, such as GLUT4 translocation, in the BAT. Future studies on how 17,18-EEQ and 19,20-EDP improve glucose metabolism through suppression of BAT inflammation are warranted.

5.4.4 Limitations and future directions

There are a few limitations in our studies. We only studied male C57BL/6J mice. Female of the C57BL/6J mice are not sensitive to a high-fat diet in our hands (our unpublished results) and may respond differently to 17,18-EEQ and 19,20-EDP. Future studies are needed to examine the effects of these epoxides on obesity and metabolic biomarkers in female mice.

In addition, sEH ablation and an n-6 epoxide analog have been reported to induce browning in the WAT [60] and 3T3-L1 adipocytes [61]. However, consistent with our previous study [15], we did not find browning of sWAT by n-3 epoxides combined with *t*-TUCB in the mice housed at 22-23°C (preliminary results, data not shown). Although we delivered 17,18-

EEQ, 19,20-EDP, and *t*-TUCB locally to the iBAT, we found the average area of adipocytes of the sWAT was significantly decreased in the T+EEQ group (preliminary results, data not shown), suggesting systemic effects of T+EEQ. Therefore, it is possible that these epoxides may improve systemic metabolism through modulating activities of other metabolic active tissues, such as WAT and muscle. Further studies are needed to examine the effects of 17,18-EEQ or 19,20-EDP combined with sEH inhibitor on WAT and muscle.

Lastly, the specific effects of 17,18-EEQ and 19,20-EDP on the BAT were extrapolated by comparing their biological effects when combined with *t*-TUCB with the effects by *t*-TUCB alone due to the quick metabolism of epoxides by the sEH. Future studies are needed to confirm the effects of 17,18-EEQ and 19,20-EDP on the BAT, using either sEH knockout mice or stable epoxide analogs [62].

5.5 Conclusion

Overall, the results demonstrate that local delivery of 17,18-EEQ or 19,20-EDP combined with *t*-TUCB by osmotic pump to iBAT may promote BAT activity by regulating the protein expression of genes involved in mitochondrial biogenesis, lipolysis, fatty acid oxidation, and inflammatory pathways, leading to increased thermogenesis and improvement of glucose and lipid metabolism in diet-induced obesity. 17,18-EEQ may be more potent than 19,20-EDP in enhancing thermogenesis by upregulating a more comprehensive set of genes involved in lipid metabolism, including CD36, in the BAT. Future studies are needed to confirm the beneficial effects of these epoxides on the BAT using either sEH knockout mice or stable epoxide analogs.

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Appendix

Table 5.1. Antibodies used in the western blot

Antibodies	Company	Catalog Number
Anti-ATGL	Cell Signaling Technology (Danvers, MA, USA)	2439
Anti-CD36	Novus Biologicals (Centennial, CO, USA)	NB400-144
Anti-CPT1A	Cell Signaling Technology (Danvers, MA, USA)	12252S
Anti-CPT1B	Proteintech North America (Rosemont, IL, USA)	22170-1-AP
Anti-ERK1/2	Cell Signaling Technology (Danvers, MA, USA)	4695
Anti-FABP4	Cell Signaling Technology (Danvers, MA, USA)	2120S
Anti-I κ B α	Cell Signaling Technology (Danvers, MA, USA)	9242S
Anti-JNK	Cell Signaling Technology (Danvers, MA, USA)	9252S
Anti-LPL	Santa Cruz Biotechnology (Dallas, TX, USA)	SC-373759
Anti-PGC1 α	Millipore (Temecula, CA, USA)	AB3242
Anti-phospho-ERK(T202/Y204)	Cell Signaling Technology (Danvers, MA, USA)	4370S
Anti-phospho-I κ B α	Cell Signaling Technology (Danvers, MA, USA)	2859S
Anti-phospho-JNK(T183/Y185)	Cell Signaling Technology (Danvers, MA, USA)	9251S
Anti-phospho-PLIN (S517)	Vala Sciences (San Diego, CA, USA)	4856
Anti-UCP1	Sigma Aldrich (St. Louis, MO, USA)	U6382
Lipolysis Activation Antibody Sampler Kit (Antibodies for phospho-HSL (Ser 563, Ser 565, and Ser 660), HSL, and PLIN)	Cell Signaling Technology (Danvers, MA, USA)	8334

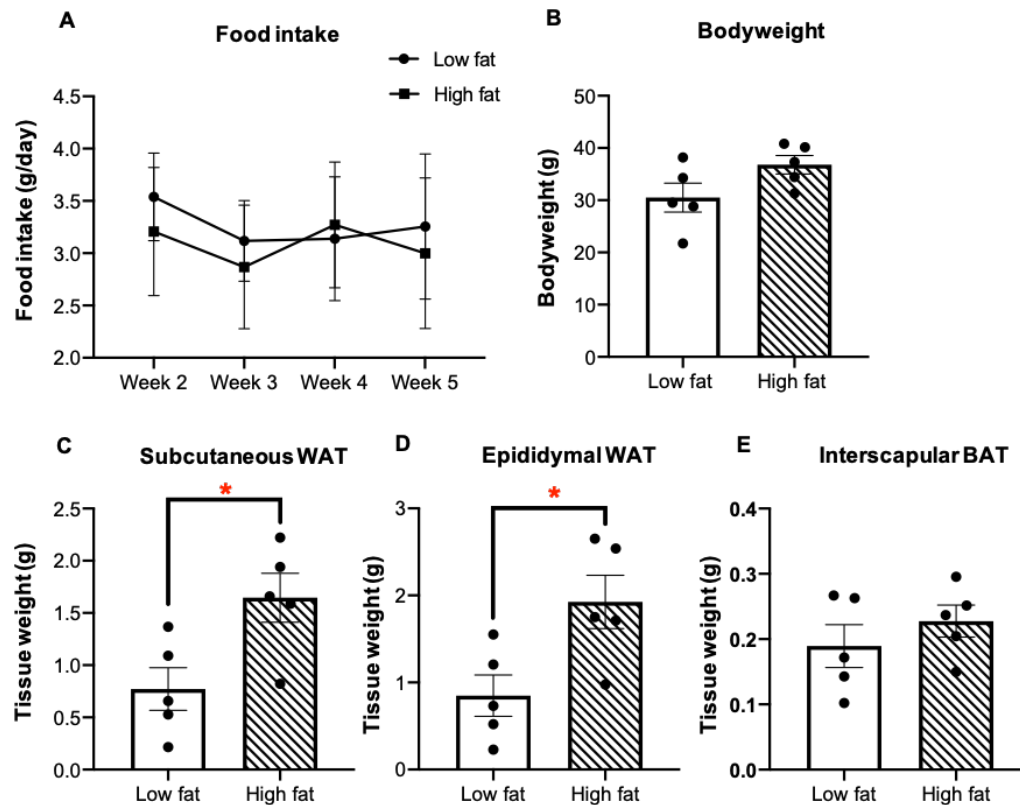


Figure 5.1. The effects of high-fat diet on food intake, body weights, fat pad weights, glucose tolerance, fasting glucose, serum TG and NEFA, core body temperature, and heat production in C57BL/6J mice. Male C57BL/6J mice were fed either a low-fat or a high-fat diet for 6 weeks. Body weight, food intake, fasting glucose were recorded, and glucose tolerance test, cold tolerance test, and indirect calorimetry were performed as described. (A) Changes in average food intake. (B) body weight at termination; (C), (D), (E) Fat pad weights at termination; (F) Fasting glucose; (G) Oral glucose tolerance test and areas under the curve; (H), (I), (J) Serum TG, NEFA, and TC levels at termination; (K) Core body temperature at termination; (L) Heat production and area under the curve; Data=Mean±SEM (n=5). *, **, ***, $p<0.05$, $p<0.01$, and $p<0.001$ compared to the low-fat group, respectively (Student's t-test).

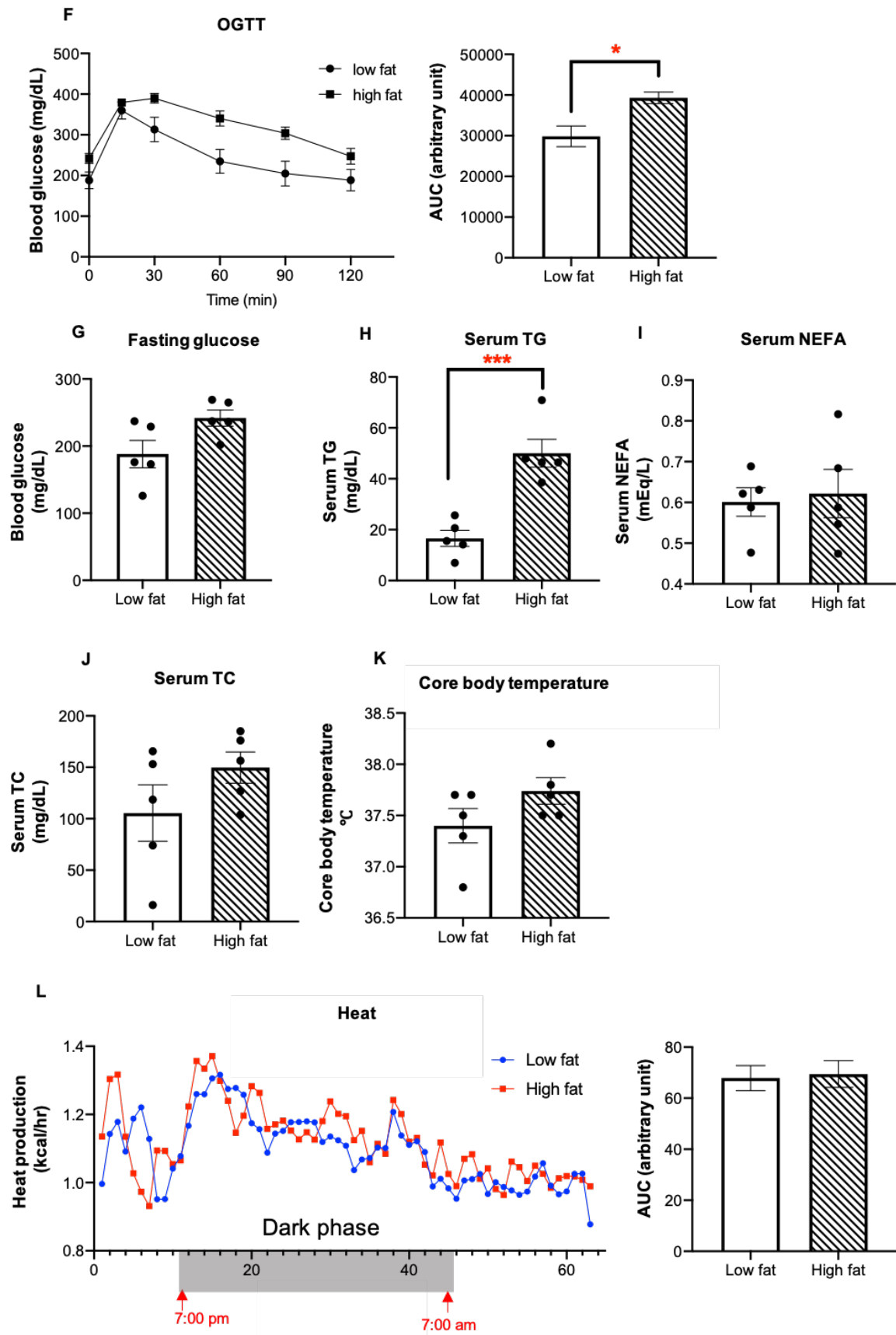


Figure 5.1 (continued)

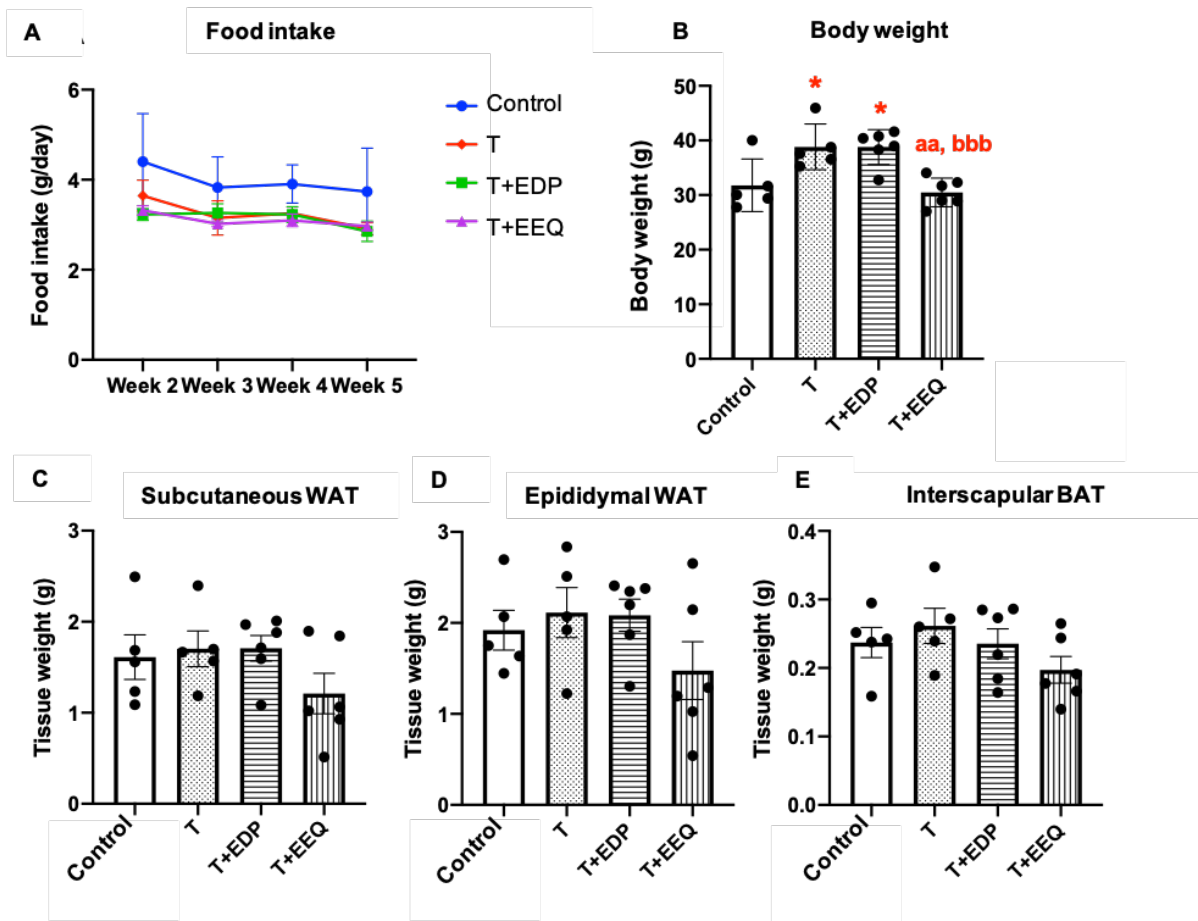


Figure 5.2. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump affected body weight differentially in diet-induced obesity. Male C57BL/6J mice fed with a high-fat diet (45% kcal from fat) were treated with *t*-TUCB (3 mg/kg/day) alone or combined with 19,20-EDP (0.05 mg/kg/day) (T+EDP) or 17,18-EEQ (0.05 mg/kg/day) (T+EEQ) by osmotic minipump implantation for 6 weeks as described in the methods section. Average daily food intake after implantation (A), body weight (B), subcutaneous WAT (C), epididymal WAT (D), and interscapular BAT weight (E) at the end of 6 weeks were shown. Data=Mean±SEM (n=5-6). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the controls, respectively. a, aa, aaa, p<0.05, p<0.01, and p<0.001 compared to the T group, respectively. b, bb, bbb, p<0.05, p<0.01, and p<0.001 compared to the T+EDP group, respectively.

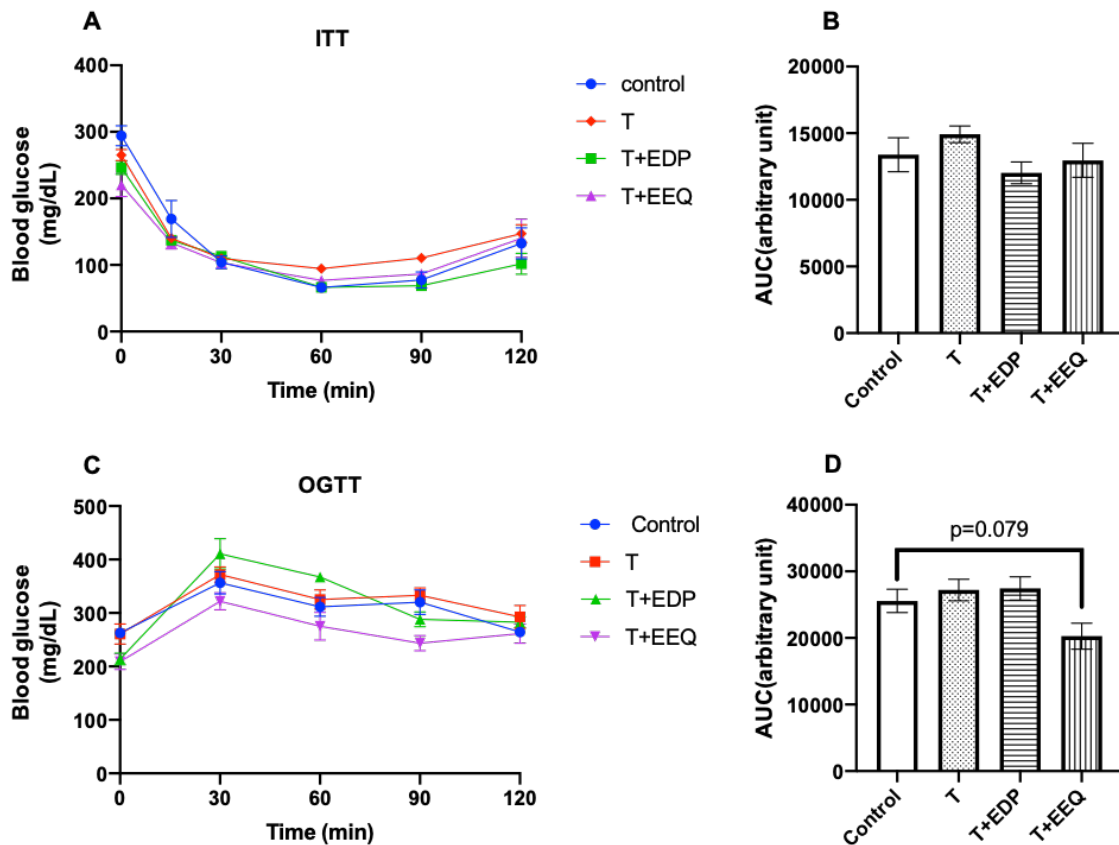


Figure 5.3. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump decreased fasting glucose and serum TG levels in diet-induced obesity. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump decreased fasting glucose and serum TG levels in diet-induced obesity. Male C57BL/6J mice fed with a high fat diet (45% kcal from fat) were treated with *t*-TUCB (3 mg/kg/day) or combined with 19,20-EDP (0.05 mg/kg/day) (T+EDP) or 17,18-EEQ (0.05 mg/kg/day) (T+EEQ) by osmotic minipump implantation for 6 weeks as described. Insulin tolerance test and the area under the curve (A-B), oral glucose tolerance test and the area under the curve (C-D), fasting glucose levels (E), serum insulin (F), serum TC (G), NEFA (H), and TG levels (I) after 6 weeks of implantation are shown; Data=Mean±SEM (n=5-6). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the controls, respectively.

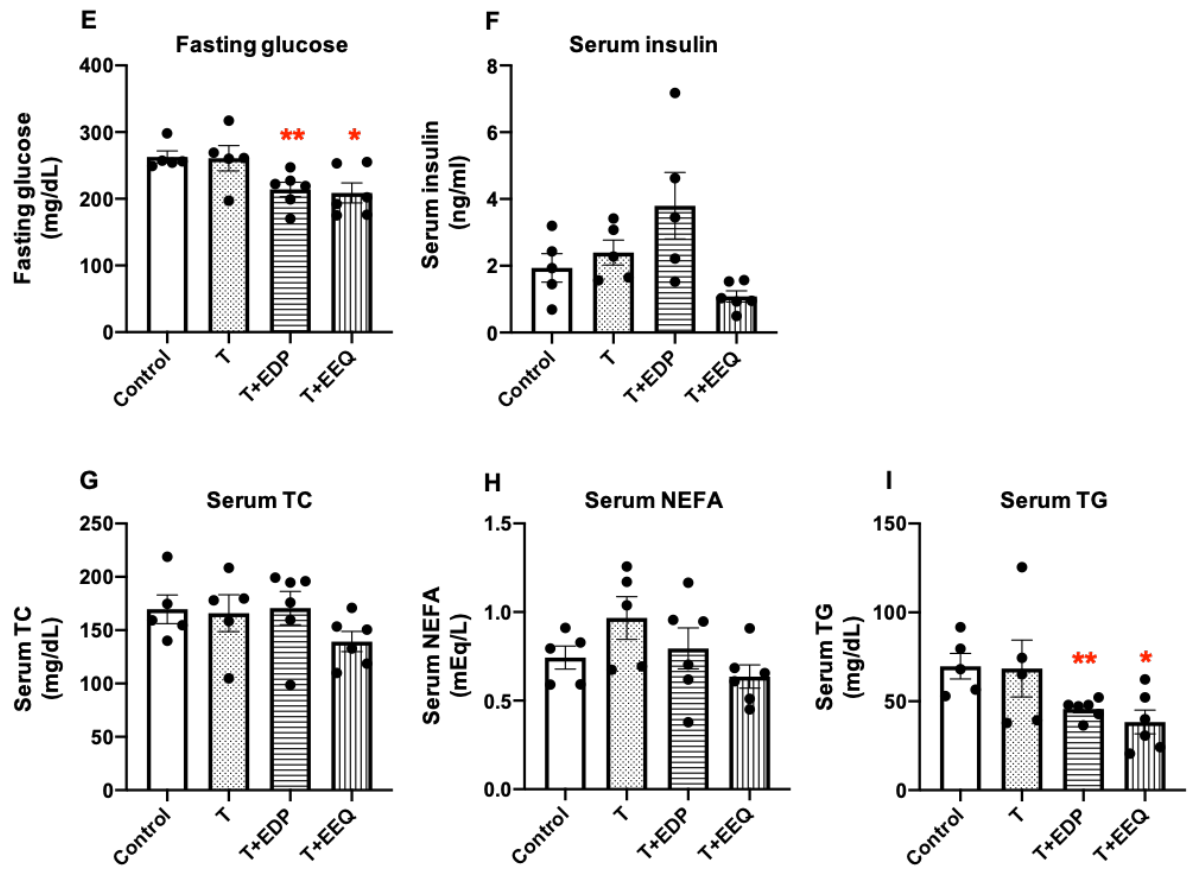


Figure 5.3 (continued)

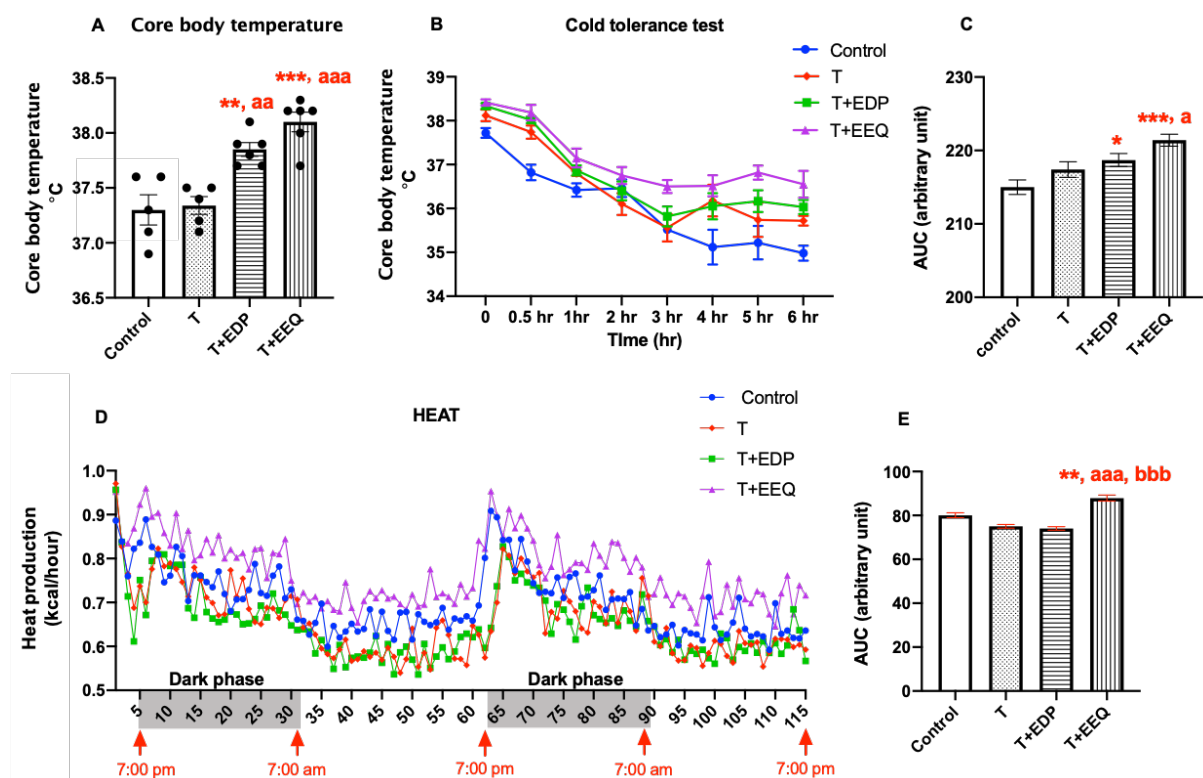


Figure 5.4. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump increased thermogenesis in diet-induced obesity. Male C57BL/6J mice fed with a high fat diet (45% kcal from fat) were treated with *t*-TUCB alone (3 mg/kg/day) or combined with 19,20-EDP (0.05 mg/kg/day) (T+EDP) or 17,18-EEQ (0.05 mg/kg/day) (T+EEQ) by osmotic minipump implantation for 6 weeks as described. Core body temperature (A); cold tolerance test and the area under the curve (B-C); heat production and the area under the curve (D-E) after 6 weeks of minipump implantation were shown; Data=Mean±SEM (n=5-6) (A-C and E) or Data=Mean (n=5-6) (D). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the controls, respectively. a, aa, aaa, p<0.05, p<0.01, and p<0.001 compared to the T group, respectively. b, bb, bbb, p<0.05, p<0.01, and p<0.001 compared to the T+EDP group, respectively.

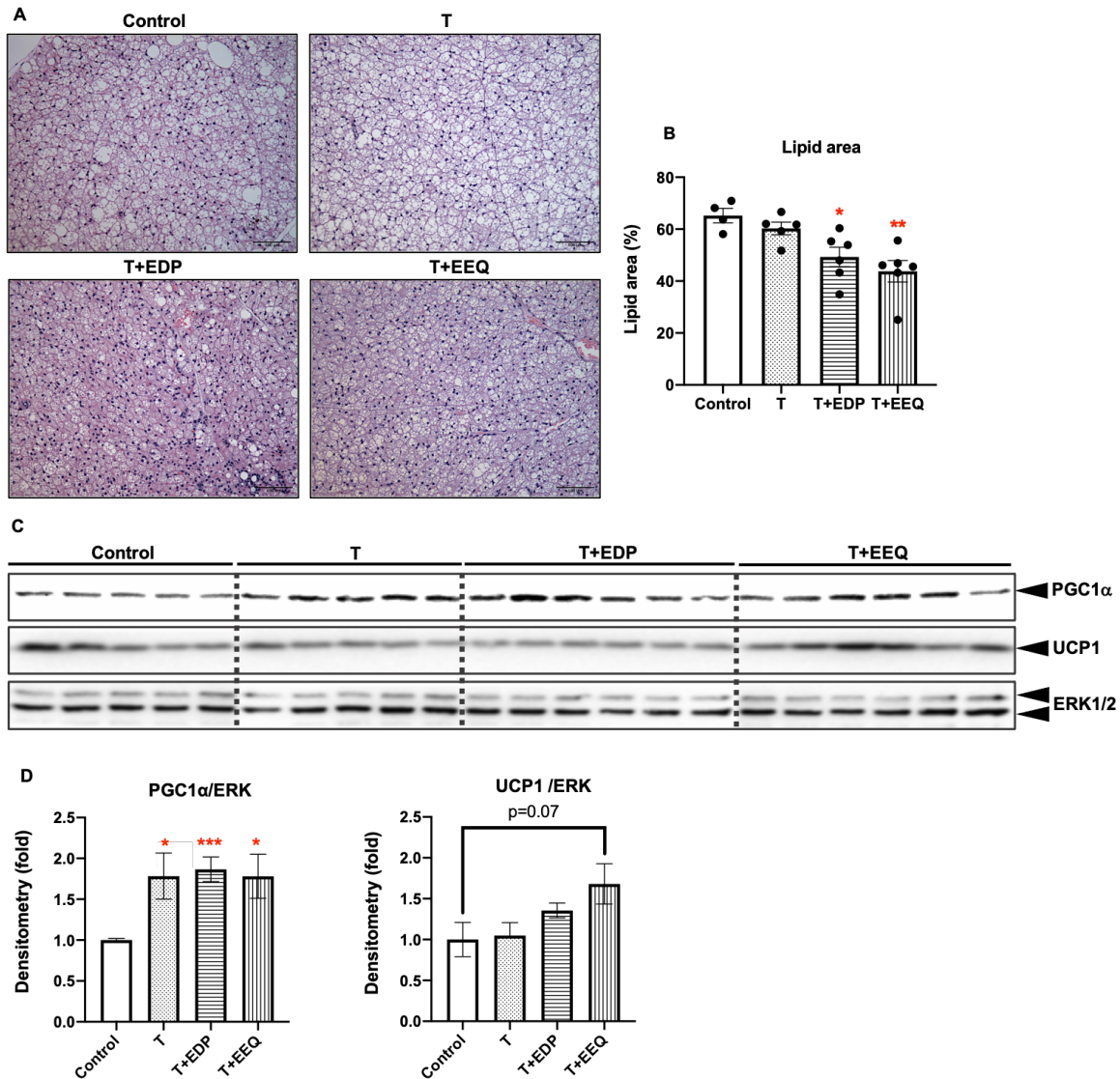


Figure 5.5. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump decreased lipid accumulation and regulated thermogenic gene expression in the iBAT of diet-induced obesity. After termination, the iBAT tissue slides from mice were stained with hematoxylin and eosin (H&E; A), and the % area occupied by lipid from 2-3 fields per slide per mouse (n=5) were analyzed using Image J software (B). Scale bars = 100 μ m. Protein expression of PGC1 α , UCP1, and the loading control ERK in the iBAT of mice in the control, T, T+EDP, and T+EEQ group (C), and their densitometry (D) are shown; Bar graphs show normalized densitometry for PGC1 α /ERK1/2 and UCP1/ERK1/2. Data=Mean $\pm$ SEM (n=5-6). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the controls, respectively.

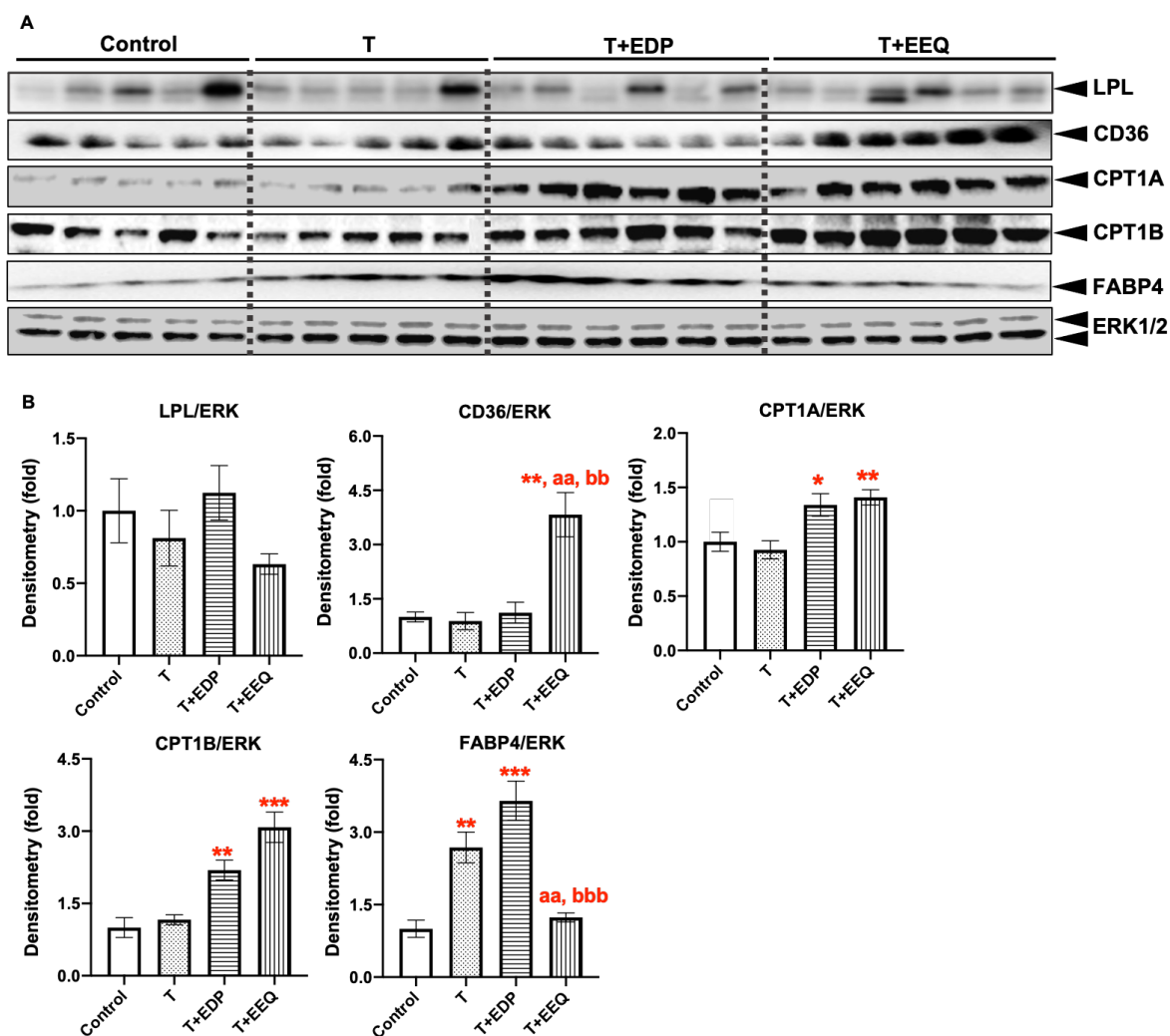


Figure 5.6. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump regulated protein expression of lipid metabolic genes in the iBAT of diet-induced obesity. Protein expression of LPL, CD36, CPT1A, CPT1B, FABP4, and the loading control ERK in the iBAT of mice in control, T, T+EDP, and T+EEQ groups (A), and their densitometry (B) are shown; Bar graphs show normalized densitometry for LPL/ERK1/2, CD36/ERK1/2, CPT1A/ERK1/2, CPT1B/ERK1/2, and FABP4/ERK1/2. Data=Mean±SEM (n=5-6). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the control, respectively. a, aa, aaa, p<0.05, p<0.01, and p<0.001 compared to the T group, respectively. b, bb, bbb, p<0.05, p<0.01, and p<0.001 compared to the T+EDP group, respectively.

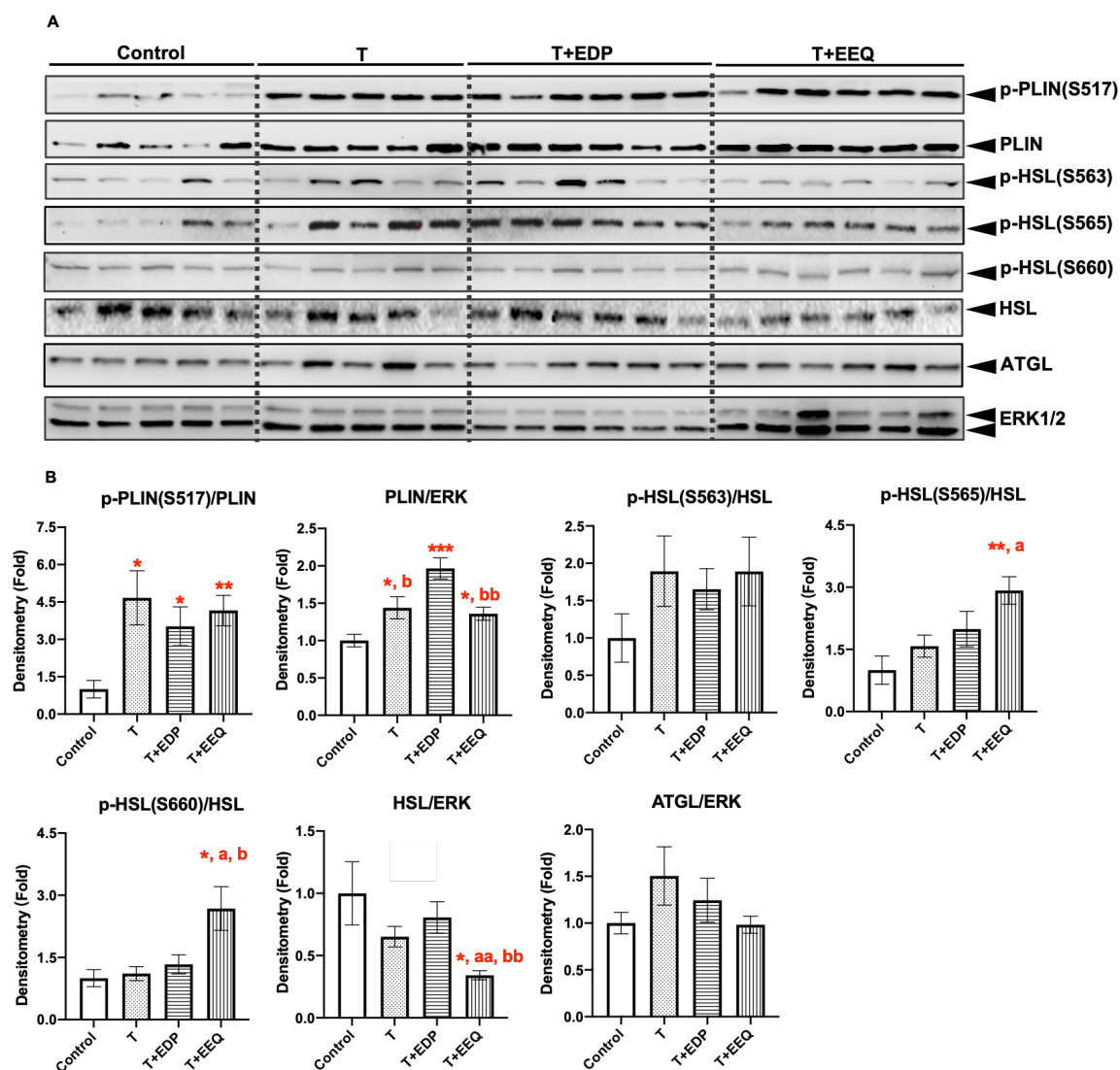


Figure 5.7. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump regulated protein expression of genes involved in lipolysis in diet-induced obesity. Protein expression of PLIN, HSL and ATGL, the phosphorylation of PLIN and HSL, and the loading control ERK in the iBAT of mice in control, T, T+EDP, and T+EEQ groups (A), and their densitometry (B) are shown; Bar graphs show normalized densitometry for p-PLIN(S517)/PLIN, PLIN/ERK1/2, p-HSL(S563)/HSL, p-HSL(S565)/HSL, p-HSL(S660)/HSL, HSL/ERK1/2, and ATGL/ERK1/2. Data=Mean±SEM (n=5-6). *, **, ***, $p<0.05$, $p<0.01$, and $p<0.001$ compared to the control, respectively. a, aa, aaa, $p<0.05$, $p<0.01$, and $p<0.001$ compared to the T group, respectively. b, bb, bbb, $p<0.05$, $p<0.01$, and $p<0.001$ compared to the T+EDP group, respectively.

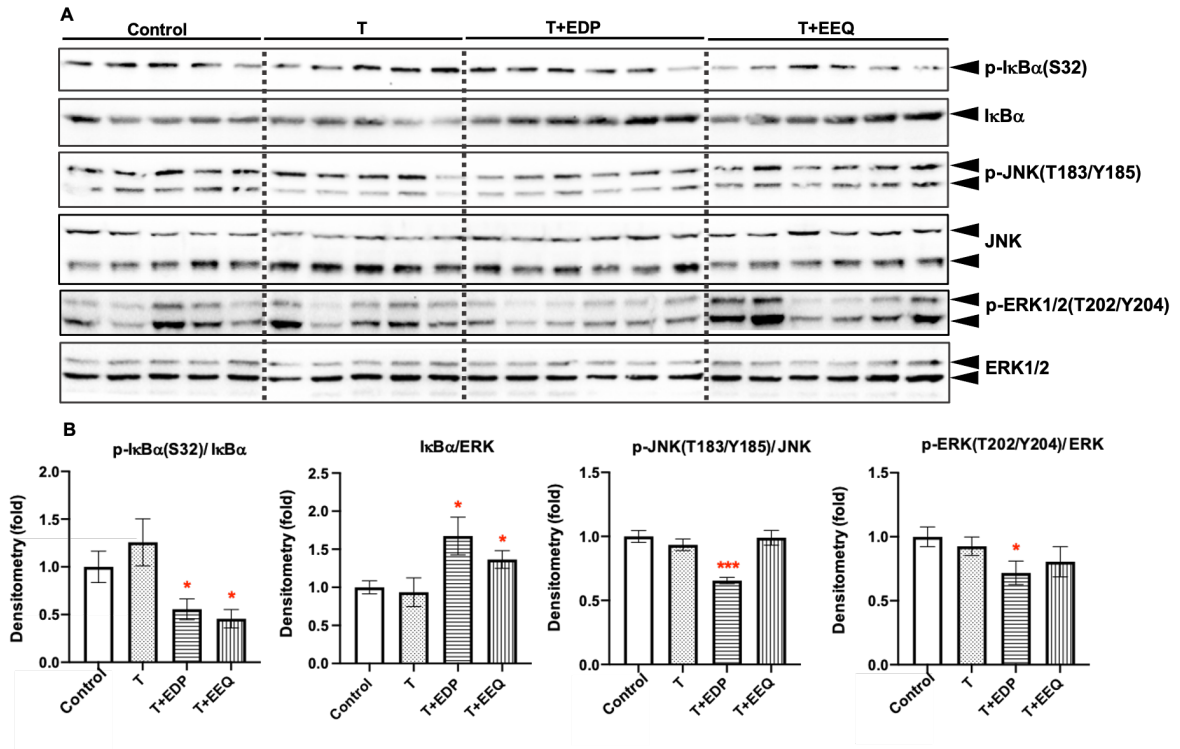


Figure 5.8. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB delivered via mini osmotic pump regulated activation of inflammatory pathways in the iBAT of diet-induced obesity. Protein expression of p-IκBα(S32), IκBα, p-JNK(T183/Y185), JNK, p-ERK1/2(T202/Y204), and ERK1/2 in the iBAT of mice in control, T, T+EDP, and T+EEQ groups (A), and their densitometry (B) are shown; Bar graphs show normalized densitometry for p-IκBα(S32)/IκBα, IκBα, p-JNK(T183/Y185)/JNK, and p-ERK(T202/Y204)/ERK. Data=Mean±SEM (n=5-6). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the control, respectively.

**Chapter VI 17,18-EEQ and 19,20-EDP Combined
with *t*-TUCB Promote Murine Brown Adipogenesis
and Mitochondrial Respiration and Uncoupling *in*
*vitro***

Abstract

N-3 EpFAs, such as 17,18-EEQ and 19,20-EDP, are bioactive metabolites produced from EPA and DHA by CYP 450 epoxygenase, respectively. Cytosolic enzymes metabolized these EpFAs to less active diols very quickly by adding a water molecule. Previously, we have demonstrated that an sEH inhibitor *t*-TUCB decreased serum TG and increased lipid metabolic protein expression in the iBAT of diet-induced obese mice (Chapter III). In addition, 19,20-EDP or 17,18-EEQ combined with *t*-TUCB prevented the high-fat diet-induced increase of fasting glucose and triglyceride levels, in part through differentially regulating protein expression involved in thermogenesis, lipid metabolism, and inflammatory pathways in the iBAT (Chapter V). Therefore, here we investigated whether n-3 epoxides combined with *t*-TUCB have direct effects on brown adipocyte differentiation and thermogenic capacity using a brown adipocyte cell model. We reported that 19,20-EDP or 17,18-EEQ combined with *t*-TUCB promote murine brown adipogenesis and mitochondrial respiration and uncoupling *in vitro*, possibly associated with PPAR γ activation. Moreover, when combined with *t*-TUCB, both 19,20-EDP and 17,18 EEQ improved mitochondrial respiration of mature brown adipocytes; however, only 17,18-EEQ combined with *t*-TUCB increased the proton leak in mature brown adipocytes. These findings are in line with the result (Chapter V) that when combined with *t*-TUCB, 17,18-EEQ had more potent effects than 19,20-EDP in increasing core body temperature and improving cold tolerance, and only 17,18-EEQ combined with *t*-TUCB significantly increased heat production in mice. Our results suggest that 17,18-EEQ may be more potent than 19,20-EDP in promoting BAT activity for obesity prevention.

6.1 Introduction

EDPs and EEQs are epoxy fatty acids produced from DHA and EPA by CYP450 epoxygenases, respectively [1]. EDPs and EEQs consist of several regioisomers, depending on which double bonds are converted to the monoepoxide ring [1]. CYP2C and 2J subfamilies are two major families of CYP epoxygenases that metabolize DHA to 19,20-EDP and EPA to 17,18-EEQ in both rodents and humans [1]. These epoxides are bioactive metabolites involved in vasodilation, angiogenesis, and inflammation; however, they are not stable and quickly metabolized into less active diols by sEH, a cytosolic enzyme expressed in various tissues, and is encoded by the EPHX2 gene in both rodents and humans [2]. Therefore, potent pharmacological sEH inhibitors are developed and widely used to study the functional effects of EpFAs, and many of the beneficial effects of EpFAs were potentiated by co-administration of an sEH inhibitor [2-4].

Recent studies indicate that higher sEH activity was associated with obesity and metabolic diseases in rodents [5]. In addition, sEH expression in subcutaneous adipose tissue of obese adults was significantly higher than that of lean subjects [6]. Moreover, sEH inhibitor significantly increased the iBAT mass in the fat-1 mice [7], which had a transgenic expression of the n-3 desaturase, leading to enriched endogenous n-3 PUFAs content and a higher n-3 EpFAs level [7]. On the other hand, dietary n-3 PUFAs supplementation has shown to increase energy expenditure and thermogenesis in animals, and n-3 PUFAs, EPA in particular, have been reported to promote brown adipogenesis and browning *in vitro* [8, 9]. These results lead us to hypothesize that n-3 EpFAs may promote BAT development or thermogenic function to combat obesity and may partially contribute to the anti-obesity effects of n-3 PUFAs. In the previous chapter (chapter IV), we demonstrated that 19,20-EDP or 17,18-EEQ combined with sEH inhibitor *t*-TUCB prevented the high-fat diet-induced increase of fasting glucose and serum triglyceride levels, in part through differentially regulating protein expression

responsible for thermogenesis, lipid metabolism, and inflammatory pathways in the BAT. In the current study, we use murine brown adipocytes to explore whether n-3 epoxides combined with *t*-TUCB have direct effects on brown adipocyte differentiation and thermogenic capacity.

6.2 Materials and methods

6.2.1 Reagents

sEH inhibitors *t*-TUCB and n-3 epoxides (17,18-EEQ and 19,20-EDP) were synthesized as described [10, 11]. Antibodies used for western blot analysis were listed as following: Anti-UCP1 antibody (Catalog# U6382) was purchased from Sigma Aldrich (St. Louis, MO); Anti-PGC-1 α antibody (Catalog# AB3242) was purchased from Millipore (Temecula, CA); Anti-CD36 (NB400-144) was purchased from Novus Biologicals (Centennial, CO).

6.2.2 Cell culture and treatment

The interscapular brown fat of newborn C57BL/6J mice was used to generate murine brown preadipocyte cell line as described [12], which was a generous gift from Dr. Johannes Klein (University of Lubeck, Lubeck, Germany). The cells were maintained in growth media containing 20% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) in Dulbecco's Modified Eagle's medium (DMEM) in a humidified incubator (maintained with 37 °C and 5 % CO₂) until they reached 100% confluence. The cells were then induced to differentiate in differentiation media (Day 0) (DMEM supplemented with 20% FBS, 1 nM T₃, and 20 nM insulin). The media was changed every 2 days for 6 days (Day6).

For experiments that investigate the effects on brown preadipocytes differentiation (During differentiation), cells were treated with the DMSO (Control), *t*-TUCB (TUCB) alone or combined with 17,18-EEQ (T+EEQ) or 19,20-EDP (T+EDP) at the indicated concentrations on day 0 (D0) of the differentiation program and replaced with each change of media until day 6 (D6). For experiments that examined the effects on mature brown adipocytes (Post differentiation), cells were induced to differentiate in differentiation media described above

and then maintained in growth media in the presence of the DMSO (Control), *t*-TUCB (TUCB) alone or combined with 17,18-EEQ (T+EEQ) or 19,20-EDP (T+EDP) at the indicated concentrations for 3 days.

6.2.3 Oil red O staining

Lipid accumulation in the differentiated brown adipocytes was assessed by oil red O (ORO) staining and ORO absorbance, as described [13].

6.2.4 Western blot analysis

1xRIPA lysis buffer (Cell Signaling, Danvers, MA) was used to collect total cell lysates. Protein concentrations were determined by the BCA assay kit (Thermo Scientific, Waltham, MA). 10% SDS-PAGE gels were used to separate protein samples (35 µg per lane). Proteins within the gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) at 25 volts for 20 hr. A blocking buffer (TBST buffer, 20 mM Tris Base, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4)) supplemented with 5% nonfat milk were used to block the membranes for 1 hr. After 3 washes by TBST buffer for 10 minutes each, membranes were then immunoblotted with the indicated primary antibodies against proteins of interest at 4 °C overnight at 1:1000 dilution. Next, membranes were washed by TBST buffer 5 times (5 minutes each) and then incubated in secondary antibodies conjugated with horseradish peroxidase at 1:4000 dilution for 1 hr. After 3 washes by TBST buffer for 15 minutes each, bands of the protein of interest on the membranes were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA), and visualized and quantified by ChemiDoc XRS+ system with Image Lab software (Bio-Rad, Hercules, CA).

6.2.5 Analysis of mitochondrial respiration

For experiments that investigate the effects of n-3 epoxides on mitochondrial respiration during differentiation, murine brown preadipocytes were differentiated in the presence or absence of the following treatments DMSO (Control), 1 µM of *t*-TUCB alone (TUCB) or

combined with 1 μ M of 17,18-EEQ (T+EEQ 1) or 19,20-EDP (T+EDP 1) or 10 μ M of 17,18-EEQ (T+EEQ 10) or 19,20-EDP (T+EDP 10) for 4 days.

For experiments that examined the effects on mature murine brown adipocytes, cells were induced to differentiate and then maintained in growth media with the treatment of DMSO (Control), 1 μ M of *t*-TUCB (TUCB) alone or combined with 10 μ M of 17,18-EEQ (T+EEQ 10) or 19,20-EDP (T+EDP 10) for 3 days.

Then the cells were reseeded onto a 24-well XFe assay plates at 2.0×10^4 cells per well. On the next day, cells in 24-well XFe assay plate were washed by XF assay media (XF assay base medium supplemented with 1 μ M sodium pyruvate, 10 μ M glucose, 2 μ M glutamax, and 2% BSA) twice with a volume of 400 μ L each. Then the assay plate was added with 525 μ L XF assay media into each well and incubated in a non-CO₂ incubator (maintained at 37 °C) for 1 hr. Cell's real-time oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at the basal level and then in response to the injections of oligomycin (1.5 μ M), carbonyl cyanide-ptrifluoromethoxyphenylhydrazone (FCCP; 6 μ M), and antimycin A/rotenone (1 μ M each) by an XFe24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). OCR and ECAR were automatically recorded by XFe24 software v1.8 provided by the manufacturer. Basal OCR, ATP production coupled OCR, proton leak coupled OCR, coupling efficiency, and maximal respiration capacity were calculated based on the manufacturer's instructions.

6.2.6 Reporter gene assays

Brown preadipocytes seeded in 24-well plates were transiently transfected with murine PPAR γ or NF κ B activation reporters and β -galactosidase control plasmid with Lipofectamine 2000 transfection reagent and Plus reagent (Thermo Fisher Scientific, Carlsbad, CA) for 24 hr. PPAR γ transactivation reporters consist of two separate systems described as following: murine PPAR γ ligand-binding domain ligated to the Gal4 DNA binding domain (DBD)

(mPPAR γ -Gal4) and a reporter construct containing an upstream activating sequence (UAS)–linked luciferase, 4xUAS-TK-Luc (TK: thymidine kinase) [13]. NF κ B activation reporter consists of an upstream NF κ B-response element–linked luciferase, NF κ B-Luc [14]. For PPAR γ activation, the cells were treated with the following treatments: DMSO (Control), 17,18-EEQ at 1 or 10 μ M (EEQ 1 or EEQ10;) or 19,20-EDP at 1 or 10 μ M (EDP 1 or EDP 10) alone, or combined with *t*-TUCB (1 μ M) for 24 hr. For NF κ B activation, the cells were pretreated with DMSO, 17,18-EEQ or 19,20-EDP alone (10 μ M) or combined with *t*-TUCB (1 μ M) for 1hr and then were treated with above treatments in the presence of LPS (100 ng/ml) for 18 hr. Cell lysates were prepared for the measurement of reporter luciferase and β -galactosidase activities by GloMax Luminometer (Promega, Madison, WI). Relative luciferase activity was normalized to β -galactosidase activity and indicated as fold compared to the DMSO group.

6.2.7 Statistical analysis

Data are presented as Mean+SEM (n=3) for oil red O staining and absorbance, western blot analysis, and reporter assay; n=3-4 for mitochondria respiration assay due to the design of XFe 24 assay plate. Statistical analysis was performed using Prism 8 (GraphPad Software, San Diego, CA). Student's t-Test was performed to determine the differences in group mean between the treatment groups and the control group. The level of significance was set at $p < 0.05$.

6.3 Results

6.3.1 17,18 EEQ and 19,20-EDP combined with *t*-TUCB differentially promoted murine brown adipogenesis, upregulated protein expression of thermogenic genes and lipid metabolic genes, and enhanced the mitochondrial respiration and uncoupling

Firstly, we investigate the effects of 17,18-EEQ and 19,20-EDP on brown adipocyte differentiation. Murine brown preadipocytes were differentiated in the presence of the following treatments: the DMSO (Control), *t*-TUCB alone (TUCB), or combined with 17,18-EEQ (T+EEQ) or 19,20-EDP (T+EDP) for 6 days as indicated in the method section. We

analyze lipid accumulation as a measure of adipocyte differentiation. Both 17,18-EEQ and 19,20-EDP significantly increased ORO absorbance at the dose of 10 μ M when combined with 1 μ M of *t*-TUCB compared to the *t*-TUCB alone ($p < 0.01$ for 19,20-EDP; $p < 0.001$ for 17,18-EEQ) or the controls ($p < 0.01$ for 19,20-EDP; $p < 0.001$ for 17,18-EEQ), indicating an enhanced differentiation (i.e., adipogenesis) in murine brown adipocytes (Fig. 6.1). All figures are shown in the Appendix.

Since 17,18-EDP and 19,20-EEQ combined with *t*-TUCB regulated the protein expression of thermogenic genes and lipid metabolic genes in the iBAT in *in vivo* studies, we further analyzed the protein expression of thermogenic genes and lipid metabolic genes *in vitro* (Fig. 6.2). Both T+EEQ and T+EDP groups but not the TUCB group showed significantly increased protein expression of PGC1 α compared to the controls ($p < 0.05$) (Fig. 6.2A-B). Only T+EEQ group presented significantly increased CD36 protein expression compared to the *t*-TUCB alone or the controls ($p < 0.05$), consistent with previous results of mice study (Fig. 6.2A-B). Similarly, UCP1 protein expression was only significantly increased in the T+EEQ group compared to the controls and the TUCB group ($p < 0.05$) (Fig. 6.2A-B).

To confirm whether 17,18-EDP or 19,20-EEQ combined with *t*-TUCB induced brown adipogenic differentiation and upregulation of thermogenic genes lead to increased thermogenic function, we performed the mitochondrial stress test to measure cellular energetics using an XFe24 Extracellular Flux Analyzer. The effects of 17,18-EEQ and 19,20-EDP on mitochondrial respiration during murine brown preadipocytes differentiation were analyzed (Fig. 6.3). Combined with *t*-TUCB, 17,18-EEQ treated brown adipocytes presented significant higher basal oxygen consumption rate (OCR) ($p < 0.01$ and $p < 0.001$ for 17,18-EEQ at 1 μ M and 10 μ M), ATP production coupled OCR ($p < 0.05$ and $p < 0.001$ for 17,18-EEQ at 1 μ M and 10 μ M) and maximal OCR ($p < 0.05$ and $p < 0.01$ for 17,18-EEQ at 1 μ M and 10 μ M) than the controls (Fig. 6.3A-B). In addition, basal OCR ($p < 0.01$ for 17,18-EEQ at 10 μ M), ATP

production coupled OCR ($p < 0.01$ for 17,18-EEQ at 10 μM), and maximal OCR ($p < 0.05$ for 17,18-EEQ at 10 μM) were significantly higher in the T+EEQ (with 10 μM of 17,18-EEQ) group than the TUCB group (Fig. 6.3A-B). Proton leak coupled OCR was only increased by 10 μM of 17,18-EEQ combined with *t*-TUCB compared to the control group ($p < 0.01$) and the TUCB group ($p < 0.05$) (Fig. 6.3A-B). Consistently, coupling efficiency was only decreased by 10 μM of 17,18-EEQ combined with *t*-TUCB compared to the controls ($p < 0.05$) (Fig. 6.3A-B).

Differentiating brown adipocytes treated with 19,20-EDP combined with *t*-TUCB presented the significant higher basal oxygen consumption rate (OCR) ($p < 0.001$), ATP production coupled OCR ($p < 0.001$), and maximal OCR ($p < 0.01$) than the controls only at the dose of 10 μM (Fig. 6.3C-D). Similar to that of 17,18-EEQ, brown adipocytes treated by 10 μM of 19,20-EDP combined with *t*-TUCB showed significantly higher basal OCR ($p < 0.001$), ATP production coupled OCR ($p < 0.001$), and maximal OCR ($p < 0.01$) than the cells treated by *t*-TUCB alone (Fig. 6.3C-D). Also, proton leak coupled OCR was only increased by 10 μM of 19,20-EDP combined with *t*-TUCB compared to the control group ($p < 0.001$) and the TUCB group ($p < 0.001$) (Fig. 6.3C-D). However, 10 μM of 19,20-EDP plus *t*-TUCB did not decrease the coupling efficiency compared to the controls (Fig. 6.3C-D). Moreover, brown adipocytes showed higher basal respiration ($p < 0.05$), ATP production, maximal respiration ($p < 0.05$), and proton leak ($p < 0.05$) when treated by 10 μM of 17,18-EEQ than by 10 μM of 19,20-EDP (Fig. 6.3C-D) combined with *t*-TUCB.

6.3.2 17,18-EEQ combined with *t*-TUCB upregulated thermogenic and lipid metabolic genes and promoted mitochondrial respiration and uncoupling in mature murine brown adipocytes

We found that 17,18-EEQ and 19,20-EDP combined with *t*-TUCB both increased thermogenesis without affecting the iBAT mass or UCP1 protein expression in the mice study

(Chapter V), suggesting the effects of 17,18-EEQ and 19,20-EDP may be independent of brown adipogenic differentiation. To investigate the effects of 17,18-EEQ and 19,20-EDP combined with *t*-TUCB on mature brown adipocyte function, we treated differentiated mature murine brown adipocytes with the DMSO (Control), *t*-TUCB alone (TUCB) or combined with 17,18-EEQ (T+EEQ) or 19,20-EDP (T+EDP) for 3 days as described in the methods section. Protein expression of thermogenic genes and lipid metabolic genes were analyzed by western blot (Fig. 6.2C-D). Similarly, PGC-1 α protein expression was significantly increased in both T+EEQ and T+EDP groups but not in the TUCB group compared to the controls ($p < 0.05$) (Fig. 6.2 C-D). Although CD36 protein expression was significantly increased in all treated groups compared to the controls ($p < 0.05$, $p < 0.05$, and $p < 0.001$ for the TUCB, T+EDP, and T+EEQ groups, respectively), T+EEQ presented the significantly higher CD36 protein expression than the TUCB and T+EDP groups ($p < 0.001$) (Fig. 6.2C-D). However, all three treated groups did not increase the protein expression of UCP1 compared to the controls, similar to the results in the previous mice study (Chapter V) (Fig. 6.2C-D).

Next, the effects of 17,18-EEQ and 19,20-EDP combined with *t*-TUCB on mitochondrial respiration of mature murine brown adipocytes were analyzed (Fig. 6.4). Among all treated groups, murine brown adipocytes treated with 10 μ M of 17,18-EEQ plus *t*-TUCB had the significantly higher basal OCR, ATP production coupled OCR, and proton leak coupled OCR than the control group ($p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively), TUCB group ($p < 0.001$), and T+EDP 10 group ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively) (Fig. 6.4). However, coupling efficiency and maximal respiration were not significantly changed by 17,18-EEQ or 19,20-EDP plus *t*-TUCB compared to the controls (Fig. 6.4).

6.3.3 17,18-EDP and 19,20-EEQ activated PPAR gamma and inhibited LPS-induced activation of NFκB

To investigate the molecular mechanisms by which 17,18-EEQ or 19,20-EDP promote murine brown differentiation, the abilities of 17,18-EEQ and 19,20-EDP to activate PPAR gamma (PPAR γ) were measured using the transactivation reporter assays since PPAR γ is one of the master regulators of brown adipogenic differentiation [15]. Either 17,18-EEQ or 19,20-EDP alone at 10 μ M or combined with *t*-TUCB significantly activated PPAR γ (Fig. 6.5A), suggesting that 17,18-EEQ and 19,20-EDP may promote brown adipocytes differentiation through, at least in part, PPAR γ activation.

In the mice study (Chapter V), we found that 17,18-EEQ or 19,20-EDP combined with *t*-TUCB increased the total amount of IκB α (i.e., prevented the degradation of IκB α) and inhibited its phosphorylation in the iBAT, suggesting a decreased NFκB-induced inflammatory response. IκB α is a protein that binds and inactivates the NFκB, which is a key transcription activator for genes that encode pro-inflammatory cytokines such as TNF α and IL-6 [16]. Upon phosphorylation and subsequent degradation of IκB α , NFκB is released and activated, leading to increased gene expression of pro-inflammatory cytokines and inflammatory response [16]. Therefore, we investigate the molecular mechanisms of the anti-inflammatory effects of 17,18-EEQ and 19,20-EDP by NFκB reporter assays (Fig. 5.5B). We found that either 17,18-EEQ or 19,20-EDP alone (at 10 μ M) or combined with *t*-TUCB significantly suppressed the NFκB activation ($p < 0.01$ and $p < 0.001$ for EDP and EDP+*t*-TUCB respectively; $p < 0.05$ for EEQ and EEQ+*t*-TUCB) (Fig. 6.5B). Our results, in line with suppressed NFκB signaling in the BAT *in vivo*, suggest that 17,18-EEQ and 19,20-EDP may improve the inflammatory response by suppressing NFκB pathways.

6.4 Discussion

In the current chapter, we demonstrate that 19,20-EDP or 17,18-EEQ, when stabilized by sEH inhibitor *t*-TUCB, significantly promote murine brown adipocyte differentiation and mitochondrial respiration and uncoupling. Moreover, 17,18-EEQ combined with *t*-TUCB also significantly promote mitochondrial respiration and uncoupling of mature brown adipocytes, which is accompanied by enhanced fatty acid transporter CD36 protein expression. Furthermore, we demonstrate that 17,18-EEQ and 19,20-EDP activate PPAR γ and suppress NF κ B activation in murine brown preadipocytes.

6.4.1 Significance

To our knowledge, this is the first report demonstrating the promoting effects of 17,18-EEQ and 19,20-EDP combined with *t*-TUCB on murine brown adipocyte differentiation and mitochondrial respiration and uncoupling in differentiating brown adipocytes. 17,18-EEQ and 19,20-EDP are major epoxy metabolites from EPA and DHA, respectively. EPA and DHA are n-3 PUFAs mainly derived from fatty fish or fish oil. Dietary n-3 PUFAs supplementation, usually provided as fish oil with the different formulations, has been reported to increased thermogenesis and heat production in rodents to protect against diet-induced obesity and associated metabolic disorders [8, 9]. In addition, n-3 PUFAs, especially EPA, are shown to promote brown adipogenesis [17, 18] and browning [19, 20] *in vitro*. However, the active metabolites that are responsible for EPA or DHA's effects are not known. Our results may add new insights to this knowledge gap by demonstrating that 17,18-EEQ and 19,20-EDP are effective metabolites that may be responsible for EPA or fish oil-induced increases in energy expenditure and thermogenesis.

6.4.2 17,18-EEQ may be more potent than 19,20-EDP in promoting brown adipocyte thermogenesis

As a mixture of EPA and DHA, fish oil has been reported to promote energy expenditure and thermogenesis in animals [8, 9]. However, whether the beneficial effects of fish oil come from EPA, DHA, or both are largely uncharacterized. In addition, few studies directly compared the effects between EPA and DHA. One study reported that EPA increased thermogenic gene expression, mitochondria function, and β -oxidation in murine brown adipocytes and promoted the browning process in inguinal white adipocytes, whereas no effects on thermogenic gene expression were observed in DHA-treated murine brown and inguinal white adipocytes [20]. In the current study, we found that when combined with *t*-TUCB, both 19,20-EDP and 17,18-EEQ promoted brown adipocyte differentiation and improved mitochondria respiration and uncoupling of brown adipocytes; however, only 17,18-EEQ combined with *t*-TUCB increased mitochondrial respiration and uncoupling in mature brown adipocytes. These findings are in line with the results (Chapter V) that when combined with *t*-TUCB, only 17,18-EEQ significantly increased heat production in mice and had more potent effects than 19,20-EDP in increasing core body temperature and improving cold tolerance. Taken together, these results suggest that 17,18-EEQ may be more potent than 19,20-EDP in promoting brown adipogenesis and brown adipocyte function for obesity prevention.

6.4.3 The role of PPAR γ and NF κ B pathways

How n-3 EpFAs activate brown adipogenesis and increase thermogenesis remains unknown. PPAR γ is one of the master regulators of brown adipogenesis and thermogenesis [15]. Several studies have reported that n-3 PUFA supplementation in mice regulated BAT thermogenesis at least in part through PPAR γ activation [8, 21]. Moreover, some of the biological effects of n-3 EpFAs have been shown to be mediated through PPAR γ in various

disease models [22, 23]. For example, 17,18-EEQ was reported to suppress inflammation in bronchi to protect lung function in a PPAR γ -dependent manner [24]. However, to date, no studies have reported the effects of n-3 EpFAs on PPAR γ activation in brown preadipocytes. Our results showed that both 17,18-EEQ and 19,20-EDP significantly activated PPAR γ in brown preadipocytes in reporter assays, suggesting that 17,18-EEQ and 19,20-EDP may promote brown adipocytes differentiation through, at least in part, PPAR γ activation. Future studies demonstrating that epoxides physically bind and activate PPAR γ are needed.

Pro-inflammatory cytokines, such as TNF α , are reported to impair the brown adipocyte thermogenic properties [25]. NF κ B is one of the major transcriptional activators for pro-inflammatory cytokines to induce inflammatory response [16]. Using reporter assays, we demonstrated that 17,18-EEQ or 19,20-EDP significantly suppressed the NF κ B activation in brown preadipocytes, suggesting that the anti-inflammatory effects of 17,18-EEQ or 19,20-EDP may contribute to their beneficial effects on thermogenesis in brown adipocytes. However, the direct changes of downstream pro-inflammatory cytokines regulated by n-3 EpFAs in brown adipocytes need further investigation.

6.4.4 Limitations and future directions

In the current study and previous *in vivo* study (Chapter V), the effects of 17,18-EEQ or 19,20-EDP on the cells or the mice were demonstrated when stabilized with the sEH inhibitor *t*-TUCB, as these epoxides are quickly metabolized by sEH [2]. In addition, these epoxides are also known to be metabolized by autooxidation, β -oxidation, esterification, and by other enzymes, such as cyclooxygenases and lipoxygenases [26]. Future confirmation of the effects of 17,18-EEQ and 19,20-EDP are needed, possibly by testing them in sEH knockout cells or using structural and metabolic stable analogs [27].

6.5 Conclusion

In conclusion, the current study suggests that when combined with sEH inhibitor *t*-TUCB, 17,18-EEQ or 19,20-EDP promote brown adipogenesis and thermogenic function *in vitro*, which may be associated with their abilities to activate PPAR γ and suppressing NF κ B activation. Moreover, 17,18-EEQ may be more potent than 19,20-EDP in promoting brown adipogenesis and brown adipocyte function for obesity treatment and prevention.

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Appendix

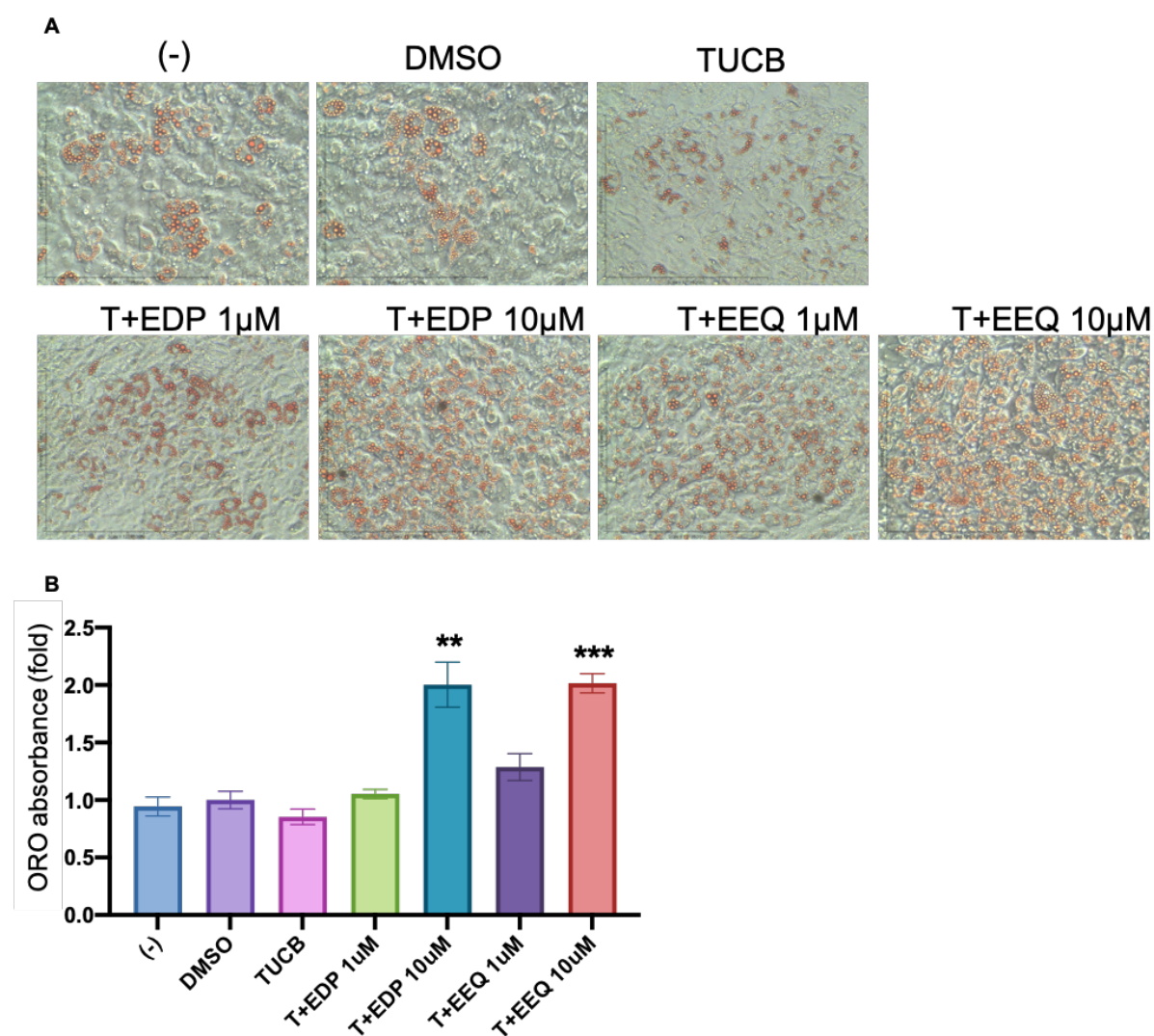


Figure 6.1. 17,18-EEQ and 19,20-EDP combined with *t*-TUCB promoted murine brown adipogenesis *in vitro*. Murine brown preadipocytes were differentiated in the presence of the following treatments: the DMSO (0.5%, Control), *t*-TUCB alone (TUCB) or combined with 17,18-EEQ (T+EEQ) or 19,20-EDP (T+EDP), as indicated for 6 days. Oil red O-stained cell morphology (A) and ORO absorbance (B) were shown. Data=Mean±SEM (n=3). *, **, ***, $p<0.05$, $p<0.01$, and $p<0.001$ compared to the controls, respectively.

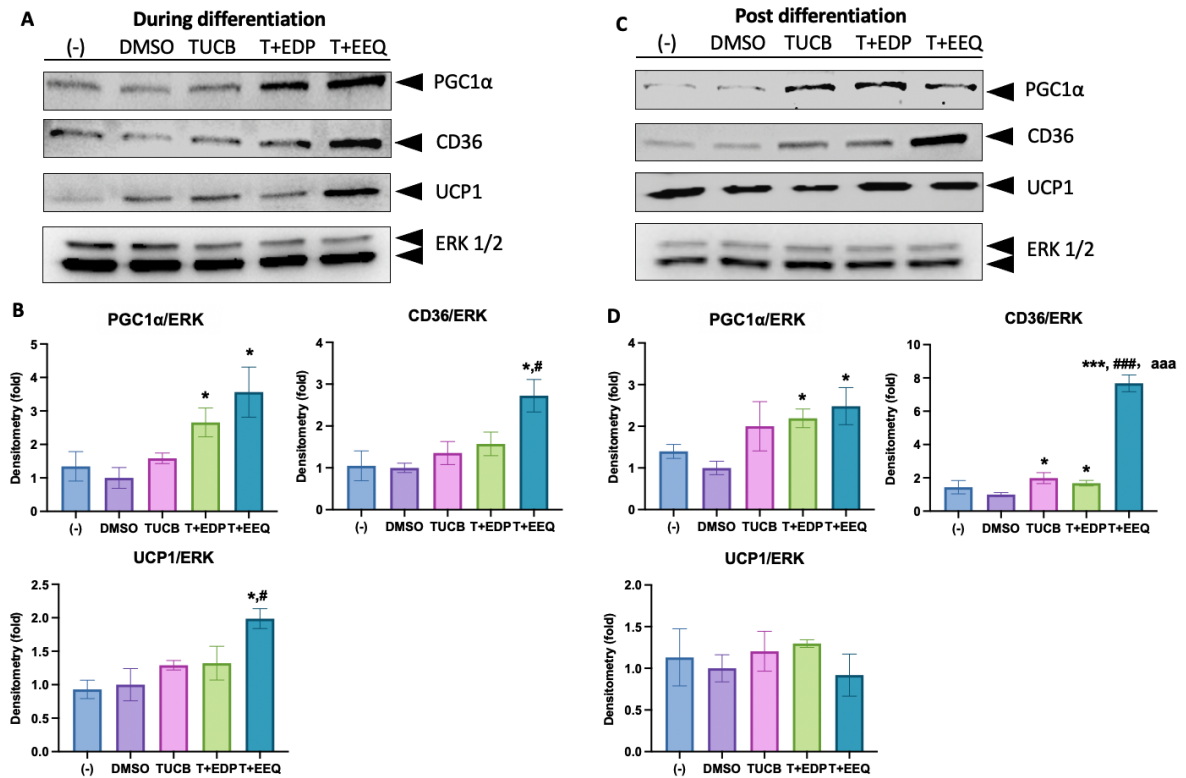


Figure 6.2. 17,18-EEQ or 19,20-EDP combined with *t*-TUCB differentially regulated protein expression of thermogenic genes and lipid metabolic genes *in vitro*. Murine brown preadipocytes were differentiated in the presence of the following treatments: the DMSO (Control), *t*-TUCB alone (TUCB) or combined with 17,18-EEQ (T+EEQ) or 19,20-EDP (T+EDP), as indicated for 6 days (During differentiation) (A-B). Mature murine brown adipocytes were maintained in growth media in the presence of the same treatments for 3 days (Post differentiation) (C-D). Protein expression of UCP1, PGC-1 α , CD36, and the loading control ERK1/2 and the densitometry are shown. Bar graphs showed the normalized densitometry for UCP1/ERK1/2, PGC1 α /ERK1/2, and CD36/ERK1/2. Data=Mean $\pm$ SEM (n=3). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to the controls, respectively. #, ##, ###, p<0.05, p<0.01, and p<0.001 compared to the T group, respectively. a, aa, aaa, p<0.05, p<0.01, and p<0.001 compared to the T+EDP 10 group, respectively.

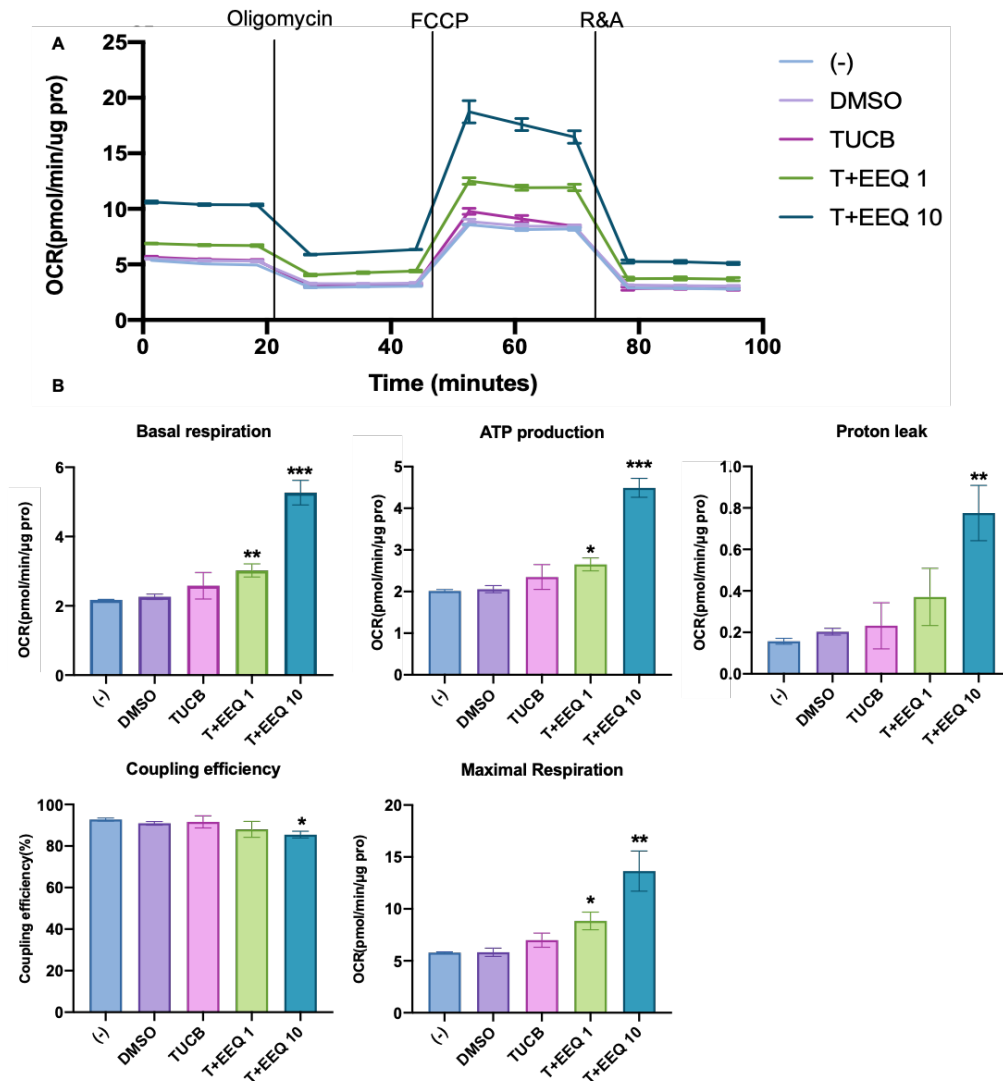


Figure 6.3. 17,18-EEQ and 19,20-EDP combined with *t*-TUCB increased mitochondrial respiration and proton leak in differentiating murine brown adipocytes. Murine brown preadipocytes were differentiated in the presence of the following treatments: the DMSO (Control), *t*-TUCB alone (TUCB) or combined with 17,18-EEQ (T+EEQ) or 19,20-EDP (T+EDP), as indicated for 4 days. Then differentiating brown adipocytes were reseeded onto a 24-well XFe assay plate at 2.0×10^4 cells per well. After 24 hr, the cells were then subjected to real-time measurements of OCR using an XFe24 Extracellular Flux Analyzer. OCR overtime during mitochondrial stress tests (A, C) and bar graphs (B, D) of the basal respiration, ATP production, proton leak, coupling efficiency (%), and maximal respiration are shown. Data=Mean $\pm$ SEM (n=3-4). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to Controls, respectively. #, ##, ###, p<0.05, p<0.01, and p<0.001 compared to the T group, respectively. a, aa, aaa, p<0.05, p<0.01, and p<0.001 compared to the T+EEQ 1 group, respectively.

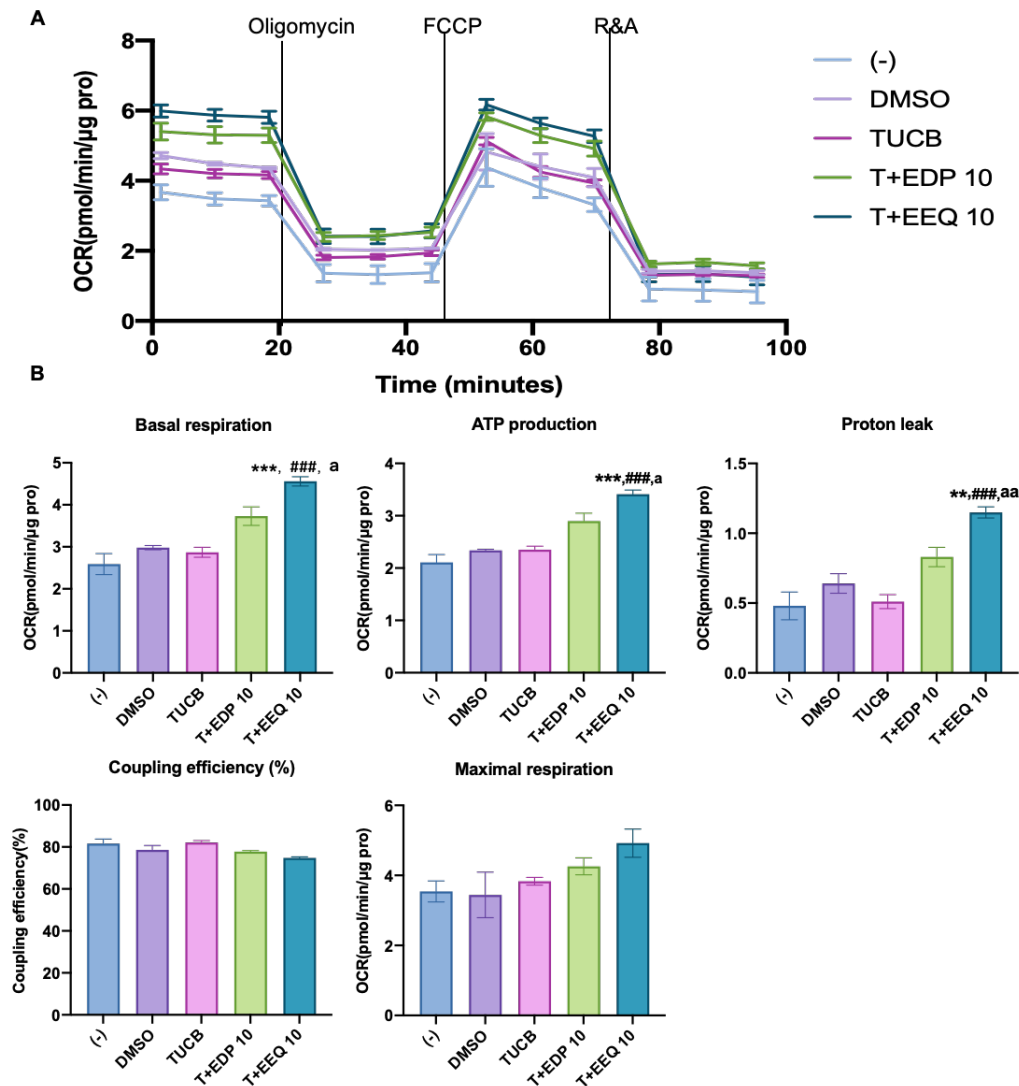


Figure 6.4. 17,18-EEQ combined with *t*-TUCB increased mitochondrial respiration and proton leak in mature murine brown adipocytes. Murine brown preadipocytes were differentiated for 6 days and then maintained in the growth condition in the presence of the following treatments: the DMSO (Control), *t*-TUCB alone (TUCB) or combined with 10 μ M of 17,18-EEQ (T+EEQ 10) or 19,20-EDP (T+EDP 10), as indicated for another 3 days. Then brown adipocytes were reseeded onto a 24-well XFe assay plate at 2.0×10^4 cells per well. After 24 hr, the cells were then subjected to real-time measurements of OCR using an XFe24 Extracellular Flux Analyzer. OCR overtime during mitochondrial stress tests (A) and the bar graphs (B) of the basal respiration, ATP production, proton leak, coupling efficiency (%), and maximal respiration are shown. Data=Mean $\pm$ SEM (n=3-4). *, **, ***, p<0.05, p<0.01, and p<0.001 compared to Controls, respectively. #, ##, ###, p<0.05, p<0.01, and p<0.001 compared to the T group, respectively. a, aa, aaa, p<0.05, p<0.01, and p<0.001 compared to the T+EDP 10 group, respectively.

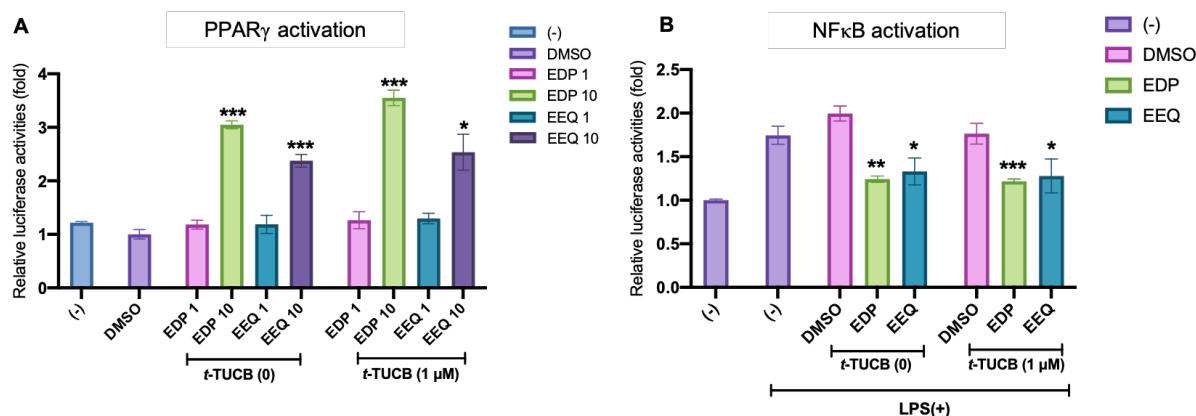


Figure 6.5. 17,18-EDP and 19,20-EEQ activated PPAR γ and inhibited LPS-induced activation of NF κ B in murine brown preadipocytes. Murine brown preadipocytes were seeded onto 24-well plates at 1.5×10^4 cells per well. On the next day, cells were transiently transfected with murine PPAR γ (A) or NF κ B (B) transactivation reporters and β -gal plasmid for 24 hr. For PPAR γ activation, the cells were treated with the following treatments: DMSO (Control), 17,18-EEQ (1 or 10 μ M) or 19,20-EDP (1 or 10 μ M) in the presence or absence of *t*-TUCB (1 μ M) for 24 hr. For NF κ B activation, the cells were pretreated with DMSO (Control), 10 μ M of 17,18-EEQ or 19,20-EDP, *t*-TUCB (1 μ M) alone or combined with 10 μ M of 17,18-EEQ or 19,20-EDP in the presence or absence of *t*-TUCB (1 μ M) for 1 hr then were co-treated with LPS overnight. Reporter gene assays were performed and normalized to β -gal activity. Relative luciferase activities were expressed as fold of the controls (set as 1). Data=Mean $\pm$ SEM (n=3). *, **, ***, $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared to the controls, respectively.

Chapter VII Conclusion and Future Direction

7.1 Conclusions

There are four main studies included in this dissertation (i.e., Chapter III, IV, V, and VI).

In Chapter III, the effects of the sEH inhibitor *t*-TUCB were studied in the obese mice via mini osmotic pump delivery. Although *t*-TUCB (3 mg/kg/day) did not change body weight, fat pad weight, or glucose and insulin tolerance, it decreased serum triglycerides level and increased protein expression of PLIN in the iBAT of diet-induced obese mice. Our results suggest that sEH inhibition may be beneficial in obesity-related hypertriglyceridemia through improving metabolic activity of BAT by regulating protein expression involved in lipid metabolism.

In Chapter IV, we further investigated whether 19,20-EDP, a prominent epoxy metabolite of DHA, has similar beneficial effects as those of sEH inhibitor *t*-TUCB (chapter III). We found that 19,20-EDP alone or combined with *t*-TUCB did not improve body weight and metabolic health; however, both treatments significantly improved cold tolerance in the diet-induced obese mice. Our results suggest that 19,20-EDP may improve cold-induced thermogenesis of BAT in obese mice. Future studies on the effective treatment time and dose of 19,20-EDP alone or combined with *t*-TUCB for obesity treatment are needed.

In Chapter V, the effects of sEH inhibitor *t*-TUCB alone or combined with 19,20-EDP or 17,18-EEQ on BAT activation in preventing diet-induced obesity were investigated through mini osmotic pump delivery. When combined with *t*-TUCB, both 19,20-EDP and 17,18-EEQ significantly improved fasting glucose and serum triglycerides levels, core body temperature, and lipids accumulation in BAT, whereas heat production was only significantly increased by 19,20-EEQ treatment. Protein expression of PGC1 α was significantly increased in all three treated groups, whereas UCP1 protein expression was unchanged. In contrast, protein expressions of CPT1A and CPT1B (rate-limiting enzymes for fatty acid β -oxidation) were only increased in the T+EDP and T+EEQ groups. Interestingly, protein expression of CD36, a

membrane transporter responsible for fatty acids uptake, was only increased in the T+EEQ group. Furthermore, inflammatory NF κ B signaling in the iBAT was decreased by the treatment of 19,20-EDP or 17,18-EEQ combined with *t*-TUCB. These results indicate that 17,18-EEQ or 19,20-EDP combined with *t*-TUCB may be beneficial in preventing high-fat diet-induced metabolic disorders, at least in part, through increasing thermogenesis, upregulating protein expression of genes involved in lipid metabolism, and suppressing inflammatory signaling in the BAT. In addition, we found that when combined with *t*-TUCB, 17,18-EEQ is more potent than 19,20-EDP in promoting thermogenesis and upregulating lipid metabolic genes in the BAT.

Finally, in Chapter VI, we investigated whether the beneficial effects of n-3 epoxides combined with *t*-TUCB in animal models are associated with improved brown adipogenic differentiation and enhanced thermogenic capacity in murine brown adipocytes. We found that 19,20-EDP or 17,18-EEQ combined with *t*-TUCB promote murine brown adipogenesis and mitochondrial respiration *in vitro*, which were accompanied by activation of PPAR γ and suppression of NF κ B. However, only 17,18-EEQ combined with *t*-TUCB increased mitochondrial respiration, including basal respiration and ATP-linked and proton-linked oxygen consumption, in mature brown adipocytes. Combined with the results of core body temperature, cold tolerance, and heat production from the mice studies (Chapter V), our results suggest that 17,18-EEQ may be more potent than 19,20-EDP in enhancing mitochondrial respiration and upregulating lipid metabolic genes in brown adipocytes, thereby increasing thermogenesis and improving obesity-associated metabolic disorders.

In summary, sEH inhibition by *t*-TUCB alone or combined with n-3 EpFAs is beneficial in obesity-associated metabolic disorders through improving BAT activity by modulating gene expression in thermogenesis and lipid metabolism. Combined with *t*-TUCB, 17,18-EEQ and 19,20-EDP differentially regulate thermogenic and lipid metabolic gene expression in the BAT

and brown adipocytes, and 17,18-EEQ is more potent than 19,20-EDP, in increasing thermogenesis and improving metabolism in mice and increasing mitochondrial respiration and upregulating thermogenic and lipid metabolic genes in brown adipocytes.

7.2 Significance of the study

Obesity is a worldwide pandemic with increased risks of multiple diseases, including type 2 diabetes, cardiovascular diseases, and cancers. In recent years, BAT has become a potential target to fight against obesity and associated metabolic diseases.

N-3 PUFAs are promising dietary factors that have been shown to be beneficial for obesity, at least in part, through targeting BAT. However, the mechanisms by which n-3 PUFAs improve BAT activity to protect against obesity remain unclear. As the downstream epoxy metabolites of n-3 PUFAs, n-3 EpFAs are thought to contribute to the beneficial effects of n-3 PUFAs in cardiovascular diseases and inflammatory diseases. In addition, sEH inhibitors are also shown to be beneficial in multiple disorders, possibly through stabilizing endogenous n-3 EpFAs. But it is undetermined whether n-3 EpFAs are responsible for the effects of n-3 PUFAs on BAT activity in obesity prevention/treatment. Results presented in the current dissertation have provided new insights on the mechanism by which n-3 PUFAs improved diet-induced obesity. The downstream epoxides derived from n-3 PUFAs, 17,18-EEQ and 19,20-EDP, when combined with *t*-TUCB modulated thermogenesis, energy expenditure, and glucose and lipid metabolism concurrent with changes in thermogenic and lipid metabolic gene expression. These results indicated that 17,18-EEQ and 19,20-EDP may be, at least in part, responsible for the anti-obesity effects reported for n-3 PUFAs, such as EPA and fish oil.

To our knowledge, there have been no studies directly comparing the effects of DHA and EPA on BAT in diet-induced obesity. We demonstrate that as EPA and DHA epoxy metabolites, 17,18-EEQ and 19,20-EDP differentially regulate lipid metabolic gene expression in the BAT and brown adipocytes, and 17,18-EEQ is more potent than 19,20-EDP in promoting

thermogenesis in the BAT and brown adipogenesis and mitochondrial respiration in brown adipocytes.

With the increasing prevalence of obesity during past decades, it is important to establish effective strategies to prevent and manage obesity. It is not always feasible to lose or maintain body weight by simply changing lifestyles (e.g., physical exercise or caloric restriction) or surgical treatments. Therefore, pharmacological agents or dietary factors are promising alternative or adjunctive strategies to lifestyle changes in preventing and treating obesity. Our studies suggest that pharmacological sEH inhibitors, such as *t*-TUCB, alone or combined with 17,18-EEQ or 19,20-EDP may be novel pharmaceutical/nutritional strategies to improve obesity-associated metabolic disorders in mice, which may be beneficial for human obesity.

7.3 Limitations and future directions

There are several limitations in the current dissertation. Only male C57BL/6J mice were studied in *in vivo* studies (Chapter III-V). Female C57BL/6J mice are not sensitive to a high-fat diet in our hands (our unpublished results) and may respond differently to 17,18-EEQ and 19,20-EDP. Effects of these epoxides on obesity and metabolic parameters in female mice need to be investigated in the future.

The duration of our *in vivo* studies was 6 weeks, which was based on the osmotic mini pump's maximal delivery capacity. However, some studies may need a longer time, for example, 8-12 weeks, to achieve optimal anti-obesity effects by improving BAT mass/activity in obese mice. Therefore, future studies are needed to determine the optimal duration of the treatment of *t*-TUCB alone or combined with n-3 epoxides in reversing obesity in mice.

In addition, it has been reported that sEH ablation and an n-6 epoxide analog induced browning in the WAT [1] and 3T3-L1 adipocytes [2]. However, we did not find browning of sWAT by *t*-TUCB alone or combined with n-3 epoxides in the mice housed at 22-23°C (maintained room temperature of the animal facility) (data not shown). We did find the average

area of adipocytes of sWAT was significantly decreased by the treatment of *t*-TUCB combined with 17,18-EEQ in the mice of diet-induced obesity in Chapter V (data not shown). These results suggest that although we delivered 17,18-EEQ, 19,20-EDP, and *t*-TUCB locally to the iBAT, the local treatment had systemic metabolic effects. Therefore, we could not fully rule out the possibility that sEH inhibition combined with epoxides can benefit systemic metabolism by regulating activities of other metabolic active tissues, such as WAT, liver, and muscle, in addition to the BAT. Investigating the effects of 17,18-EEQ or 19,20-EDP combined with sEH inhibitor on WAT, liver, or muscle in fighting against obesity is needed in future studies.

Moreover, we speculated the specific effects of 17,18-EEQ and 19,20-EDP on the BAT by comparing biological effects of these epoxides when combined with *t*-TUCB with the effects of *t*-TUCB alone since the epoxides are quickly metabolized by the sEH. Effects of 17,18-EEQ and 19,20-EDP on the BAT need to be confirmed by using either sEH knockout mice or stable epoxide analogs [3] in future studies.

In addition, there are other major epoxy regioisomers produced from n-3 PUFAs, particularly from DHA, such as 7,8-EDP and 13,14-EDP. Also, major epoxy metabolites are produced from n-6 PUFA, such as arachidonic acid. The effects of other epoxy regioisomers from DHA and from arachidonic acid on BAT and brown adipocytes warrant future investigation.

Finally, to translate our findings into novel and effective strategies for human obesity treatment and prevention, future studies on the effects of *t*-TUCB alone or combined with 17,18-EEQ or 19,20-EDP on human brown adipocyte differentiation and mitochondrial respiration are warranted.

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Vita

Yang Yang was born to Xiang he and Dejun Yang in Hami, Xinjiang province, China. She graduated from Hami District High School in Hami in 2007 and got her bachelor's and master's degree at Southeast University in China in 2012 and 2015.

In 2018, Yang started her doctoral program in the lab of Dr. Ling Zhao in the Department of Nutrition at the University of Tennessee, Knoxville. During her time at UTK, Yang acted as a graduate research assistant in Dr. Zhao's lab and completed her dissertation research. She defended her dissertation on July 28th, 2021. Following graduation, Yang plans to look for postdoctoral training for her future academic career.