Octacosanol prevents high fat diet-induced obesity by activating energy expenditure and thermogenesis in brown and beige fats

(オクタコサノールは褐色脂肪細胞およびベージュ細胞のエネルギー消費および熱産生を活性化させることにより高脂肪食誘導性の肥満を抑制する)

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Abbreviations

Abbreviations

BMI: Body mass index

WAT: White adipose tissue

BAT: Brown adipose tissue

eWAT: epididymal white adipose tissue

iWAT: Inguinal white adipose tissue

NALFD: Non-alcoholic fatty liver disease

TGs: Triglycerides

UCP1: Uncoupling protein 1

β3AR: β3 adrenergic receptor

cAMP: Cyclic adenosine monophosphate

PPARγ: Peroxisome proliferator-activated receptor γ

OAB: Overactive bladder

IBS: Irritable bowel syndrome

GPCR: G-protein coupled receptor

GUI: Glucose utilization index

GLUT4: Glucose transporter type 4

FGF21: Fibroblast growth factor 21

EPA: Eicosapentaenoic acid

DHA: Docosahexaenoic acid

CHD: Coronary heart disease

HDL: High density lipoprotein

LDL: Low density lipoprotein

FAs: Fatty acid

Elovl: Elongation of very long chain fatty acid

DEXA: Dual-energy x-ray absorptiometry

TC: Total cholesterol

FFA: Free fatty acid

H&E: Hematoxylin & Eosin

TH: Tyrosine hydroxylase

Adrb3: Adrenergic β3 receptor

Aldh3a2: Aldehyde dehydrogenase

Cs: Citrate synthase

FFAR4: Free fatty acid receptor-4

Ppargc1α: Peroxisome proliferative activated receptor, gamma, co-activator 1 alpha

PRDM16: PR domain containing 16

Cidea: Cell death inducing DNA fragmentation factor, alpha subunit like effector A

HFD: High fat diet

BW: Body weight

Srebp1c: Sterol regulatory element binding protein 1 c

Srebf2: Sterol regulatory element binding transcription factor 2

FAS: Fatty acid synthase

SCD1: Stearoyl-CoA desaturase-1

Gpam: Glycerol-3-phosphate acyltransferase, mitochondrial

Pparα: Peroxisome proliferator activated receptor alpha

Cpt1a: Carnitine palmitoyltransferase 1a

Acadm: Acyl-Coenzyme A dehydrogenase, medium chain

LDLR: Low density lipoprotein receptor

Hmgcs1: 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1

Hmgcr: 3-hydroxy-3-methylglutaryl-Coenzyme A reductase

FALDH: Fatty aldehyde dehydrogenase

PUFA: Poly unsaturated fatty acid

Chapter 1. Introduction

1.1. Obesity and its risk

The presence of obesity and diabetes in the modern world are devastating. More than 1 billion adults are either overweight (Body mass index (BMI) >25) or obese (BMI>30), and more than 150 million adults have diabetes, most of which is type 2 diabetes (T2D). Also, about 25% of children in the USA alone are overweight or obese which puts them into the risk of developing T2D. Worrisome is that these numbers are expected to double by the year 2025 worldwide, with particularly drastic impact in developing countries [1]. Obesity develops when energy intake exceeds energy expenditure, resulting in the accumulation of white adipose tissue (WAT) in the body (Figure 1). Most commonly it is usually the result of an imbalance between the calorie dense food intakes, reduced physical activity (lifestyle), and sometimes inability of the brain central nervous system (CNS) to suppress the appetite [2].

Obesity possesses a strong risk factor for several metabolic diseases; such as T2D, hyperlipidaemia, atherosclerosis and non-alcoholic fatty liver disease (NALFD). Several current anti-obesity drugs that aim to reduce food intake cause severe psychiatric or cardiovascular side effects [3,4]. Therefore, alternative safety therapeutic strategies are urgently needed to combat obesity.

1.2. Brown adipose tissue (BAT): A novel target to combat obesity

Based on the function, cell structure, location and colour, adipose tissue are divided into two major types: White adipose tissue (WAT) and BAT. WAT is the primary site of fat (energy) storage in the form of triglycerides (TGs), whereas BAT contains multiocular lipid droplets and has higher mitochondrial content, which gives it the colour brown. BAT is capable of burning fat and is specialised in heat production and therefore energy expenditure [5]. BAT is necessary for thermogenesis and energy balance in small mammals, and BAT induction in mice promotes energy expenditure, which leads to reduced adiposity and also protects mice from diet-induced obesity [6,7]. Also, the ablation of BAT activity results in reduced energy expenditure and increased weight gain in response to high-fat diet [8]. Mitochondria in the brown adipocytes express uncoupling protein-1 (UCP-1), which uses lipids and carbohydrates to generate heat by uncoupling electron transport from oxidative phosphorylation [9] (Figure 2). Activation of brown adipocytes results in fatty acid (FAs) oxidation for which lipids (fats) and carbohydrates are utilized from outside the cell [10].

Recent studies have revealed the ability of certain WAT depots to activate thermogenesis upon exposure to cold and hormonal stimuli [11,12]. A subpopulation of cells in inguinal WAT (iWAT), known as “beige” cells, expresses UCP-1 and carries out thermogenesis. Thus, promoting the development and function of brown and beige fat appears as an attractive treatment for obesity and obesity-related metabolic diseases [13].

1.3. Pharmacological approach towards obesity

Until now the therapeutic strategies intended towards reducing the hunger or increasing the satiety have not been successful. Calorie restriction in combination with exercise is effective in short-term, but long-term weight maintenance is difficult and weight regains, sometimes rising above previous weight is common [14-16].

Appetite suppressant drugs such as sibutramine and rimonabant have both been withdrawn from the market due to their severe short and long-term side effects like cardiovascular disorders, stroke, and psychiatric disorders. A gastrointestinal lipase inhibitor, orlistat, which increases satiety, is available. However, this drug has limited efficacy and causes serious side effects. Hence, an alternative strategy is to increase energy expenditure, via BAT activation, as a means to prevent weight gain and to induce weight loss.

1.4. Drugs activating BAT: enhancing thermogenesis

Norepinephrine contained in sympathetic neuronal fibres in BAT interacts with β3 adrenergic receptors (β3 AR) present in the adipocyte cell surface [17]. Activation of β3AR increases the cyclic adenosine monophosphate (cAMP), which subsequently results in overexpression of UCP-1 resulting in the increased glycolysis [18]. Thus, one approach is the agonist-mediated activation of β3AR on brown adipocyte as a strategy for studying BAT biology, and as a potential therapeutic approach for diabetes and obesity. Apart from this elevating norepinephrine levels, adenylyl cyclase and peroxisome proliferator-activated receptor γ (PPARγ) are other possible strategies to combat obesity and related disorders [19]. Based on this, the drugs used for BAT activation can be classified broadly into four categories (Figure 3).

Category 1: β3 AR agonist, category 2: Norepinephrine altering drugs, category 3: PPARγ activators, and category 4: Other drugs/ natural products.

1.4.1 Category 1 drug: β3 adrenergic receptor agonists

Currently, a β3AR agonist is approved only for the treatment of overactive bladder (OAB) [20]. The β3AR are G-protein coupled receptors (GPCR) and are found abundantly on brown adipocytes [21-24].

BAT is innervated by sympathetic nerves containing catecholamine norepinephrine, which activate β3AR. A significant attempt has been made to assess β3AR selective agonists as potential therapeutic agents for the treatment of obesity [25].

Table 1 shows the list of some selective β3AR agonists. BRL37344 a selective β3AR agonist is known to be selective for adipocyte lipolytic response [26]. Also, prolonged treatment with BRL37344 causes a 34-fold increase in basal glucose utilisation index (GUI) of BAT specifically [27]. CL316,243 is another highly selective β3AR agonist [28]. In previous studies, CL316,243 activated BAT [29] and promoted BAT mitochondrial proliferation and energy expenditure [30]. In initial human studies with CL316,243 energy expenditure after eight weeks in lean young males did not differ from baseline [31] and because of its poor bioavailability in humans, CL 316,24 trials were discontinued in humans.

Apart from CL316,243, there are three closely related derivatives, rafabegron, mirabegron and solabegron which are being pursued clinically for use in OAB and irritable bowel syndrome (IBS) [20]. Rafabegron exhibited some increase in energy expenditure at the highest dose in obese men and women [32]. Solabegron which is studied for its effect in IBS has not been studied for effects on energy expenditure. However, mirabegron a β3AR selective agonist [33] is approved for use in OAB only [34]. Mirabegron was shown to activate rat [35] BAT and human [36] BAT activity.

Talibegron and ZD7114 are selective β3AR drugs which increase energy expenditure via non-shivering thermogenesis [37]. In contrast, ZD7114 has been reported to have antagonist properties towards β3AR in isolated rat ileum [38]. Also, ZD7114 did not affect 24-hour energy expenditure in obese women and men, while talibegron had a very minimal stimulatory effect on

energy expenditure [39]. Their role in weight loss or diabetes is therefore not satisfactory. ICID7114 has been reported to stimulate BAT activity and oxygen consumption in canine studies [40,41]. However, no further studies on its effect on weight loss or diabetes have been published. Also, another selective β3AR drug L-796568 did not show any significant effect in lipolysis or thermogenic activity in nondiabetic men [42]. Other clinically used β3AR agonists, amibegron [43,44] have also been discontinued.

1.4.2 Category 2 drugs: Norepinephrine altering drugs

Norepinephrine stimulates β3AR and cold temperatures may increase metabolism indirectly by raising norepinephrine levels [45,46]. The BAT capacity of thermogenesis is enhanced with raise in norepinephrine level [47]. List of drugs, which elevate norepinephrine levels, is listed in table 2.

1.4.3 Category 3 drugs: PPARγ activators (glitazones)

Activation of PPARγ by the glitazones class of drugs affects carbohydrate and lipid metabolism by several mechanisms and have been studied for its effect in the treatment of T2D [48]. Knowing the significant role of brown adipocytes in the increase of energy expenditure, an increase of brown fat adipogenesis by glitazones could add to the advantageous effects of these drugs on insulin sensitivity in humans. Table 3 shows the different types of glitazones and their status.

Rosiglitazone promotes brown pre-adipocyte cell differentiation and increases rat BAT mass. Also, treatment of human pre-adipocytes with rosiglitazone caused an increase in levels of UCP-1 mRNA [49]. Previous studies have shown increased BAT activity in rodents treated with troglitazone [50]. On the other hand, ciglitazone reduced blood glucose, triglycerides, and food consumption without affecting body weight in obese hyperglycemic mice. However, it did show a reduction in human blood sugar but is currently not used in any medication form [51]. Troglitazone increases glucose transporter type 4 (GLUT4) expression in obese T2D rat models

and increases insulin sensitivity in non-insulin-dependent diabetes mellitus, but this drug comes with grave liver-damaging side effects [52]. It was used as an anti-diabetic but has now been discontinued due to its severe side effects. Pioglitazone is used for the treatment of diabetes mellitus, but it causes severe urinary bladder side effects in some cases [53,54]. Balaglitazone reduced blood glucose levels in obese rats [53,54].

Rivaglitazone also reduces blood glucose levels by increasing insulin sensitivity in diabetic animal models. The drug also improves glycemic control in T2D patients. The drug is under trial for the treatment of T2D [55,56]. Darglitazone showed an increase in BAT activity [57], but, clinical development of darglitazone has been discontinued.

1.4.4 Category 4 drugs: Other products/Natural products

Nicotine causes the release of catecholamine, including norepinephrine, which initiates thermogenesis in BAT for energy expenditure [58]. Nicotine also promotes the resting metabolic rate, all of which contribute to the reduction of obesity [59].

Forskolin is another BAT activator known for inducing thermogenesis [60]. It stimulates the adenylyl cyclase enzyme directly and enhances the intracellular levels of cAMP [61]. Caffeine raises BAT temperature with limited impact on core temperature and oxygen consumption in BAT mitochondria suggesting caffeine activates BAT thermogenesis [62]. Adenosine receptors, A2A have also been suggested to play a role in BAT activation [63]. Previous studies have shown a significant decrease in adiposity after continued ingestion of capsinoids (capsaicin) in humans. BAT is connected in the capsinoid-induced boost in energy expenditure. Raised UCP1 expression was also noted in rats administered with capsinoids for two weeks [64]. Capsinoid ingestion enhances energy expenditure through the activation of brown adipose tissue in humans [65].

Curcumin is a yellow pigment found in turmeric and has been studied as a treatment for obesity-related conditions. It interacts directly with adipocytes, pancreatic cells, macrophages, and muscle

cells. Curcumin has been used to reverse insulin sensitivity, hyperglycaemia, hyperlipidaemia, and other symptoms linked to obesity. It also has the capability of binding to PPARγ in order to stimulate differentiation of human adipocytes [66]. It has been further demonstrated to increase cold tolerance in mice and to promote β3AR gene expression in inguinal WAT. Elevation of plasma norepinephrine levels was enhanced with curcumin treatment [67]. A list of natural products, which have the potential to activate BAT and energy expenditure, is provided in table 4.

1.5 Octacosanol: A long chain fatty alcohol

Over the past 10 years, nutritional supplements, or nutraceuticals, have become increasingly popular among the general public. One such supplement, policosanol, has been the subject of numerous studies.

Policosanol is a mixture of very-long-chain saturated fatty alcohols purified from natural sources such as rice bran, wheat, sugarcane and beeswax, whose main component is octacosanol ($CH_3(CH_2)_{26}CH_2O_{14}$), a high molecular weight primary aliphatic alcohol [68,69].

Octacosanol has a number of indications for its use, many of which are currently being researched. Previous studies have demonstrated that octacosanol lowers blood cholesterol [70-74], suppresses platelet aggregation [75,76], reduces inflammation [77-79], increases athletic performance [80-83], protects cells [84], alleviates stress and restores stress-affected sleep [85]. Thus, octacosanol (policosanol) could be used as a drug or food supplement without any side effects.

1.5.1 Lipid lowering effect of octacosanol

A lot of research investigating the potential of policosanol in lowering blood cholesterol had been published in the recent years. Effect of policosanol and octacosanol has been studied in a wide range of subjects including experimental animals, healthy volunteers, and elderly patients with hypercholesterolemia.

Cholesterol is an essential component in the body as it is a vital component of cell membranes, but high cholesterol levels can cause hypercholesterolemia and eventually atherosclerosis leading to coronary heart disease (CHD). There are six important lipoprotein transporters involved in the transportation of cholesterol in the body high-density lipoproteins (HDLs), low-density lipoproteins (LDLs), intermediate-density lipoproteins, very low-density lipoproteins, chylomicron remnants, and chylomicrons. High levels of HDL are desirable as it plays a major role in maintaining cholesterol homeostasis in the body by transporting cholesterol from the

peripheral tissues to the liver [86]. Transportation of cholesterol in the blood plasma is performed by LDL, which helps incorporate cholesterol into cell membranes. LDL also acts as an essential precursor during biosynthesis of steroids.

LDL receptors on cells take up the LDL via a mechanism known as endocytosis. Also, there are cells on the hepatocytes that bind to the LDL and remove LDL from the blood. More the number of LDL receptors, more LDL will be removed, which is desirable. Increase in LDL can cause deficiency in the binding mechanisms, known as type II hypercholesterolemia. That is why treatment for hypercholesterolemia targets towards increasing HDL and decreasing total and LDL cholesterol [87,88].

During a motor endurance experiment researchers observed altered hepatic and serum lipid concentration in mice receiving octacosanol [89] and began to investigate the role of octacosanol as a cholesterol-lowering agent.

Being a natural compound, policosanol has shown to be a very safe agent in rodents and monkeys, without any major side effects [90].

A study was published in 1995, highlighting the beneficial effects of octacosanol supplementation in rats fed a high fat [71]. In the experiment, rats were fed a high-fat or normal fat diet for 4 weeks; one group on each diet was also given octacosanol. The results showed that supplementation of the high fat diet with octacosanol causes a significant decrease in the weight of perirenal adipose tissue. In further experiments, effect of octacosanol on the enzymes involved in lipid metabolism was investigated and it was found that the rate-limiting step in the esterification of fatty acid into triacylglycerol was decreased by octacosanol in rats fed a high-fat diet. This indicated that a step in the cholesterol biosynthetic pathway was inhibited by octacosanol, which was dependent on dietary fat content.

In order to check the tolerability of policosanol, a study was conducted in which healthy volunteers were given policosanol (10 or 20 mg) or placebo in two divided doses for 20 days.

Researchers observed that the subjects taking policosanol had a significant decrease in their serum cholesterol levels. Similarly, subjects receiving higher dose of policosanol (20mg) also had significant decrease in their LDL levels and increase in HDL levels. Surprisingly, subjects receiving placebo showed an increase in serum cholesterol and LDL levels. Also, there was good tolerance in all subjects taking the policosanol [91].

Ideally, high cholesterol should be managed with a low-fat diet; if there is little improvement, a statin is often added. Normal levels are those less than 6.5 mM although other risk factors such as family history, obesity, diabetes and smoking must be taken into account when determining the risk of developing atherosclerosis.

A variety of statins are available such as atorvastatin, fluvastatin, pravastatin, and simvastatin. All of these work by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase in the process of cholesterol synthesis [92].

Statins have many side effects such as myositis and other muscle effects, headache, gastrointestinal effects, rash, angioedema, altered liver function, and hepatitis. These drugs therefore should not be used in patients with renal failure or in people with a high alcohol intake whose liver function may be compromised.

Recently, one of the most popularly prescribed statins, cerivastatin, was withdrawn from the market after 31 deaths occurred due to rhabdomyolysis, a severe form of myositis, causing myoglobinuria and acute renal failure [93]. Policosanol therefore could be a useful alternative to this group of drugs because it inhibits the earlier steps of cholesterol biosynthesis.

In a double blind randomized study [94], effect of policosanol and pravastatin on lipid profile of older hypercholesterolemic patients of both sexes was investigated. Patients started a 6-week cholesterol lowering diet and lipid-lowering medication was stopped at the recruitment stage. Patients received 10 mg of policosanol and pravastatin for 8 weeks. It was found that the policosanol was more effective in lowering LDL ratio and the ratios of LDL to HDL and total

cholesterol to HDL compared to pravastatin. Moreover, patients receiving pravastatin had significantly higher serum transaminase and serum creatine phosphokinase levels, suggesting risk of hepatotoxicity and myositis. In another study, effect of policosanol with those of fluvastatin was compared [95]. Seventy elderly women received 20mg/day fluvastatin and 10mg/day policosanol. This study also identified increased transaminase, suggesting muscle deterioration and safety issues with the use of statins.

Another comparative study between policosanol and lovastatin found similar results [96]. Many placebo studies have investigated the effects of policosanol on hypercholesterolemia [97-102] and in all cases the effects seen from policosanol were similar: LDL cholesterol decreased and HDL cholesterol increased slightly.

From these studies octacosanol appear to be a good lipid-lowering agent without any serious side effects. Combination of octacosanol with lower doses of statins could be a possibility in patients with high cholesterol levels. Also, octacosanol may be used as a prophylactic treatment as one study observed lowered cholesterol levels in young healthy volunteers supplemented with octacosanol [103]. Hence, octacosanol may also be useful in healthy people.

1.5.2 Does octacosanol possess anti obesity property? Hypothesis and aim

A study investigating the biodistribution and metabolism of radiolabeled octacosanol in rats after oral administration has demonstrated higher octacosanol radioactivity in the liver, muscle and adipose tissue, especially BAT [104]. Moreover, previous studies have demonstrated that shortened saturated fatty acids (FAs; myristic, palmitic and stearic) and unsaturated FAs (oleic, palmitoleic) are formed after oral administration of policosanol to monkeys [105]. These FAs might be utilized for energy expenditure via β-oxidation [106]. Policosanol, a long chain fatty alcohol is converted into fatty acid and the reaction is catalysed by fatty alcohol: NAD oxidoreductase (FAO) [107]. FAO is a membrane-bound multi-component enzyme complex that consists of fatty alcohol dehydrogenase (FADH) and fatty aldehyde dehydrogenase (FALDH), which acts sequentially to convert fatty alcohol to aldehyde and fatty acids [108,109] (Figure 4).

The β-oxidation of FAs is critical for the function of BAT and beige fat and hence thermogenesis [110,111]. Recent studies using experimental models and humans suggest that the long chain saturated FAs, mainly stearic acid (C18:0), regulate mitochondrial function [112,113]. Moreover, the ELOVL family member 6 (Elovl6), a microsomal enzyme responsible for converting C16 FAs into C18 species, has been suggested to regulate BAT thermogenic capacity [114].

Considering that the longer fatty acids could be preferred substrates for thermogenesis and the abovementioned effects of octacosanol or policosanol on lipid metabolism, their role in the prevention or treatment of obesity and in the thermogenic function of brown and beige adipocytes has not yet been established. In the present study, we investigated anti-obesity and thermogenic function of octacosanol and policosanol in mice.

Chapter 2. Materials and methods

2.1 Animals and growth conditions

All animal husbandry and animal experiments complied with regulations of the University of Tsukuba for animal experiments and were approved by the Animal Experiment Committee of the University of Tsukuba. Seven-week-old C57BL/6 mice were obtained from CLEA Japan (Tokyo, Japan). Mice were housed in colony cages under a 12 h light/12 h dark cycle, with unlimited access to food and water. At 10 weeks of age, mice were randomly divided into the following four groups: chow group (fed on standard chow with vehicle (10 mg/ml Acacia-gum water), HFD group (fed with vehicle treatment), the HFD + octacosanol group (60 mg/kg/day; Sigma-Aldrich, Tokyo, Japan), and HFD + policosanol group (60 mg/kg/day). Octacosanol, policosanol, or the same volume of vehicle was administered via oral gavage once every four weeks. At the end of four weeks, all mice were sacrificed in the early light phase in a non-fasting state.

2.2 Dual-energy x-ray absorptiometry (DEXA) analysis

PIXImus2 DEXA (GE Medical Systems Lunar) was used to measure weight and percent of lean body tissue and fat mass.

2.3 Metabolic measurements

Glucose, insulin, TG, TC and FFA levels in plasma and TG and TC levels in the liver were determined, as described previously [115]. Bloos samples were taken during the light phase after food deprivation for four hours.

2.4 Body temperature measurement

Core body temperature was measured between 5:00 PM and 6:00 P.M. using a rectal probe attached to a digital thermometer (RET-3 rectal probe, BRC BDT-100 thermometer; Physitemp, Clifton, NJ).)

2.5 Histological analysis

Interscapular BAT (iBAT), iWAT, and eWAT were removed, fixed in 10% buffered formalin, embedded in paraffin, cut into 4 μm thick sections and used for hematoxylin and eosin (H&E) staining and immunohistochemistry as described previously [116]. Immunohistochemical staining for UCP-1 (1:400, ab10983; Abcam, Cambridge, UK) and CD68 (1:400, MCA1957GA; Bio-Rad, Hercules, USA) primary antibodies and detection with secondary antibodies (anti-rabbit IgG, Alexa Fluor 555 conjugate, 1:500, #4413; Cell Signalling Technology Japan, Tokyo, Japan or goat abti-rat IgG, Alexafluor 488 conjugate, 1:500, ab150157; Abcam, Cambridge, UK) was performed as previously described [117]. All images were acquired using a BZ-X710 microscope (Keyence, Osaka, Japan) and data were analysed using a BZ-H3 Analyzer (Keyence, Osaka, Japan) and data were analysed using a BZ-H3 Analyzer (Keyence, Osaka, Japan).. Adipocyte cell size was measured using ImageJ software (National Institutes of Health, Bethesda, MD) and at least 300 cells were counted in each sample.

2.6 Western blotting

Western blotting was performed as previously described [115]. Aliquots of whole cell lysate (30 μg) proteins extracted from BAT or iWAT were loaded onto 10% SDS-PAGE gels and transferred to PDVF membranes (Millipore, Darmstadt, Germany). Membranes were probed with anti-UCP-1 (1:1000, ab10983; Abcam, Cambridge, UK) and anti-GAPDH (1:5000, sc-32233; Santa Cruz Biotechnology, Dallas, Texas, USA) followed by horseradish peroxidase (HRP)-conjugated anti-

mouse or anti-rabbit IgG (Cell Signaling Technology Japan, Tokyo, Japan). Immune complexes were visualized using enhanced chemiluminescence (GE Healthcare Japan, Tokyo, Japan).

2.7 RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from BAT, iWAT, eWAT and liver and used for cDNA synthesis and qRT-PCR as described previously [115-118]. Primer sequences for *Adrb3*, *Aldh3a2*, *Cs*, *Elovl3*, *Fgf21* and *Ffar4* are listed in Table 5. The mRNA levels of these genes were normalized relative to cyclophilin mRNA and expressed relative to the appropriate experimental control using the ΔΔCT method.

2.8 Statistical analysis

All data are expressed as the mean ± SEM. Data were compared using Student's *t*-test (between two groups) or analysis of variance (ANOVA; more than two groups) followed with scheffe post hoc analysis and were considered statistically significant if $p < 0.05$.

Chapter 3. Results

3.1 Octacosanol and policosanol prevent HFD-induced obesity

To investigate the effect of octacosanol and policosanol on the development of obesity, male C57BL/6 mice were fed on normal chow, HFD, or HFD treated with octacosanol or policosanol ($HFD_{O/P}$) for four weeks. The body weight (BW) of HFD-fed mice was significantly higher than that of chow-fed mice. Treatment with octacosanol or policosanol suppressed the HFD-induced BW gain (Fig. 5A, B). The fat mass gain was significantly less in mice fed on $HFD_{O/P}$ compared with HFD-fed mice (Fig. 5C). The lean body mass tended to reduce in mice fed on $HFD_{O/P}$ compared with chow- or HFD-fed mice (Fig. 5D). Total body fat percentage was also significantly lower in mice fed on $HFD_{O/P}$ (Fig. 5E). The weight of epididymal WAT (eWAT) (Fig. 5F), iWAT) (Fig. 5G) and BAT (Fig. 5H) was significantly reduced in HFD-fed mice supplemented with octacosanol and policosanol.

3.2 Octacosanol or policosanol treatment reduces plasma insulin levels in HFD-fed mice

Figure 6 compares plasma metabolic parameters in four dietary groups; Chow fed, HFD fed, HFD treated with octacosanol or policosanol on the 4h fasted condition. Plasma glucose levels did not show a significant increase in the HFD group compared with the chow group (Fig. 6A). The treatment of $HFD_{O/P}$ did not change plasma glucose levels. Plasma insulin levels were markedly increased in the HFD group (Fig. 6B), demonstrating that HFD causes insulin resistance. Conversely, insulin resistance induced by the HFD was efficiently attenuated by the $HFD_{O/P}$, as reflected in significantly decreased plasma insulin levels than in the HFD group. Previous studies have shown that octacosanol supplementation alters hepatic and serum lipid concentrations in mice [73,90]. However, HFD-fed mice treated with octacosanol or policosanol showed no significant reduction in the plasma levels of TG, TC), and FFA (Fig. 6C–E).

3.3 Octacosanol and policosanol activate BAT in HFD-fed mice

Previous studies have shown that orally administered octacosanol was distributed to adipose tissue, especially BAT, and is partly oxidised and degraded to FAs through β-oxidation [104-106]. Thus, we investigated the effects of octacosanol and policosanol on BAT (Fig. 7). Histological analysis revealed that BAT of HFD-fed mice showed markedly enlarged adipocytes compared with that of chow-fed mice (Fig. 7A). However, the $HFD_{O/P}$ prevented HFD-induced enlargement of adipocytes in BAT, indicating reduced lipid accumulation within the tissue probably because they were used more as a metabolic fuel. Western blot analysis showed that protein levels of UCP-1 were significantly increased by the $HFD_{O/P}$ (Fig. 7B,C). Moreover, UCP-1 immunohistochemistry further revealed higher levels of UCP-1 in the BAT of $HFD_{O/P}$–fed mice compared with that of chow- or HFD fed mice (Fig. 7D). The expression of thermogenic genes, including UCP-1, peroxisome proliferative activated receptor, gamma, co-activator 1α (*Ppargc1a*), β3-adrenergic receptor (*Adrb3*), FFA receptor 4 (*Ffar4*) and *Elovl3* were significantly increased in BAT of mice fed on $HFD_{O/P}$ (Fig. 7E). Furthermore, mice fed on $HFD_{O/P}$ exhibited a higher core body temperature than chow- or HFD-fed mice (Fig. 7F). These results suggest that octacosanol and policosanol activate adaptive thermogenesis and energy expenditure through the upregulation of thermogenic gene expression.

3.4 Octacosanol and policosanol promote beiging of iWAT in HFD-fed mice

Beiging of iWAT has been highlighted as a new possible therapeutic target for obesity, diabetes and lipid metabolic disorders because browning of iWAT is known to increase energy expenditure and reduce adiposity. Since our results suggest that octacosanol and policosanol activate non-shivering thermogenesis in BAT and reduce body fat, we assessed the effect of octacosanol and policosanol on iWAT. Histological analysis showed large adipocytes in iWAT in HFD-fed mice; however, mice fed on $HFD_{O/P}$ showed significantly smaller adipocytes in iWAT (Fig. 8A,B). Next,

we examined whether the diminished adipocyte size in mice fed on $HFD_{O/P}$ could be the result of increased beiging of iWAT. As expected, we found that the $HFD_{O/P}$ increased the expression of genes involved in adaptive thermogenesis, such as *Ppargc1a,* PR domain containing 16 (*Prdm16*), *Ppara*, cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (*Cidea*) and *Ffar4,* as well as citrate synthase (*Cs*), a crucial component of the tricarboxylic (TCA) cycle of mitochondria (Fig. 8C). However, we unexpectedly found that the mRNA levels of UCP-1 and its transcriptional regulator PPAR gamma (*Pparg*) and the protein levels of UCP-1 was not increased in the iWAT of $HFD_{O/P}$ –fed mice (Fig. 8C-E). These data suggest that octacosanol and policosanol activate energy combustion but do not fully promote the iWAT beiging in HFD-fed mice.

3.5 Octacosanol and policosanol reduce adipocyte size and lower inflammation in epididymal WAT (eWAT) in HFD-fed mice

We also examined the effect of octacosanol and policosanol on eWAT. Histological analysis showed reduced adipocyte size in eWAT of mice fed on HFD treated with octacosanol or policosanol (Fig. 9A, B). Since octacosanol possesses anti-inflammatory activity [77-79], we examined the effects of octacosanol and policosanol on HFD-induced adipose tissue inflammation. Immunohistochemical staining of eWAT sections for CD68, a marker of infiltrated macrophage revealed that crown-like structures in mice fed on $HFD_{O/P}$ were markedly reduced relative to mice fed on HFD (Fig. 9C). Moreover, the expression of genes associated with inflammation including *F4/80*, *CD68*, tumor necrosis factor α (*Tnfa)* and interleukin 1 β *(Il1b)* was markedly upregulated in the eWAT of mice fed on HFD (Fig. 9D). The induction of these genes was suppressed in mice fed on $HFD_{O/P}$. These results suggest that, besides preventing fat accumulation in WAT, octacosanol and policosanol also prevent HFD-induced adipose tissue inflammation.

3.6 Octacosanol and policosanol attenuate fatty liver in HFD-fed mice

Liver regulates energy metabolism in the body through the dynamic control of glucose and lipid metabolism. Therefore, we investigated the effect of octacosanol and policosanol on the liver. The weight of liver in HFD-fed mice was lower than in chow-fed mice (Fig. 10A); however, HFD markedly increased hepatic levels of TG and TC (Fig. 10B, C). While the liver weights were similar between HFD-fed mice and $HFD_{O/P}$ –fed mice (Fig. 10A) hepatic levels of TG and TC were significantly lower in $HFD_{O/P}$ group compared with the HFD group (Fig. 10B, C). These results suggest that octacosanol or policosanol supplementation attenuates the increase in hepatic TG and TC levels caused by HFD. The expression of genes involved in lipogenesis and β-oxidation was analysed by quantitative PCR (qPCR) (Fig. 10D). Expression levels of sterol regulatory element-binding protein-1c (*Srebf1c)* and its downstream genes involved in lipogenesis such as FA synthase (*Fasn*), *Elovl6*, stearoyl-CoA desaturase-1 (*Scd1*) and glycerol-3-phosphate acyltransferase, mitochondrial (*Gpam*) were significantly increased in the HFD-fed mice compared with the chow-fed mice. Expression levels of Elovl6 and *Gpam* in mice fed on $HFD_{O/P}$, and of *Srebf1c* and Fasn in mice fed on HFD treated with policosanol were significantly lower than in HFD-fed mice. Expression levels of peroxisome proliferator activated receptor alpha (*Pparα*) and its target genes involved in β-oxidation, including carnitine palmitoyltransferase 1a (*Cpt1a*) and acyl-Coenzyme A dehydrogenase, medium chain (*Acadm*), were significantly increased in the HFD-fed mice compared with the chow-fed mice. $HFD_{O/P}$ significantly reduced *Pparα* expression; it also reduced the expression levels of *Cpt1a* and *Acadm*, although the reduction was non-significant. We also examined the expression levels of genes involved in cholesterol biosynthesis and uptake. Results showed that the treatment of octacosanol or policosanol significantly suppressed HFD-induced increase in the expression of low-density lipoprotein receptor (*Ldlr*) (Fig. 10E). Moreover, the treatment of $HFD_{O/P}$ tended to reduce the

expression levels of *Srebf2*, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (*Hmgcs1*) and octacosanol treatment significantly reduced 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (*Hmgcr*) expression. The expression levels of lipoprotein lipase (*Lpl*), the rate-limiting enzyme to catalyse the hydrolysis of TG in circulation, are similar in all groups. Collectively, these results suggest that octacosanol and policosanol ameliorate the development of HFD-induced fatty liver by suppressing the biosynthesis of FAs and TGs and uptake of cholesterol by the liver.

3.7 Octacosanol activates BAT and promotes beiging in normal chow-fed mice

To examine whether the higher BAT and iWAT function in HFD-fed mice treated with octacosanol is not solely due to HFD feeding, we investigated the effect of octacosanol on standard chow-fed mice. Mice fed on standard chow supplemented with octacosanol for six days showed significantly higher mRNA levels of genes involved in BAT function, including *Ucp-1*, *Ppargc1a*, *Adrb3*, *Ffar4* and fibroblast growth factor 21 (*Fgf21*) (Fig. 11A). Similarly, higher mRNA levels of *Ucp-1* and other beiging marker genes were observed in the iWAT of mice fed on standard chow treated with octacosanol (Fig. 11B). Moreover, expression levels of *Elovl6* and *Elovl3*, genes encoding FA elongases involved in the biosynthesis of long chain and very long chain saturated FAs, were significantly increased in BAT and tended to increase in iWAT, respectively, following octacosanol treatment. These data suggest that octacosanol activates BAT and iWAT not only in mice fed on HFD but also in mice fed on chow.

Table 1. List of selective β3AR agonist drugs

Drug	Status
BRL37344	Improves glucose utilization. No human studies
CL316,243	Animal studies are still continued. Human studies are discontinued.
Rafabegron	Treatment of overactive bladder
Mirabegron	Treatment of overactive bladder. BAT activated in rats and humans.
Solabegron	Treatment of overactive bladder
Amibegron	Discontinued
ICID7114	No effect on humans
ZD7114	No effect on BAT
Talibegron (ZD2079)	Increase in energy expenditure is very minimal
L-796568	Minimal effect on energy expenditure

Table 2. Norepinephrine elevators

Drug Name	Specific target(s)	Current status
Norepinephrine (NE)	↑NE	Used in the treatment of critically low blood pressure. BAT activation in mice
Ephedrine	↑NE-like activity	BAT activated in humans and mice
Atomoxetine	↑NE levels	BAT activation in mice
Nisoxetine	↑NE levels	BAT activated in mice. No human studies
Sibutramine	Serotonin-norepinephrine uptake inhibitor	Reduces appetite and promote Weight loss. Serious cardiovascular side-effect
Milnacipram	Serotonin-norepinephrine uptake inhibitor	Control weight gain in fibromyalgia patients

Table 3. List of glitazones

Drugs	Status
Rosiglitazone	↑BAT lipogenesis. Serious side effects
Ciglitazone	↓blood glucose, triglycerides, and food intake. Not in use
Troglitazone	Used as an anti-diabetic drug. Severe liver damage, discontinued
Pioglitazone	Treatment of T2D Severe bladder damage in some cases
Balaglitazone	↓ glucose levels.
Rivoglitazone	↓glucose levels by improving insulin resistance Under trial
Darglitazone	BAT size increased in rats. Serious side-effects

Table 4. Category 4 drugs: natural and other products

Drug Name	Specific target(s)	Current status
Nicotine	Nicotine receptor agonist	BAT activated in rats
Forskolin	Adenylate cyclase activator to produce cAMP	BAT activated in mice
Caffeine	Potential mechanism via adenosine receptors	Activates BAT thermogenesis
Capsacin	Increases UCP1 expression	Increases energy expenditure by activation of BAT
Curcumin	Various mechanism including PPAR-y	Stimulates human adipocyte differentiation
Rimonabant	Cannabinoid CB-1 receptor inverse agonist	CNS side effects. Drug discontinued
SHK186	Potassium ion channel blocker	BAT activation in mice
ibroblast growth factor 21 (FGF2	Endocrine factor present in liver, pancreas and adipose tissue	Energy expenditure may be associated with BAT

Table 5. Primer Sequence information for the quantitative real-time PCR analysis

Gene	Forward primer	Reverse primer
Adrb3	CCTTGGGCGAAACTGGTG	GTTGGTGACAGCTAGGTAGCG
Aldh3a2	TTCTCGTAACAATAAGCTCATCAAACG	CAGCATCCCCAGCCTTCCTTTGTTG
Cs	GGACAATTTTCCAACCAATCTGC	TCGGTTCATTCCCTCTGCATA
Elovl3	TTCTCACGCGGGTTAAAAATGG	GAGCAACAGATAGACGACCAC
Ffar4	ACCAAGTCAATCGCACCCAC	GTGAGACGACAAAGATGAGCC

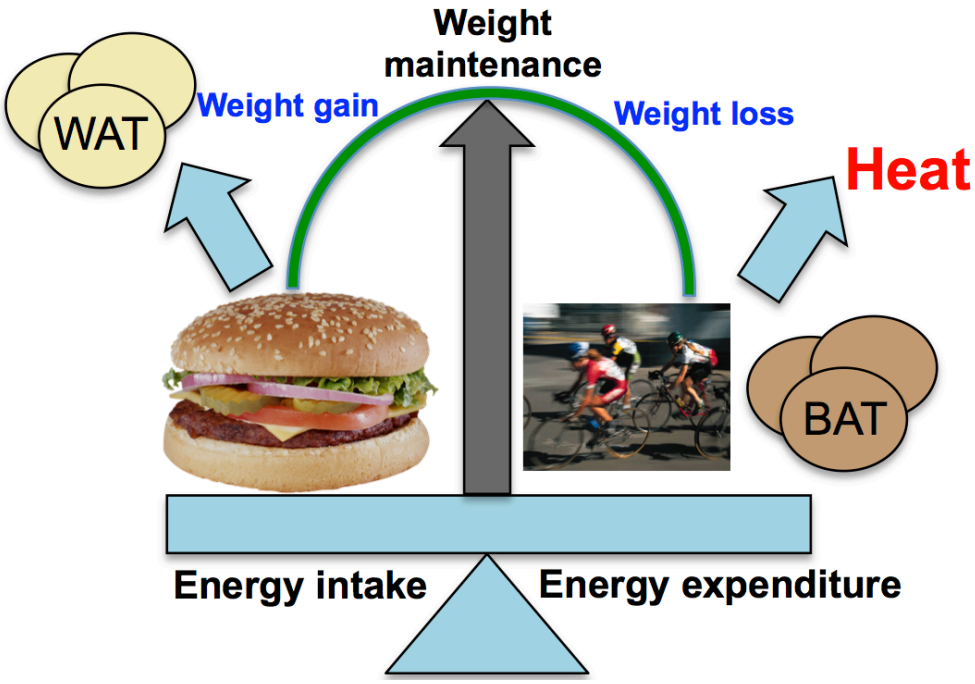


Figure 1. Obesity : a imbalance between energy intake and energy expenditure
Obesity is the consequence of imbalance in energy intake and energy expenditure. Energy intake comesfrom food consumption, whereas the vital givers to expenditure are exercise and basic metabolic processes. Energy is stored in the form of TGs in the WAT while the stored energy is utilised by brown adipose tissue to generate heat.

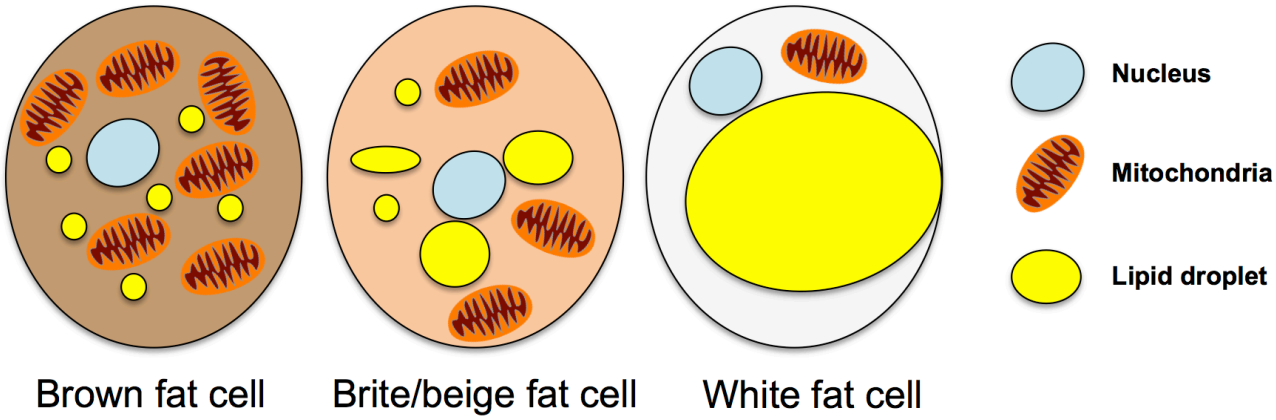


Figure 2. The three distinct fat cells.
Brown fat cell fat: converts chemical energy to heat and burns fat, brite fat cells: White fat cells that can act like brown fat cell and burns fat to dissipate energy as heat, white fat cells: most common fat cell, used to store fat.

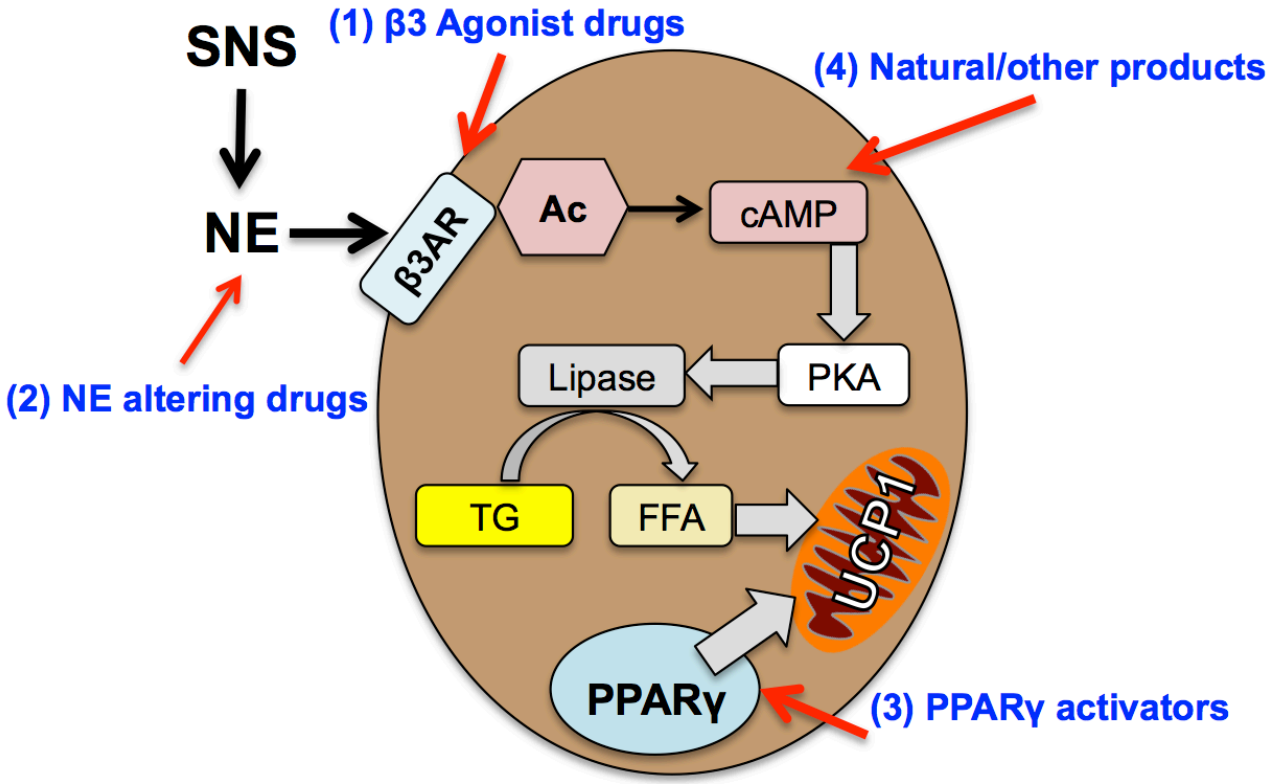


Figure 3. Possible drug sites on BAT for its activation
First category of drugs act on the cell membrane bound β3AR, which triggers the cascade of events via cAMP. Category 2 drugs acts on the norepinephrine transporter (NET) and increases norepinephrine (NE) content, which stimulates β3AR. Category 3 drugs activate PPAR- γ and category 4 drugs acts on various other pathways within BAT.

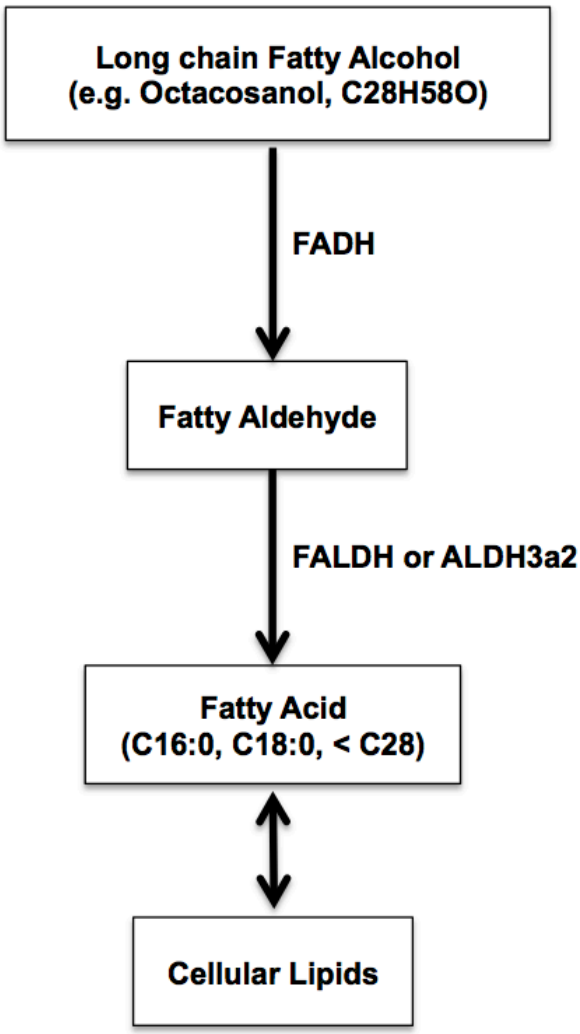


Figure 4. Conversion of fatty alcohol into fatty acids.
Fatty alcohol dehydrogenase (FADH) and fatty aldehyde dehydrogenase (FALDH) catalyse the conversion of fatty alcohol into fatty acid.

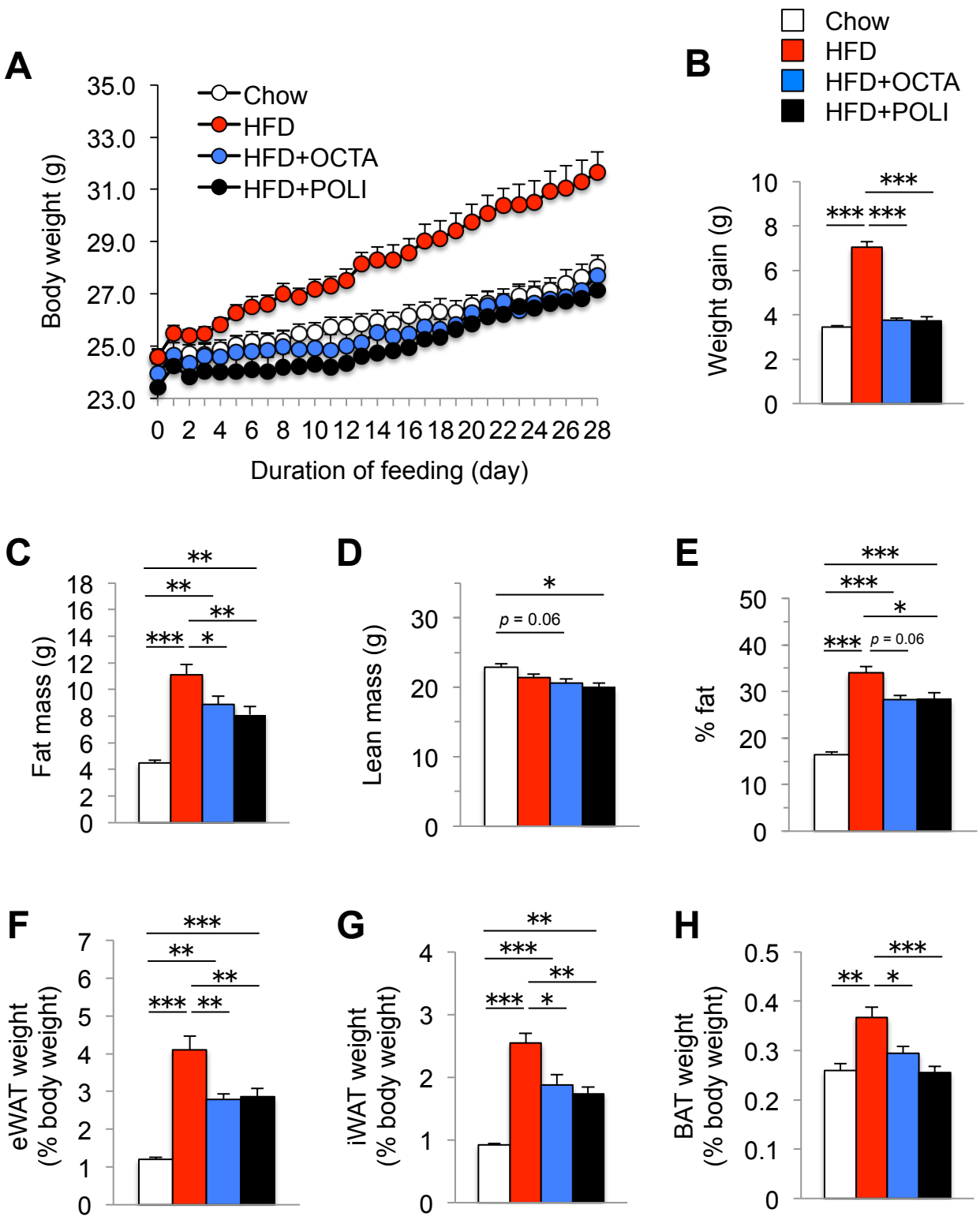


Figure 5. Effect of octacosanol and policosanol on body weight, body composition and fat accumulation in mice fed on chow, high fat diet (HFD) and HFD treated with octacosanol or policosanol. (**A, B**) Body weight (**A**) and body weight gain (**B**) of mice fed on chow, HFD and HFD treated with octacosanol or policosanol (60 mg/kg/day) for four weeks (n = 7–8). (**C–E**) Changes in fat mass (**C**), lean mass (**D**) and percent fat (**E**) measured by dual-energy X-ray absorptiometry (DEXA) analysis (n = 5). (**F–H**) Weight of epididymal white adipose tissue (eWAT) (**F**), inguinal WAT (iWAT) (**G**), brown adipose tissue (BAT) (**H**) of mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 7–8). Values represent mean ± standard error of mean (SEM). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by using one-way ANOVA followed by scheffe post hoc test.

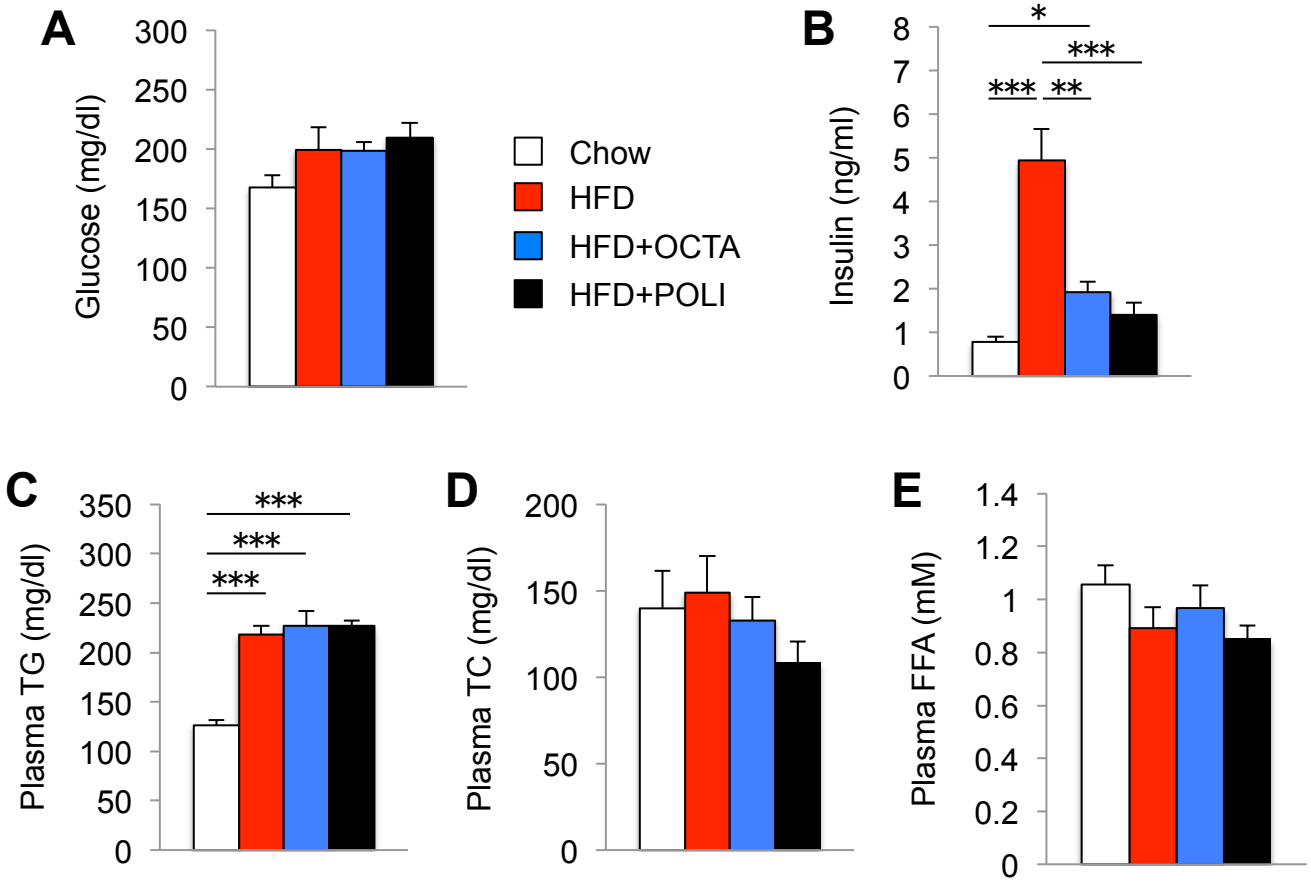
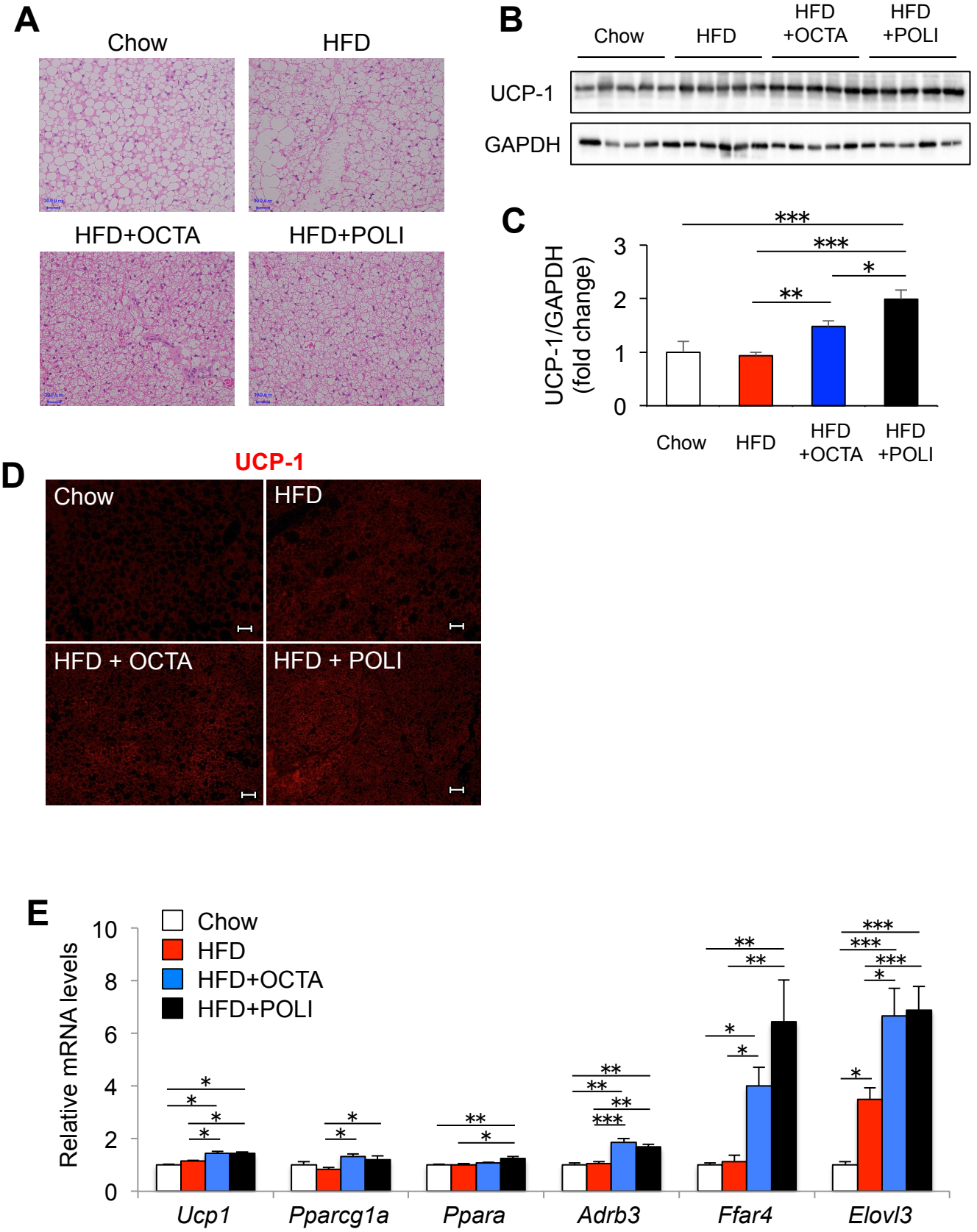


Figure 6. Effect of octacosanol and policosanol on plasma metabolic parameters of mice fed on chow, HFD and HFD treated with octacosanol or policosanol. (**A–E**) Concentrations of blood glucose (**A**), plasma insulin (**B**), plasma triglycerides (TGs) (**C**), plasma total cholesterol (TC) (**D**) and plasma free fatty acids (FFA) (**E**) of mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5). Values represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by using one-way ANOVA followed by scheffe post hoc test.


A
Chow
HFD
HFD+OCTA
HFD+POLI
B
Chow
HFD
HFD +OCTA
HFD +POLI
UCP-1
GAPDH
C
UCP-1/GAPDH (fold change)
0
1
2
3
***
***
*
**
Chow
HFD
HFD +OCTA
HFD +POLI
D
UCP-1
Chow
HFD
HFD + OCTA
HFD + POLI
E
Relative mRNA levels
0
2
4
6
8
10
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HFD
HFD+OCTA
HFD+POLI
Ucp1
Pparcg1a
Ppara
Adrb3
Ffar4
Elovl3

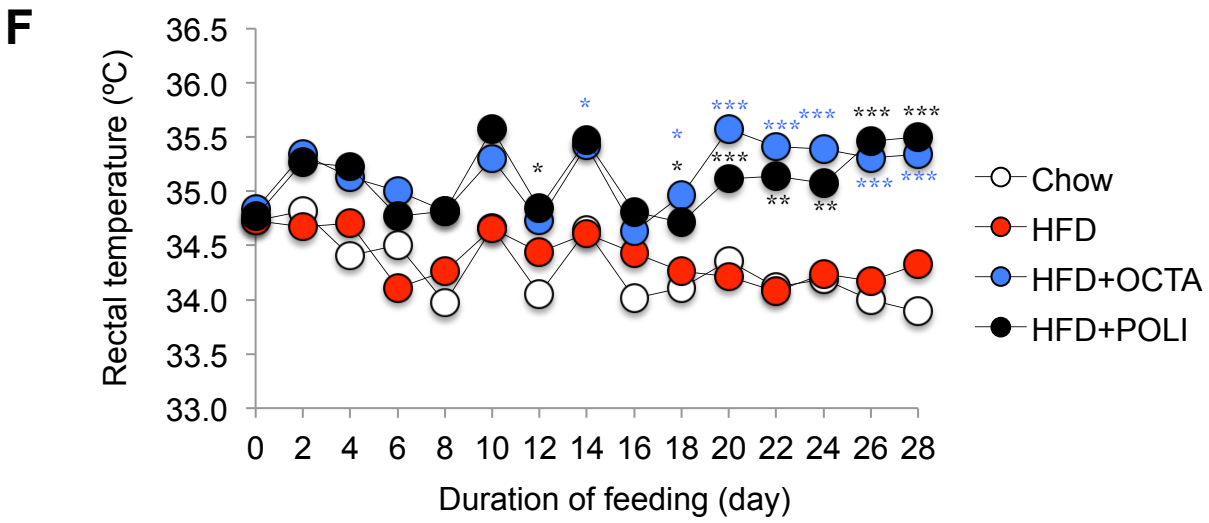

Figure 7. Effect of octacosanol and policosanol on brown adipose tissue (BAT) of mice fed on chow, HFD and HFD treated with octacosanol or policosanol. (**A**) Representative hematoxylin and eosin (H&E)-stained sections of BAT harvested from mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks. (**B**) Western blotting of UCP-1 and GAPDH in BAT (n = 5). GAPDH was used as a loading control. (**C**) Protein expression of UCP-1 determined by densitometry analysis (n = 5). (**D**) Representative immunohistochemical staining for UCP-1 in BAT sections of mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks , scale bar 30um. **(E)** Quantitative real-time PCR (qRT-PCR) analysis of genes involved in thermogenesis of BAT in mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5–8). (**F**) Rectal temperature of mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5). Values represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by using one-way ANOVA followed by scheffe post hoc test.

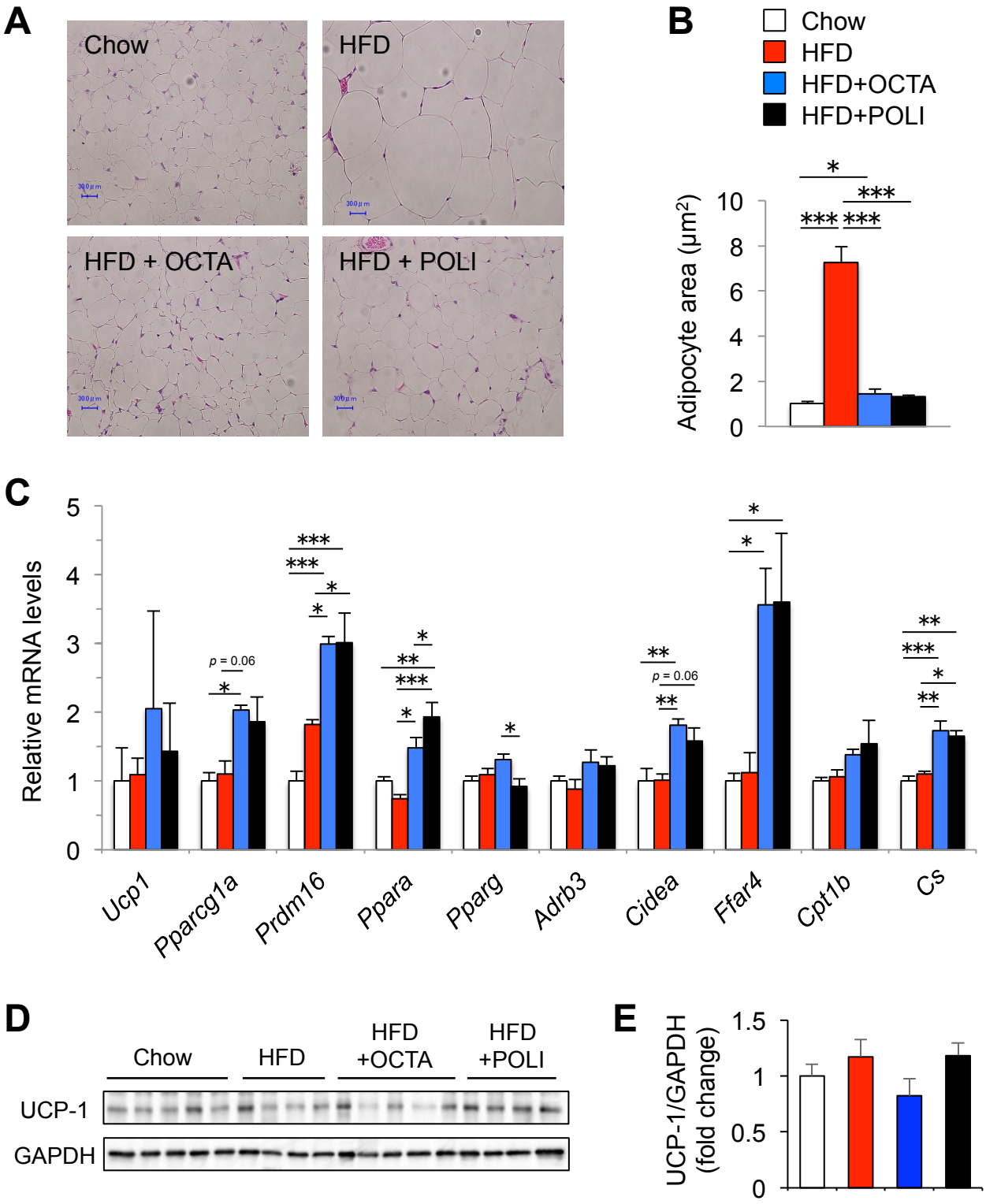

Figure 8. Effect of octacosanol and policosanol on inguinal white adipose tissue (iWAT) of mice fed on chow, HFD and HFD treated with octacosanol or policosanol. (**A**) Representative H&E-stained sections of iWAT harvested from mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks. Scale bar = 30 μm. (**B**) Average adipocyte size in iWAT of mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5). (**C**) qRT-PCR analysis of beige fat markers in iWAT harvested from mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5–8). (**D**) Western blotting of UCP-1 and GAPDH in iWAT (n = 4–5). GAPDH was used as a loading control. (**E**) Protein expression of UCP-1 determined by densitometry analysis (n = 4–5). Values represent mean ± SEM **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by using one-way ANOVA followed by scheffe post hoc test.

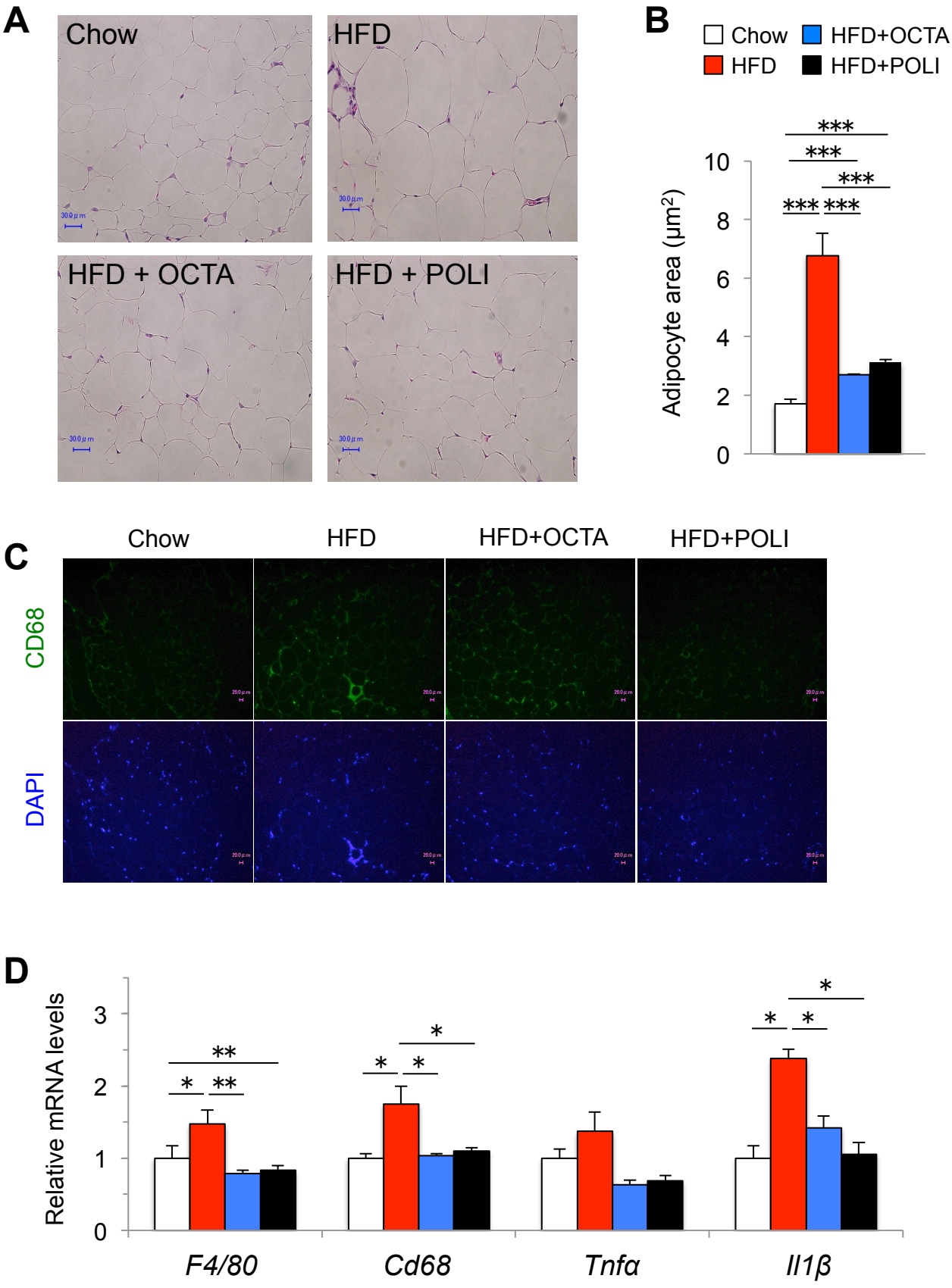
A
Chow
HFD
HFD + OCTA
HFD + POLI
B
Chow
HFD+OCTA
HFD
HFD+POLI
Adipocyte area (μm²)
0
2
4
6
8
10
***
***
***
***
***
C
Chow
HFD
HFD+OCTA
HFD+POLI
CD68
DAPI
D
Relative mRNA levels
0
1
2
3
F4/80
Cd68
Tnfα
Il1β

Figure 9. Effect of octacosanol and policosanol on epididymal WAT (eWAT) of mice fed on chow, HFD and HFD treated with octacosanol or policosanol. (**A**) Representative H&E-stained sections of eWAT harvested from mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks. Scale bar = 30 μm. (**B**) Average adipocyte size in eWAT of mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5). (**C**) Representative immunohistochemical staining for CD68 in eWAT sections of mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks, scale bar 20um. (**D**) qRT-PCR analysis of genes involved in the inflammation of iWAT harvested from mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5). Values represent mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by using one-way ANOVA followed by scheffe post hoc test.

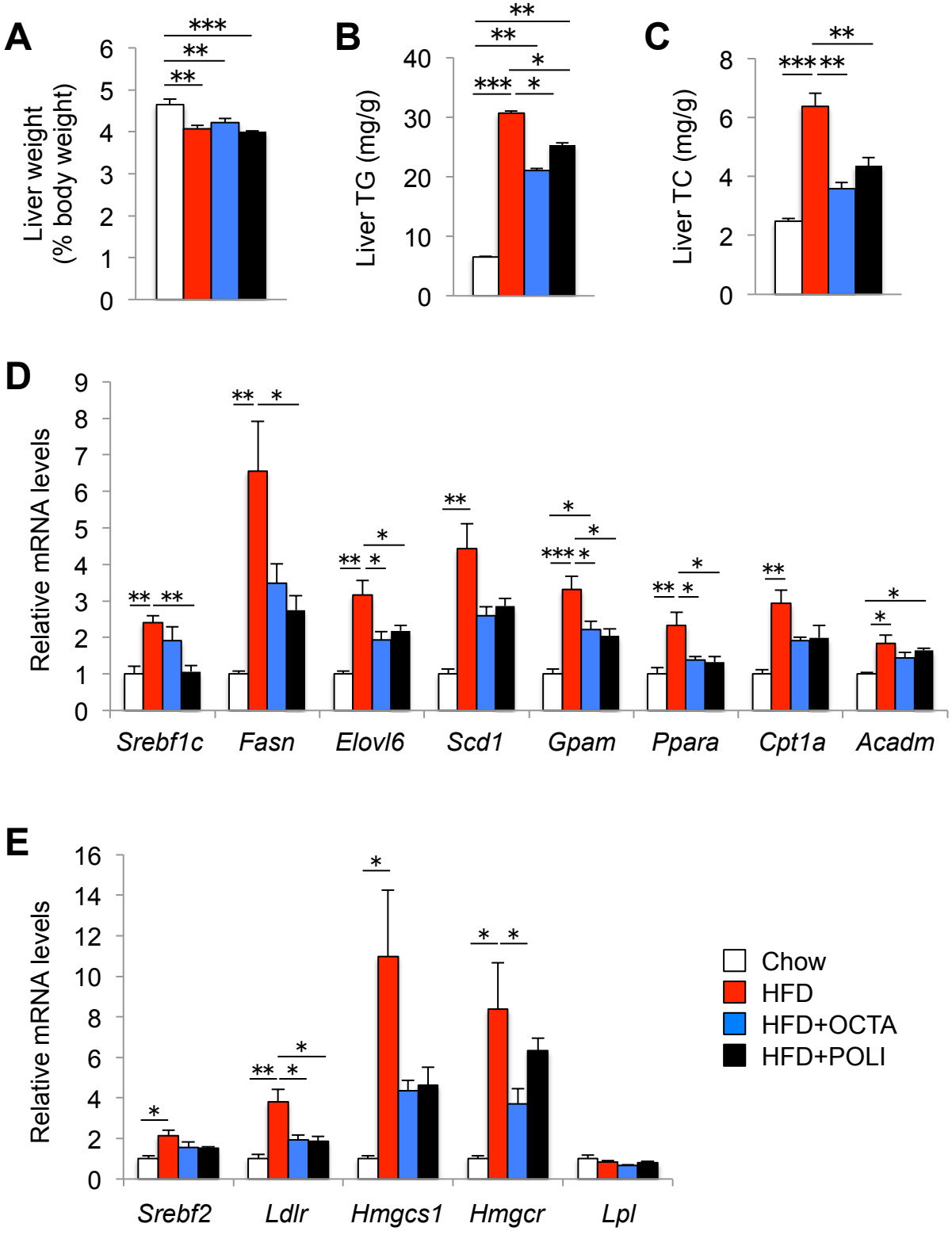

Figure 10. Effect of octacosanol and policosanol on the liver of mice fed on chow, HFD and HFD treated with octacosanol or policosanol. (**A–C**) Liver weight (**A**) and hepatic levels of TGs (**B**) and TC (**C**) in mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5–8). (**D, E**) qRT-PCR analysis of genes involved in fatty acid (FA) metabolism (**D**) and cholesterol biosynthesis (**E**) in livers harvested from mice fed on chow, HFD and HFD treated with octacosanol or policosanol for four weeks (n = 5). Values represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by using one-way ANOVA followed by scheffe post hoc test.

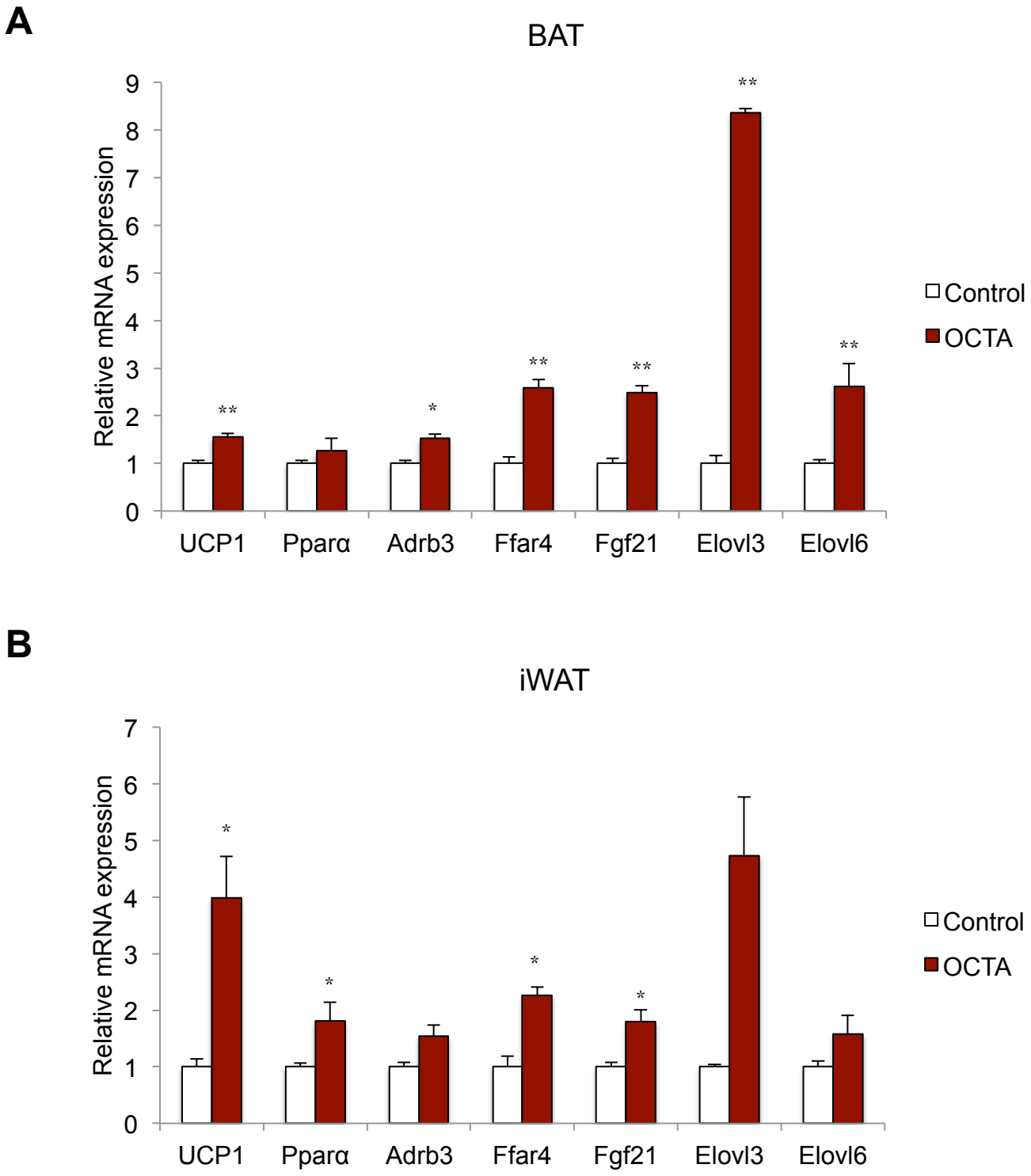

Figure 11. Effect of octacosanol on gene expression in BAT and iWAT harvested from mice fed a chow diet. (**A, B**) qRT-PCR analysis of genes involved in thermogenesis and energy expenditure of BAT (**A**) and iWAT (**B**) in mice fed on chow, with or without octacosanol treatment, for seven days (n = 5). Values represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. control (vehicle-treated) mice by using student-t-test.

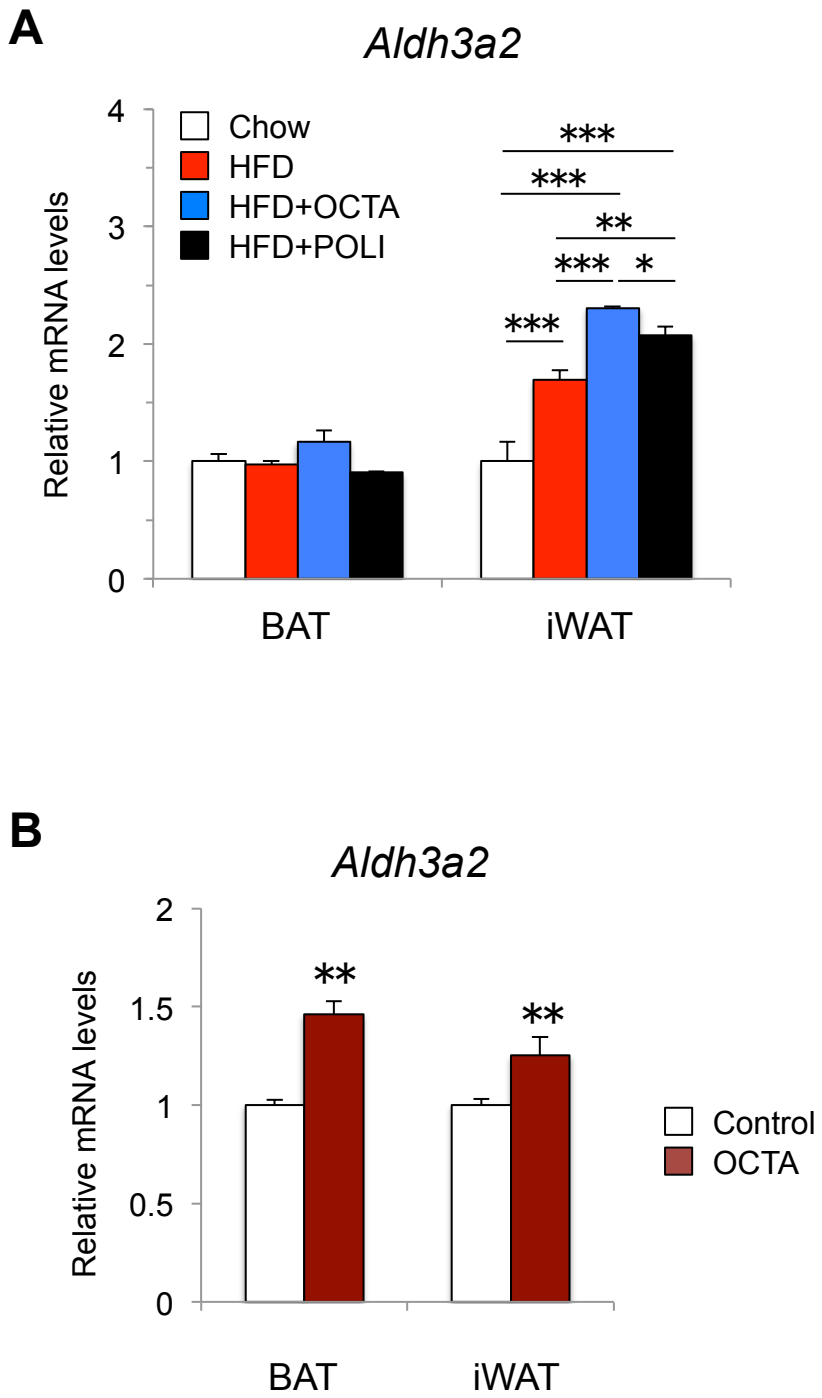

Figure 12.
Effect of octacosanol and policosanol on the expression of *Aldh3a2* in BAT and iWAT from mice fed a normal chow or HFD.
(A) Quantitative real-time PCR analysis of *Aldh3a2* in BAT and iWAT from chow-fed mice treated with or without octacosanol or policosanol for 4 weeks. Values represent means ± SEM (n=5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(B)** Quantitative real-time PCR analysis of *Aldh3a2* in BAT and iWAT from chow-fed mice treated with or without octacosanol for 7 days. Values represents means ± SEM (n=5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs vehicle-treated control

Chapter 4. Discussion

This study aimed to determine whether purified octacosanol and policosanol have beneficial effects on HFD-induced obesity in mice. We showed that both octacosanol and policosanol significantly ameliorated HFD-induced fat gain and fatty liver. Furthermore, octacosanol and policosanol activated signalling pathways that regulate thermogenesis and enhance energy expenditure in BAT and iWAT.

Several factors are involved in energy expenditure and thermogenesis in BAT and beige fat [11,12]. Policosanol is a fatty alcohol, which is converted to saturated and monounsaturated FAs by fatty aldehyde dehydrogenase (FALDH, alternatively known as Aldh3a2) [108,109]. Fatty aldehydes are also implicated in thyroid function [119], cell proliferation [120], adipogenesis and signalling pathways involving PPARγ [121]. In this study, we also observed higher expression of *Aldh3a2* in BAT and iWAT of chow- or HFD-fed mice treated with octacosanol or policosanol (Fig. 12), suggesting the conversion of octacosanol and policosanol into FAs. Previous studies have suggested that orally administrated octacosanol and policosanol may be oxidised and degraded into shortened saturated and monounsaturated FAs via β-oxidation *in vivo* [104-106]. Additionally, long chain and very long chain saturated and monounsaturated FAs synthesized via Elovl6 and Elovl3 regulate mitochondrial function and thermogenic capacity in BAT and iWAT [112-114] [122,123]. Our findings suggest that shortened saturated and unsaturated FAs metabolized from octacosanol and policosanol perform cellular functions important for thermogenesis and energy expenditure in BAT and WAT. Further studies are needed to confirm this possibility.

Recent studies have shown that FFAR4 (GPR120), a receptor for polyunsaturated FAs (PUFAs), plays a pivotal role in the thermogenic activity of BAT and browning of WAT [124-126]. Activation of FFAR4 by *n*-3 PUFA or a selective agonist increases β-oxidation and reduces body

weight and fat mass by inducing BAT activity and promoting the browning of WAT. In this study, we showed that the treatment of HFD with octacosanol or policosanol induced *Ffar4* expression in BAT and iWAT of mice and decreased lipid content in BAT and WAT. This suggests that the treatment of HFD with octacosanol or policosanol triggers brown/beige fat activation by upregulating *Ffar4* expression in BAT and WAT of HFD-induced mice.

Besides improving metabolism, octacosanol also possesses anti-inflammatory properties [77-79]. In obesity, a paracrine loop between adipocytes and macrophages augments chronic inflammation of adipose tissue, thereby inducing systemic insulin resistance and ectopic lipid accumulation [127]. Since adipose tissue in obese individuals is characterized by adipocyte hypertrophy and chronic inflammation, we also investigated the effects of octacosanol and policosanol on the inflammatory state of WAT. Treatment of HFD with octacosanol or policosanol lowered the expression levels of pro-inflammatory genes including F4/80, *CD68*, *Tnfα* and *Il1b* in eWAT. Previously, a high concentration of octacosanol has been shown to inhibit the mitogen-activated protein kinase/nuclear factor-kappa B/ activator protein-1 signalling pathway involved in the regulation of inflammation in macrophages [79]. This anti-inflammatory effect of octacosanol and policosanol might contribute to the improvement of systemic insulin resistance induced by HFD. Moreover, octacosanol and policosanol-induced FFAR4 expression might contribute toward the anti-inflammatory effect of octacosanol and policosanol because FFAR4 activation by *n*-3 PUFA triggers a broad spectrum of anti-inflammatory effects in macrophages [128,129].

Apart from the main component octacosanol (C28) (60-70%), policosanol also contains other fatty alcohols such as Hexacosanol (C26) (3-10%), triacontanol (C30) (10-15%) and dotricontanol (C32) (5-10%) [68]. However, most pharmacological effects of policosanol, including cholesterol reduction, have been proven for octacosanol, not for other constituent [130]. Previous studies investigating octacosanol biodistribution using radiolabelled octacosanol have reported that the highest amount of radioactivity from octacosanol was in adipose tissue, especially BAT followed

with perirenal adipose tissue, eWAT and liver [104]. In the liver, octacosanol may be degraded to FAs, which then incorporated into TGs, sterols and phospholipids. However, the presence of radioactivity in adipose tissue suggests that either octacosanol itself or its metabolites (FAs) were transported to be utilized for thermogenesis via β-oxidation.

Chapter 5. Conclusion

In conclusion, we show that octacosanol and policosanol exert beneficial metabolic effects by activating thermogenic changes in HFD-induced obesity in mice. Although precise molecular mechanisms underlying the involvement of octacosanol and policosanol in the thermogenic activity of BAT and browning of WAT are unknown, our data shown that octacosanol is a potent dietary anti-obesity molecule, which increases the thermogenic activity of BAT and iWAT, thereby increasing energy expenditure and reducing fat mass.

Acknowledgement

I would like to express my sincere gratitude to my advisors Prof. Hitoshi Shimano and Prof. Takashi Matsuzaka for their continous support in my Ph.D study and related research, for their patience, motivation, and immense knowledge. Their guidance helped me in all the time of research and writing of this thesis. I could not have imagined having better advisors and mentors for my Ph.D study.

References

1. Haslam DW, James WP. Obesity. Lancet. 2006;366:1197–1209.
2. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature. 2006;444:847–853.
3. Manning, S., Pucci, A. & Finer, N. Pharmacotherapy for obesity: novel agents and paradigms. *Ther. Adv. Chronic. Dis.* **5,** 135-148 (2014).
4. Shettar, V., Patel, S. & Kidambi, S. Epidemiology of Obesity and Pharmacologic Treatment Options. *Nutr. Clin. Pract.* **32,** 441-462 (2017).
5. S. Gesta, Y.H. Tseng, C.R. KahnDevelopmental origin of fat: tracking obesity to its source. Cell, 131 (2007), pp. 242-256
6. Ghorbani M, Claus TH, Himms-Hagen J. Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of diet-induced obesity in rats treated with a beta3-adrenoceptor agonist. Biochem Pharmacol. 1997;54:121–131
7. Guerra C, Koza RA, Yamashita H, et al. Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. J Clin Invest. 1998;102:412–420.
8. Lowell BB, Susulic V, Hamann A, et al. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. Nature. 1993;366:740–742
9. Nicholls DG, Locke RM: Thermogenic mechanisms in brown fat. Physol Rev 1984;64: 1-64
10. Watanabe M, Yamamoto T, Mori C, et al. Cold induced changes in gene expression in brown adipose tissue: implications for the activation of thermogenesis. Biol Pharm Bull 2008; 31: 775-84
11. Cohen, P. & Spiegelman, B.M. Brown and Beige Fat: Molecular Parts of a

Thermogenic Machine. *Diabetes* **64,** 2346-2351 (2015).

12. Kajimura, S., Spiegelman, B.M. & Seale, P. Brown and Beige Fat: Physiological Roles beyond Heat Generation. *Cell Metab.* **22,** 546-559 (2015).
13. Cannon B, Nedergaard J. Thermogenesis challenges the adipostat hypothesis for body‐weight control. Proc Nutr Soc 2009; 68: 401–407
14. Wu T, Gao X, Chen M, *et al.* Long‐term effectiveness of diet‐plus‐exercise interventions vs. diet‐only interventions for weight loss: a meta‐analysis. Obes Rev 2009; 10: 313–323
15. Barte JCM, Ter Bogt NCW, Bogers RP, *et al.* Maintenance of weight loss after lifestyle interventions for overweight and obesity, a systematic review. Obes Rev 2010; 11: 899–906
16. Franz MJ, VanWormer JJ, Crain AL, *et al.* Weight‐loss outcomes: a systematic review and meta‐analysis of weight‐loss clinical trials with a minimum 1‐year follow‐up. J Am Diet Assoc 2007; 107: 1755–1767
17. De Matteis R, Ricquier D, Cinti S. 1998. TH-, NPY-, SP-, and CGRP-immunoreactive nerves in Interscapular brown adipose tissue of adult rats acclimated at different tempreatures: an Immunohistochemical study. J Neurocyto1 1998; 27:877-86.
18. Inokuma K, Ogura-Okamatsu Y, Toda C, Kimura K, Yamashita H, Saito M: Uncoupling protein 1 is necessary for norepinephrine induced glucose utilization in brown adipose tissue. Diabetes 2005; 54: 1385-91.

19. Poher A-L, Altirriba J, Veyrat-Durebex C, Rohner-Jeanrenaud F. Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance. Front Physiol 2015; 6:4. Doi: 10.3389/fphys. 2015. 00004.
20. Michel MC, Korstanje C. β3-Adrenoceptor agonists for overactive bladder syndrome: Role of translational pharmacology in a repositioning clinical drug development project. Pharm. Therapeutics 2016; dx.doi.org/10.1016/j.pharmthera.2016.01.007
21. Arch JR, Ainsworth AT, Cawthorne MA, et al. Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. Nature 1984;309:163-65
22. Arch JR. β3-adrenoceptor agonists: Potential pitfalls and progress. Eur J Pharmacol 2002; 440:99-107
23. Ursino MG, Vasina V, Raschi E, Crema F, DePonti F. The β3-adrenoceptor as a therapeutic target: Current perspectives. Pharm Res 2009; 59:221-34
24. Michel MC, Ochodnicky P, Summers RJ. Tissue functions mediated by β3-adrenoceptors-findings and challenges. Naunyn Sch Arch Pharmacol 2010;382:103-8
25. Sen A, Nichani N. Exploring beta3 adrenoceptors for potential clinical applications. Int J Pharm Sci Rev Res 2010;5: 55-8
26. Wilson C, Wilson S, Piercy V, Sennitt MV, Arch JR. The rat lipolytic β adrenoceptor: studies using novel β-adrenoceptor agonists. Eur J Pharm 1984; 100: 309-19
27. Liu YL, Stock MJ. Acute effects of the β3 adrenoceptor agonists, BRL 35135, on tissue glucose utilization. Br J Pharm 1995; 114: 888-94.

28. Yoshida T, Sakane N, Wakabayashi Y, Umekawa T, Kondo M: Anti-obesity effect of CL 316,243, a highly specific β3-adrenergic receptor agonist, in yellow kk mice. Life sci 1994; 54: 491-98.
29. Bloom JD, Dutia MD, Johnson BD, et al. Disodium (R,R)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]1,3-benzodioxole-2,2-dicarboxylate (CL 316,243). A potent beta adrenergic agonist virtually specific for beta 3 receptors. A promising antidiabetic and antiobesity agent. J Med Chem 1992; 35:3081-84.
30. Lowell BB, Flier JS: Brown adipose tissue, beta 3-adrenergic receptors, and obesity. Annu Rev med 1997; 48: 307-16.
31. Weyer C, Tataranni PA, Snitker S, Danforth E, Jr., Ravussin E. Increase in insulin action and fat oxidation after treatment with CL 316,243, a highly selective beta3-adrenoceptor agonist in humans. Diabtes 1998; 7: 1555-1561.
32. Redman LM, de jonge I, fang X, et al. Lack of an effect of novel beta3-adrenoceptor agonists, TAK-677, on energy metabolism in obese individuals: a double blind, placebo controlled randomized study. J Clin Endocrinol Metab 2007; 92: 527-531.
33. Takasu T, Ukai M, Sato S, et al. Effect of (R)-2-(2-aminothiazol-4-yl)-4-{2-[(2-hydroxy-2-phenylethyl)-amino]ethyl}acetanilide (YM178), a novel selective β3-adrenoceptor agonist, on bladder function. J Pharm Exp Ther 2007; 321: 642-647.
34. Australian public assessment report for Mirabegron, Australian Government, Department of Health. AusPAR 2014; PM-2012-01928-3-3.

35. Mirbolooki MR, Schade KN, Constantinescu CC, Pan M-L, Mukherjee J. Enhancement of ^{18}F-fluorodeoxyglucose metabolism in rat brain frontal cortex using β3 adrenoceptor agonist. Synapse 2015; 69: 96-8.
36. Cypess Am, Weiner LS, Roberts-Toler C, et al. Activation of human brown adipose tissue by β3-adrenergic receptor agonist. Cell Metab 2015; 21: 33-8
37. Savontaus E, Pesonen U, Rouru J, Huupponen R, Koulu M. Effects of ZD7114, a selective β3-adrenoceptor agonists, on neuroendocrine mechanisms controlling energy balance. Eur J Pharm. 1998; 347: 265-74.
38. Growcott JW, Holloway B, Green M, Wilson C. Zeneca ZD7114 acts as an antagonist at β3-adrenoceptors in rat isolated ileum. Br J Pharm 1993; 110: 1375-80
39. Buemann B, Toubro S, Astrup A. Effects of the two beta3-agonists, ZD7114 and ZD2079 on 24 hour energy expenditure and respiratory quotient in obese subjects. Int J Obes Relat metab Disord 2000; 24: 1553-60.
40. Holloway BR, Howe R, Rao BS, et al. ICI D7114 a novel selective beta-adrenoceptor agonist selectively stimulates brown fat and increases whole body oxygen consumption. Br J Pharm 1991; 104: 97-104.
41. Champigny O, Ricquier D, Blondel O, Mayers Rm, Briscoe MG, Holloway BR. β3-Adrenergic receptor stimulation restores message and expression of brown-fat mitochondrial uncoupling protein in adult dogs. Proc Natl Acad Sci 1991; 88: 10774-7.
42. Larsen TM, Toubro S, Van Baak MA, et al. Effect of a 28-day treatment with L-796568, a novel bta3-adrenergic receptor agonist, on energy expenditure and body composition of obese men. Am J Clin Nutr 2002; 76:780-8.

43. Bianchetti A, manara L. In Vitro inhibition of intestinal motility by phenylethanolaminotetralines: evidence of atypical beta adrenoceptors in rat colon. Br J Pharm 1990; 100: 831-9.
44. Stemmelin J, Cohen C, Yalcin I, Keane P, Griebel G. Implication of β3 adrenoceptors in the antidepressant-like effects of amibegron using Adrb3 knockout mice in the chronic mild stress. Behave Brain Res 2009; 206: 310-2.
45. Chernogubova E, Cannon B, Bengtsson T. Norepinephrine increases glucose transport in brown adipocytes via beta3 adrenoceptors through a cAMP, PKA, and PI3-kinase-dependent pathway stimulating conventional and novel PKCs. Endocrinology 2004; 145: 269-80.
46. Vijgen GH, Bouvy ND, Teule GJ, Brans B, Schrauwen P, Van MarkenLichtenbelt WD. Brown adipose tissue in morbidly obese subjects. PLoS One 2011, 6:217247.
47. Matthias A, Kerstin BE, Ohlson J, et al. Thermogenic Responses in Brown fat Cells Are Fully UCP1-dependent UCP2 or UCP3 cannot substitute for UCP1 in adrenergically or fatty acid-induced thermogenesis. J Biol Chem 2000; 275: 25073-081.
48. Salomone S. Pleiotropic effects of glitazones: double edge sword ? Eront Pharm 2011; 2: 1-6.
49. Digby JE, Mantague CT, Sewter CP, et al. Thiazolidinedione exposure increases the expression of uncoupling protein 1 in cultured human Preadipocytes. Diabetes 1998; 47: 138-41.
50. Breider MA, Gough AW, haskins JR, Sobocinski G, De La Iglesia FA. Troglitazone-Induced Heart and Adipose Tissue Cell Proliferation in Mice. Toxicol Pathol 1999; 27: 545-52.

51. Chang AY, Wyse BM, Gilchrist BJ, Peterson T, Diani AR. Ciglitazone, a new hypoglycemic agent. Studies in ob/ob and db/db mice, diabetic Chinese hamsters, and normal and streptozotocin diabetic rats. Diabetes 1983; 32: 830-38.

52. Yamamoto Y, Nakajima M, Yamazaki H, Yokoi T. Cytotoxicity and apoptosis produced by troglitazone in human hepatoma cells. Life Sci 2000; 70: 471-82.

53. Henriksen K, Byrjalsen I, Nielsen RH, et al. A comparison of glycemic control, water retention, and musculoskeletal effects of balaglitazone and pioglitazone in diet-induced obese rats. Eur J Pharm. 2009; 616: 340-45.

54. Henriksen K, Byrjalsen I, Qvist P, et al. BALLET Trial Investigators. Efficacy and safety of the PPARy partial agonist balaglitazone compared with pioglitazone and placebo: a phase III, randomized, parallel-group study in patients with type 2 diabetes on stable insulin therapy. Diabetes Metab Res Rev 2011; 27: 392-401.

55. Kanda S, Nakashima R, Takahashi K et al. potent antidiabetic effects of rivoglitazone, a novel peroxisome proliferator-activated receptor-gamma agonist, in obese diabetic rodent models. J Pharm Sci 2009; 111: 155-66.

56. Koffarnus RL, Wargo KA, Phillippe HM. Rivoglitazone: A new Thiazolidinedione for the treatment of type 2 diabetes mellitus. Ann Pharmacother 2013; 47: 877-85.

57. Aleo MD, Lindeen GR, Blackwell DK, et al. Mechanism and implications of brown adipose tissue proliferation in rats and monkeys treated with the thiazolidinone darglitazone, a potent peroxisome-activated receptor-gamma agonist. J Pharm Exp Ther 2003; 305: 1173-82.

58. Mano-Otagiri A, Iwasaki-Sekino A, Ohata H, Arai K, Shibasaki T. nicotine suppresses energy storage through activation of sympathetic outflow to brown adipose tissue via corticotropin-releasing factor type 1 receptor. Neurosci Lett 2009; 455: 26-29.
59. Yoshida T, Yoshioka K, Hiraoka N, Kondo M. Effect of nicotine on norepinephrine turnover and thermogenesis in brown adipose tissue and metabolic rate in MSG obese mice. J Nutr Sci Vitaminol (Tokyo) 1990; 36: 123-30.
60. Scarpace PJ, Matheny M. Thermogenesis in brown adipose tissue with age: post-receptor activation by forskolin. Eur J Physiol 1996; 431: 388-94.
61. De Jesus LA, Carvalho SD, Ribeiro MO, et al. The type 2 iodothyronine deiodinase is essential for adaptive thermogenesis in brown adipose tissue. J Clin Invest 2001; 108: 1379-85.
62. Yoshida T, Yoshioka K, Hiraoka N, Kondo M. Caffeine activates brown adipose tissue thermogenesis and metabolic rate in mice. J Nutr Sci Vitaminol (Tokyo) 1990; 36: 173-8.
63. Gnad T, Scheiber S, Kugelgen IV, et al. Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. Nature 2014; 516: 395-99.
64. Yoneshiro T, Aita S, Kawai Y, Iwanaga T, Saito M. Nonpungent capsaicin analogs (capsinoids) increase energy expenditure through the activation of brown adipose tissue in humans. Am J Clin Nutri 2012; 95: 845-50.
65. Leung FW. Capsaicin as an anti-obesity drug. Prog Drug Res. 2014; 68: 171-9.
66. Aggarwal, BB. Targeting inflammation induced obesity and metabolic diseases by curcumin and other nutraceuticals, Annu Rev Nutr 2010; 30: 173-99.

67. Wang S, Wang X, Zichen Y, et al. Curcumin promotes browning of white adipose tissue in a norepinephrine dependent way. Biochem Biophys Res Commun 2015; 466: 247-53.

68. Taylor, J.C., Rapport, L. & Lockwood, G.B. Octacosanol in human health. *Nutrition* **19,** 192-195 (2003).

69. Irmak, S. & Dunford, N.T. Policosanol contents and compositions of wheat varieties. *J. Agric. Food Chem.* **53,** 5583-5586 (2005).

70. Arruzazabala, M.L.*, et al.* Cholesterol-lowering effects of policosanol in rabbits. *Biol. Res.* **27,** 205-208 (1994).

71. Kato, S.*, et al.* Octacosanol affects lipid metabolism in rats fed on a high-fat diet. *Br. J. Nutr.* **73,** 433-441 (1995).

72. Lin, Y.*, et al.* Wheat germ policosanol failed to lower plasma cholesterol in subjects with normal to mildly elevated cholesterol concentrations. *Metabolism* **53,** 1309-1314 (2004).

73. Singh, D.K., Li, L. & Porter, T.D. Policosanol inhibits cholesterol synthesis in hepatoma cells by activation of AMP-kinase. *J. Pharmacol. Exp. Ther.* **318,** 1020-1026 (2006).

74. Keller, S., Gimmler, F. & Jahreis, G. Octacosanol administration to humans decreases neutral sterol and bile acid concentration in feces. *Lipids* **43,** 109-115 (2008).

75. Arruzazabala, M.L., Carbajal, D., Mas, R., Garcia, M. & Fraga, V. Effects of Policosanol on platelet aggregation in rats. *Thromb. Res.* **69,** 321-327 (1993).

76. Arruzazabala, M.L., Molina, V., Carbajal, D., Valdes, S. & Mas, R. Effect of policosanol on cerebral ischemia in Mongolian gerbils: role of prostacyclin and thromboxane A2. *Prostaglandins Leukot Essent Fatty Acids* **49,** 695-697 (1993).

77. Fernandez-Arche, A.*, et al.* Long-chain fatty alcohols from pomace olive oil modulate the release of proinflammatory mediators. *J. Nutr. Biochem.* **20,** 155-162 (2009).

78. de Oliveira, A.M.*, et al.* Antinociceptive and anti-inflammatory effects of octacosanol from the leaves of Sabicea grisea var. grisea in mice. *Int. J. Mol. Sci.* **13,** 1598-1611 (2012).

79. Guo, T.*, et al.* Octacosanol Attenuates Inflammation in Both RAW264.7 Macrophages and a Mouse Model of Colitis. *J. Agric. Food Chem.* **65,** 3647-3658 (2017).

80. Consolazio, C.F., Matoush, L.O., Nelson, R.A., Isaac, G.J. & Hursh, L.M. Physiological and biochemical evaluation of potential antifatigue drugs. III. The effect of octacosanol, wheat germ oil and vitamin E on the performance of swimming rats. *Rep US Army Med Res Nutr. Lab. Denver*, 1-2 (1963).

81. Consolazio, C.F., Matoush, L.O., Nelson, R.A., Isaac, G.J. & Hursh, L.M. Effect of Octacosanol, Wheat Germ Oil, and Vitamin E on Performance of Swimming Rats. *J. Appl. Physiol.* **19,** 265-267 (1964).

82. Stusser, R., Batista, J., Padron, R., Sosa, F. & Pereztol, O. Long-term therapy with policosanol improves treadmill exercise-ECG testing performance of coronary heart disease patients. *Int. J. Clin. Pharmacol. Ther.* **36,** 469-473 (1998).

83. Kim, H., Park, S., Han, D.S. & Park, T. Octacosanol supplementation increases running endurance time and improves biochemical parameters after exhaustion in trained rats. *J Med Food* **6,** 345-351 (2003).

84. Carbajal, D., Molina, V., Valdes, S., Arruzazabala, L. & Mas, R. Anti-ulcer activity of higher primary alcohols of beeswax. *J. Pharm. Pharmacol.* **47,** 731-

733 (1995).

85. Kaushik, M.K., Aritake, K., Takeuchi, A., Yanagisawa, M. & Urade, Y. Octacosanol restores stress-affected sleep in mice by alleviating stress. *Sci. Rep.* **7,** 8892 (2017).
86. Rang HP, Dale MM, Ritter JM. Pharmacology, 4th ed. London: Churchill Livingstone, 1990:830.
87. Castano G, Mas R, Fernandez L et al. Effects of policosanol on postmenopausal women with type II hypercholesterolemia. Gynecol Endocrinol 2000;14:187.
88. Craig CR, Stitzel RE. Modern pharmacology, 4th ed. Boston: Little, Brown & Co, 1994-907.
89. Shimura S, Hasegawa T, Takano S, et al. Studies on the effect of octacosanol on motor endurance in mice. Nutr Rep Int 1987;36:1029.
90. Arruzazabala ML, Carbajal D, Mas R, et al. Cholesterol-lowering effects of policosanol in rabbits. Biol Res 1994;27:205.
91. Hernandez F, Illait J, Mas R, et al. Effect of policosanol on serum lipids and lipoproteins in healthy volunteers. Curr Ther Res 1992;51:568.
92. BMA/RPSGB. British National Formulary, 41st ed. London: British Medical association, 2001;124
93. Bellingham C. Cerivastatin withdrawal:the impacts. Pharm J 2001;267:222
94. Castano G, Mas R, Arruzazabala M de L, et al. Effects of policosanol and pravastatin on lipid profile, platelet aggregation and endothelimia in older hypercholesterolemic patients. Int J Clin Pharm Res 1999;4:105
95. Fernandez JC, Mas R, castano G, et al. Comparison of the efficacy, safety and tolerability of policosanol versus fluvastatin in elderly hypercholesterolaemic women. Clin Drug Invest 2001;21:103

96. Crespo N, Illnait J, Mas R, et al. Comparative study of the efficacy and tolerability of policosanol and lovastatin in patients with hypercholesterolemia and non insulin dependent diabetes mellitus. Int J Clin Pharm Res 1999;19:117

97. Pons P, Rodriguez M, Mas R, et al. One-year efficacy and study of policosanol in patients with type II hypercholesterolaemia, Curr Ther Res 1994;55:1084

98. Aneiros E, Mas R, Calderon B, et al. Effect of policosanol in lowering cholesterol levels in patients with type II hypercholesterolaemia. Curr Ther Res 1995;56:176

99. castano G, mas R, Nodarse M, et al. One year study of the efficacy and safety of policosanol (5mg twice daily) in treatment of type II hypercholesterolaemia. Curr Ther Res 1995;56:296

100. Castano G, Canetti M, Moreira M et al. Efficacy and tolerability of policosanol in elderly patients with type II hypercholesterolaemia: a 12-month study. Curr ther Res 1995;56:819

101. Castano G, Tula L, Canetti M, et al. Effects of policosanol in hypertensive patients with type II hypercholesterolaemia. Curr Ther Res 1996;57:691

102. Mas R, castano G, Illnait J, et al. Effects of policosanol in patients with type II hypercholesterolaemia and additional coronary risk factors. Clin pharmacol ther 1999;65:439

103. Arruzazabala ML, Valdes S, Mas R, et al. Comparative study of policosanol, aspirin and the combination therapy policosanol-aspirin on platelet aggregation in healthy volunteers. Pharmacol Res 1997:36.

104. Kabir, Y. & Kimura, S. Biodistribution and metabolism of orally administered octacosanol in rats. *Ann. Nutr. Metab.* **37,** 33-38 (1993).

105. Menendez, R.*, et al.* In vitro and in vivo study of octacosanol metabolism.

Arch. Med. Res. **36,** 113-119 (2005).

106. Kabir, Y. & Kimura, S. Tissue distribution of (8-14C)-octacosanol in liver and muscle of rats after serial administration. *Ann. Nutr. Metab.* **39,** 279-284 (1995).

107. T. Lee, Characterization of fatty alcohol: NAD+ oxidoreductase from rat liver. J. Biol. Chem. 254 (1979) 2892-2896

108. Ichihara, K., Kusunose, E., Noda, Y. & Kusunose, M. Some properties of the fatty alcohol oxidation system and reconstitution of microsomal oxidation activity in intestinal mucosa. *Biochim. Biophys. Acta.* **878,** 412-418 (1986).

109. Ichihara, K., Noda, Y., Tanaka, C. & Kusunose, M. Purification of aldehyde dehydrogenase reconstitutively active in fatty alcohol oxidation from rabbit intestinal microsomes. *Biochim. Biophys. Acta.* **878,** 419-425 (1986).

110. Lee, J., Ellis, J.M. & Wolfgang, M.J. Adipose fatty acid oxidation is required for thermogenesis and potentiates oxidative stress-induced inflammation. *Cell Rep.* **10,** 266-279 (2015).

111. Gonzalez-Hurtado, E., Lee, J., Choi, J. & Wolfgang, M.J. Fatty acid oxidation is required for active and quiescent brown adipose tissue maintenance and thermogenic programing. *Mol. Metab.* **7,** 45-56 (2018).

112. Senyilmaz, D.*, et al.* Regulation of mitochondrial morphology and function by stearoylation of TFR1. *Nature* **525,** 124-128 (2015).

113. Senyilmaz-Tiebe, D.*, et al.* Dietary stearic acid regulates mitochondria in vivo in humans. *Nat. Commun.* **9,** 3129 (2018).

114. Tan, C.Y.*, et al.* Brown Adipose Tissue Thermogenic Capacity Is Regulated by Elovl6. *Cell Rep.* **13,** 2039-2047 (2015).

115. Matsuzaka, T.*, et al.* Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. *Nat. Med.* **13,** 1193-1202 (2007).

116. Ohno, H., *et al.* Transgenic Mice Overexpressing SREBP-1a in Male ob/ob Mice Exhibit Lipodystrophy and Exacerbate Insulin Resistance. *Endocrinology* **159,** 2308-2323 (2018).

117. Zhao, H., *et al.* Elovl6 Deficiency Improves Glycemic Control in Diabetic db/db Mice by Expanding beta-Cell Mass and Increasing Insulin Secretory Capacity. *Diabetes* **66,** 1833-1846 (2017).

118. Nakagawa, Y., *et al.* Hepatic CREB3L3 controls whole-body energy homeostasis and improves obesity and diabetes. *Endocrinology* **155,** 4706-4719 (2014).

119. Dugrillon, A. Iodolactones and iodoaldehydes--mediators of iodine in thyroid autoregulation. *Exp. Clin. Endocrinol. Diabetes* **104 Suppl 4,** 41-45 (1996).

120. Muzio, G., Maggiora, M., Paiuzzi, E., Oraldi, M. & Canuto, R.A. Aldehyde dehydrogenases and cell proliferation. *Free Radic. Biol. Med.* **52,** 735-746 (2012).

121. Yu, Y.H., *et al.* PKC-ALDH2 Pathway Plays a Novel Role in Adipocyte Differentiation. *PLoS One* **11,** e0161993 (2016).

122. Westerberg, R., *et al.* ELOVL3 is an important component for early onset of lipid recruitment in brown adipose tissue. *J. Biol. Chem.* **281,** 4958-4968 (2006).

123. Zadravec, D., *et al.* Ablation of the very-long-chain fatty acid elongase ELOVL3 in mice leads to constrained lipid storage and resistance to diet-induced obesity. *FASEB J.* **24,** 4366-4377 (2010).

124. Quesada-Lopez, T., *et al.* The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes. *Nat. Commun.* **7,** 13479 (2016).

125. Kim, J., *et al.* Eicosapentaenoic Acid Potentiates Brown Thermogenesis through FFAR4-dependent Up-regulation of miR-30b and miR-378. *J. Biol.*

Chem. **291,** 20551-20562 (2016).

126. Schilperoort, M.*, et al.* The GPR120 agonist TUG-891 promotes metabolic health by stimulating mitochondrial respiration in brown fat. *EMBO Mol. Med.* **10**(2018).

127. McNelis, J.C. & Olefsky, J.M. Macrophages, immunity, and metabolic disease. *Immunity* **41,** 36-48 (2014).

128. Oh, D.Y.*, et al.* A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. *Nat. Med.* **20,** 942-947 (2014).

129. Oh, D.Y. & Walenta, E. Omega-3 Fatty Acids and FFAR4. *Front Endocrinol. (Lausanne)* **5,** 115 (2014).

130. Gouni-Berthold, I. & Berthold, H.K. Policosanol: clinical pharmacology and therapeutic significance of a new lipid-lowering agent. Am Heart J 143, 356-365 (2002).