

Effect of 8-Methyl Nonanoic Acid, a Degradation By-Product of Dihydrocapsaicin, on Energy and Glucose Homeostasis in Diet-Induced Obese Mice

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Purpose: Consumption of chili with capsaicinoids, such as dihydrocapsaicin (DHC), offers metabolic benefits to humans. However, their spiciness and rapid degradation prevent it from being used as a treatment for metabolic syndrome (MetS), including obesity, insulin resistance (IR), and hyperglycemia. During the degradation process of capsaicinoids, DHC is metabolized to non-pungent 8-methyl nonanoic acid (8-MNA), a methylated medium-chain fatty acid (MCFA). However, the metabolic functions of 8-MNA and its therapeutic potential for MetS have been unknown in animals. As other MCFAs improve metabolic status when added to obesogenic diets, we hypothesize that 8-MNA may improve energy and glucose metabolism in diet-induced obese (DIO) mice that exhibit MetS-like metabolic derangements.

Methods: C57BL/6NJcl mice were fed a normal diet, or a high-fat diet (HFD) supplemented with triacylglycerols, which consisted of 8-MNAs or isocaloric soybean oil (SBO) for 18 weeks. Food intake, body weight, and blood chemicals were assessed, and glucose and insulin tolerance tests (GTT and ITT, respectively) were performed. Tissues and organs collected at the end of the experiments were used for biochemical analyses of metabolic determinants.

Results: Compared with HFD + SBO-fed mice, 8-MNA feeding resulted in reduced caloric intake and body weight gain in DIO mice ($p < 0.05$) in association with overall weight loss in several tissues and organs as well as transcriptional downregulation of orexigenic agouti-related protein in the hypothalamus. Despite no improvement in GTT and ITT, during the early experimental period, 8-MNA supplementation delayed the onset of HFD-induced IR.

Conclusion: We conclude that 8-MNA slows the development of MetS in DIO mice. Furthermore, these findings suggest that 8-MNA derived from DHC accounts, in part, for the metabolic benefits of consuming chili and may represent a promising non-pungent nutraceutical for preventing MetS.

Keywords: chili peppers, capsaicinoids, metabolic syndrome, insulin resistance, obesity, prediabetes

Introduction

Metabolic syndrome (MetS) is a medical condition consisting of a broad spectrum of metabolic defects, including visceral obesity, insulin resistance (IR), elevated blood glucose (BG), dyslipidemia, and hypertension.¹ The prevalence of these diseases has increased over the last few decades in developed and developing countries.^{2,3} Patients with MetS have an increased risk of developing life-threatening complications, such as coronary heart disease, stroke, type 2 diabetes (T2D, if uncontrolled), and loss of renal function.^{1,4} Currently, treatment for MetS involves improving lifestyle, particularly diet and physical activity; however, promoting sustainable efforts and compliance in lifestyle modification

is a burden for many patients.⁵ If ineffective or unsuccessful, patients may receive pharmacological treatment, although no simple cures are available for MetS-associated complications.⁶ Therefore, preventative approaches are needed for people who are at high risk for developing MetS. For example, in daily life, we can regularly consume bioactive food ingredients (or nutraceutical products) that are considered preventative treatments for these metabolic disorders.⁷

The consumption of chili peppers offers many benefits to individuals prone to developing MetS by promoting weight loss as well as improving glycemic control.⁸ These effects primarily arise from capsaicin (CAP) and dihydrocapsaicin (DHC), which are the major bioactive capsaicinoids in chili fruits; however, the bioavailability of these capsaicinoids is limited because of their unfavorable pungent flavor and rapidly degradative properties.^{9–14} The former characteristic causes discomfort to the gastrointestinal tract in humans.^{10,11} The latter was demonstrated in an HPLC study in rats, in which a time-dependent rapid clearance of CAP was observed in blood and tissues, whereas 24.4% of the administered CAP at 1 h after injection was reduced to 1.24% and 0.057% at 24 h and 48 h, respectively.^{10,12} For DHC, most of the tritiated DHC administered to rats was absorbed via the jejunum. In the portal blood, 85% of the radioactivity was derived from the DHC tracer, whereas 15% of it was metabolized to 8-methyl nonanoic acid (8-MNA).¹³ Besides the jejunum, rat liver homogenates also convert DHC to 8-MNA through hydrolysis.¹⁴ These results suggest that 8-MNA is a ubiquitous degradation by-product of DHC in animals. 8-MNA is a naturally occurring methyl-branched medium-chain fatty acid (MCFA) that is a precursor for capsaicinoid biosynthesis in plants.¹⁵ Therefore, similar to other MCFAs, this metabolite may be used as an energy source and as a functional lipid;¹⁶ however, the biological roles of 8-MNA in animals are still unknown.

Previous studies have shown that consuming other MCFAs, such as octanoic acid (C8:0) and decanoic acid (C10:0), enhances insulin sensitivity,^{17–20} the anti-inflammatory response,^{18,19} energy expenditure (EE),^{21–23} and satiety,^{24,25} along with a reduction in ectopic fat accumulation,^{26–28} which slows the development of MetS.^{16,29} Apart from these even-numbered MCFAs, nonmethylated nonanoic acid (C9:0) may act as a selective agonist of the nuclear receptor, peroxisome proliferator-activated receptor γ (PPAR γ), which is the target of the insulin-sensitizing glitazones for T2D.³⁰ Moreover, intravenous administration of C9:0 into anesthetized dogs appears to acutely enhance whole-body insulin sensitivity.³¹ Recently, our *in vitro* study demonstrated that 8-MNA exerts metabolic effects on 3T3-L1 adipocytes by inhibiting *de novo* lipogenesis and lipolytic responses to a β -agonist, while increasing insulin-dependent glucose uptake.³² Collectively, these lines of evidence suggest that 8-MNA may improve MetS in a similar manner to MCFAs in animals. However, there have been no *in vivo* studies that evaluate the metabolic effects of 8-MNA and its therapeutic potential for the prevention of MetS, despite the *in vitro* evidence showing the anti-lipogenic and insulin-sensitizing effects of 8-MNA.³² To explore these potential benefits of 8-MNA *in vivo*, we examined the effects of 8-MNA on energy and glucose metabolism in diet-induced obese (DIO) mice that exhibit MetS-like pathology without hypertension.³³

Materials and Methods

Chemical Preparation

Propane-1,2,3-triyl tris(8-methylnonanoate), a triacylglycerol that consists of three 8-MNAs esterified to one molecule of glycerol, was chemically synthesized as follows (Figure 1): Amberlite IR 120 (H form ion-exchange resin), 4-dimethylaminopyridine 99% (DMAP), *N,N'*-dicyclohexylcarbodiimide 99% (DCC), and DL-1,2-isopropylideneglycerol were purchased from Acros Organics (Cat#10098951, France), Aldrich (Cat#107700, USA), Aldrich (Cat#D80002, China), and Aldrich (Cat#122696, Spain), respectively. ¹H NMR and ¹³C NMR spectra were recorded on a 400 MHz NMR spectrometer (Avance, Bruker, Switzerland). Chemical shifts (δ) were recorded in parts per million (ppm) relative to either tetramethylsilane or the residual protonated solvent signal as a reference. Mass spectra were measured in positive ion mode using a high-resolution mass spectrometer (micrOTOF, Bruker, Switzerland) that was calibrated using sodium formate. Infrared spectroscopy was conducted using an FT-IR spectrometer (Model 1600, Perkin Elmer, USA) within a wave number range of 4000–400 cm^{−1}. 8-MNA (**1**) was synthesized as described in our previous report.³² DL-1,2-isopropylideneglycerol (**2**, 92.06 g, 696.58 mmol), 8-MNA (**1**, 100.00 g, 580.48 mmol), and DMAP (3.56 g, 29.02 mmol) were dissolved in dichloromethane (DCM, 500 mL) and cooled in an ice bath. DCC (143.73 g, 696.58 mmol) solution in DCM (150 mL) was added dropwise using cannula tubing at 0°C followed by stirring

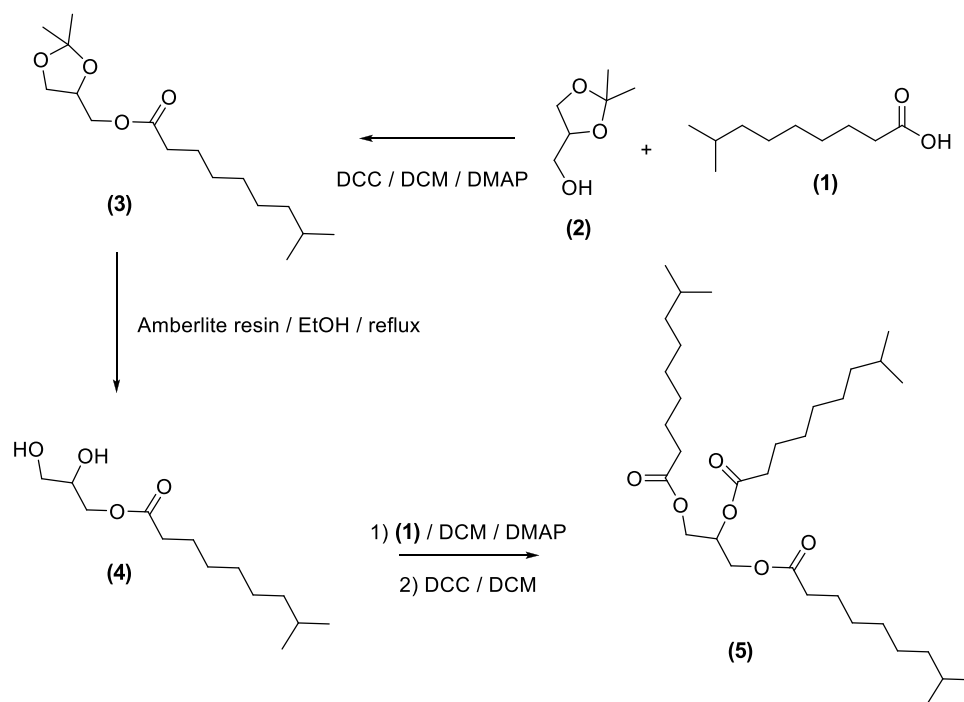


Figure 1 Chemical synthesis of propane-1,2,3-triyl tris(8-methylnonanoate). (1) 8-methyl nonanoic acid (8-MNA), (2) DL-1,2-isopropylideneglycerol, (3) intermediate product, (4) 2,3-dihydroxypropyl 8-methylnonanoate, and (5) propane-1,2,3-triyl tris(8-methylnonanoate).

overnight. The mixture was filtered and concentrated under reduced pressure to yield an intermediate compound (3). Amberlite IR 120 (100.00 g) was added to ethanol (300 mL) in which the product (3, 200.00 g) was dissolved. The deprotection reaction was completed by stirring for 3.5 h. The reaction mixture was filtered and concentrated to yield crude 2,3-dihydroxypropyl 8-methylnonanoate (4). The crude product was treated with EtOH/water (8:2 v/v, 1 L) and hexane (2 × 200 mL) for extraction. The EtOH/water layer was concentrated in vacuo to produce 2,3-dihydroxypropyl 8-methylnonanoate (4) as a light-yellow oil (111.00 g, 76% yield) with an $R_f = 0.12$ (25% EtOAc in hexane). Next, 2,3-dihydroxypropyl 8-methylnonanoate (4, 50.00 g, 202.96 mmol), 8-MNA (1, 76.92 g, 446.52 mmol), and DMAP (3.01 g, 24.56 mmol) were dissolved in DCM (500 mL) and cooled in an ice bath. DCC (92.13 g, 446.52 mmol) solution in DCM (150 mL) was added dropwise using cannula tubing at 0°C, followed by stirring overnight. The mixture was filtered, and concentrated to yield crude propane-1,2,3-triyl tris(8-methylnonanoate) (5). The crude product was purified by column chromatography (5% EtOAc in hexane) to yield propane-1,2,3-triyl tris(8-methylnonanoate) (5) as a light-yellow oil (57.25 g, 51% yield), $R_f = 0.45$ (5% EtOAc in hexane); ^1H NMR (400 MHz, CDCl_3) δ (ppm): 0.85 (d, $J = 6.6$ Hz, 18H), 1.19–1.09 (m, 6H), 1.35–1.21 (m, 18H), 1.49 (dq, $J = 13.2, 6.6$ Hz, 3H), 1.65–1.54 (m, 6H), 2.30 (td, $J = 7.5, 2.5$ Hz, 6H), 4.14 (dd, $J = 11.9, 5.9$ Hz, 2H), 4.29 (dd, $J = 11.9, 4.3$ Hz, 2H), 5.29–5.22 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 22.75, 24.99, 27.33, 28.06, 29.23, 29.64, 34.18, 34.34, 39.06, 62.22, 68.99, 77.35, 173.42. FT-IR (ATR) (cm^{-1}): (ATR) (cm^{-1}): 1741 (C=O stretching), 1153 (C-O stretching). HRMS (ESI-TOF) m/z : $[\text{M} + \text{Na}]^+$ calculated for $\text{C}_{33}\text{H}_{62}\text{O}_6\text{Na}$, 577.4439; found, 577.4437.

Dextrose (Cat#000045-B00, 50%, ANB laboratories, Thailand) and human recombinant insulin (Cat#00169-1833-11, 100 U/mL, Novo Nordisk, USA) were diluted with sterile saline solution (Cat#LS-3-101 R3, Thai Nakorn Patana Co., Ltd., Thailand) prior to use.

Animals

The animal procedures were approved by the Mahidol University Institutional Animal Care and Use Committee (MU-IACUC, Protocol numbers: MUSC64-013-562 and F02-66-010 approved as of June 15th, 2021 and March 1st, 2023, respectively, Animal license number: U1-07952-2562). For the welfare of the laboratory animals, we followed the Guide

for the Care and Use of Laboratory Animals (8th Edition) and the AVMA Guidelines for the Euthanasia of Animals (2020 Edition) with supplementary recommendations from the FELASA and CCAC guidelines. This study was conducted in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0.³⁴ To determine the sample size ($n = 42$), a power analysis was performed using G*Power 3.1 with an effect size of 0.5, alpha error of 0.05, power of 0.8, and 3 groups.^{35,36} Six-week-old male C57BL/6NJcl mice (21.7 ± 0.8 g on arrival estimated from <https://nomura-siam.com/en/animals/c57bl6njcl-en/>) were purchased from Nomura Siam International (Thailand) and acclimatized for two weeks at the Central Animal Facility, Faculty of Science, Mahidol University (MUSC-CAF), an AAALAC International-accredited facility. C57BL/6 mice are susceptible to developing metabolic derangements when being treated with a high-fat diet (HFD)^{33,37} and were used to study the beneficial effects of MCFAs on the metabolism in previous studies.^{18,22,24,26–28} Eight-week-old mice (22.9 ± 0.1 g) were singly housed and maintained under a 12:12 h light/dark cycle with ad libitum access to water. The mice were randomly divided into three groups using GraphPad Random number generator (<https://www.graphpad.com/quickcalcs/randomize1/>)³⁴ and fed a normal diet (ND, Cat#082G, Perfect Companion Group, Thailand, [Supplementary Figure 4](#) for the formulation) provided by MUSC-CAF or a HFD containing 60 kcal% fat (Cat#D12492, Research Diets, Inc., USA, <https://researchdiets.com/formulas/d12492> for the formulation) supplemented with 1.84% of soybean oil (HFD + SBO, Cat#S7381, Sigma-Aldrich, USA) or 2% of propane-1,2,3-triyl tris(8-methylnonanoate) (HFD + 8-MNA). This feeding design is based on the gross energy values of medium-chain and long-chain triacylglycerols determined by previous bomb calorimetric studies³⁸ to supplement HFD with the isocaloric amount of 8-MNA or soybean oil which is a fat constituent of HFD.³⁹ A study using C9:0-fed rodents showed low toxic effects at this dose.⁴⁰ Body weight (BW) and food intake were measured weekly using an electronic balance (KERN572, Germany). The caloric intake of individual mice was calculated by converting grams of consumption to calories. The mice were sacrificed by CO₂ inhalation-induced euthanasia at week 18. Following decapitation, trunk blood was collected into an EDTA-coated tube. Tissues and organs were harvested, weighed, snap-frozen in liquid nitrogen, and stored at -80°C until use.

Assessment of Glucose Homeostasis

Nonfasting blood glucose (BG) concentrations in the tail tip were measured using a hand-held glucometer (Roche, USA) at the indicated times. An intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT)⁴¹ were carried out on mice at weeks 16 and 17, respectively. For IPGTT, the mice received an IP injection of 20% dextrose solution (1.5 g of glucose/kg of BW) following overnight fasting. The BG levels in the tail tip were measured at 0, 15, 30, 60, 90, and 120 min following injection. For IPITT, 4-h fasted mice received an IP injection of recombinant human insulin (0.75 IU insulin/kg of BW) and the BG data were collected at the same time points as those for IPGTT.

Measurements of Insulin and Interleukin-6 (IL-6) Concentrations

Plasma samples were collected from nonfasting mice at weeks 9 and 18. Insulin and IL-6 concentrations were measured using insulin and IL-6 ELISA kits^{42–44} (Cat#90080, Crystal Chem and Cat#88-7064-22, Thermo Fisher, respectively, USA).

Measurements of Triacylglycerol (TAG) Content

TAG concentrations in plasma, liver, and aorta samples at week 18 were measured using a triglyceride assay kit (Cat#ab65336 Abcam, UK). Liver and aorta tissues (100 mg) were homogenized in 1 mL of 5% NP-40 in ddH₂O and heated at 90°C on a heat block until the TAG layer was solubilized. The homogenates were centrifuged for 2 min at $13,000 \times g$, and the supernatant was diluted 10-fold with ddH₂O. The diluted samples were further diluted 2- to 25-fold with Assay Buffer before adding Reaction Mix. Following 60-min incubation at room temperature in dark, the optimal density at 570 nm was measured with a plate reader.

Western Blot Analysis

Protein expression in brown adipose tissue (BAT) was measured by Western blot analysis. A sample of BAT (~ 300 mg) was homogenized in lysis buffer (pH 7.5, 1 mL) containing 50 mM Tris-Cl, 0.1 mM EDTA, 0.1 mM EGTA, 1% w/v deoxycholic acid, 1% v/v NP-40, 0.1% v/v SDS, 1 mM PMSF, and 1% protease/phosphatase inhibitor cocktail

(Cat#5872, Cell Signaling Technology, USA) on ice. The homogenates were centrifuged at $14,000 \times g$ for 30 min at 4°C . The protein concentration in the supernatant was measured using a DC protein assay kit (Cat#5000112, Bio-Rad, USA). The protein samples (20 μg) were incubated with 4X loading buffer containing 10% 2-mercaptoethanol at 95°C for 5 min. The denatured proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Cat#1620115, Bio-Rad, USA). After blocking with 5% nonfat milk (Cat#1706404, Bio-Rad, USA), the membranes were cut into several strips as a protein of interest was included in the middle based on its molecular size.^{32,45} Each of the membrane strips was incubated with a primary antibody against UCP1 (1:1000, Cat#14,670, Cell Signaling Technology, USA, RRID: AB_2687530), AMPK (1:1000, Cat#2532, Cell Signaling Technology, USA, RRID: AB_330331), p-AMPK (1:1000, Cat#2531, Cell Signaling Technology, USA, RRID: AB_330330), or GAPDH (1:10,000, Cat#5174, Cell Signaling Technology, USA, RRID: AB_10622025) at 4°C overnight. The membrane strip was washed three times with 1x Tris-buffered saline containing 0.1% Tween 20 for 5 min on a rocker, followed by incubation with anti-rabbit IgG, HRP-linked secondary antibody (1:2000, Cat#7074, Cell Signaling Technology, USA, RRID: AB_2099233) for 1 h at room temperature. The signals were developed using a western ECL substrate (Cat#1705062, Bio-Rad, USA), visualized with Amersham ImageQuant 800 (Cytiva, UK), and quantitated using ImageJ software.

RNA Extraction, cDNA Synthesis, and Real-Time PCR

Hypothalamic tissues were ground in a microcentrifuge tube with liquid nitrogen using a plastic pestle. Total RNA was extracted using Trizol reagent (Cat#15596026, Invitrogen, USA) based on the manufacturer's protocol and then cleaned using a Monarch RNA cleanup kit (Cat#T2010S, New England Biolabs, USA) together with DNase I treatment. cDNA was synthesized from 1 μg of total RNA using the iScript Reverse Transcription kit (Cat#1708840, Bio-Rad, USA). Real-time PCR was performed with the Luna Universal Probe qPCR Master Mix (Cat#M3004, New England Biolabs, USA). TaqMan probes (Thermo Fisher Scientific, USA) were obtained for POMC (Mm00435874_m1), agouti-related protein (AgRP) (Mm00475829_g1), IL-6 (Mm00446190_m1), and β -actin (Mm02619580_g1). A CFX96 Real-Time system (Bio-Rad, USA) was used for the assays. Relative quantitation of gene expression was done using the $\Delta\Delta\text{Ct}$ method.⁴⁶

Statistics

The data are expressed as the mean $\pm$ SEM and analyzed using Prism 10 software (GraphPad, USA). One-way ANOVA or two-way repeated measures ANOVA tests were used to compare the three groups with one or two independent variables, respectively. Student's *t*-test was used to compare the two groups. A *p*-value less than 0.05 was considered statistically significant.

Results

Effect of 8-MNA on Body Weight and Food Intake in HFD-Fed Mice

To determine the metabolic response to 8-MNA in DIO mice, we fed wild-type male mice a ND or a HFD for 18 weeks. The ND group served as a control to ensure the obesogenic effect of HFD. The HFD-fed animals were subdivided into two groups: HFD supplemented with soybean oil (HFD + SBO) containing long-chain fatty acids (LCFAs) or propane-1,2,3-triyl tris(8-methylnonanoate), which contained three 8-MNAs esterified to one molecule of glycerol (HFD + 8-MNA). BW and food intake were measured weekly in the individually housed mice. Within 18 weeks of the experimental period, no mice died ($n = 42$ in total) or showed any health issues irrespective of diet and supplement types, indicating that 8-MNA was nontoxic. The mice fed with HFD+SBO or HFD+8-MNA weekly gained more weight compared with the ND group in the first 7 weeks or weeks 2–8, respectively (Figure 2A). Over the following ~ 10 weeks, except for week 8 when HFD + 8-MNA mice gained more weight compared with ND and HFD + SBO mice, no significant variations in weekly weight gain were observed among the three groups. Consistent with the extent of weekly weight gain, HFD + SBO and HFD + 8-MNA mice were significantly heavier compared with the ND mice (Figure 2B). In the HFD groups, 8-MNA-fed mice had significantly less BW compared with the SBO-fed mice (Figure 2B). Correspondingly, Figure 2C indicates that the weekly caloric intake of HFD-fed mice was significantly greater compared with that of the ND-fed group throughout the entire experimental period. There was no significant difference in weekly

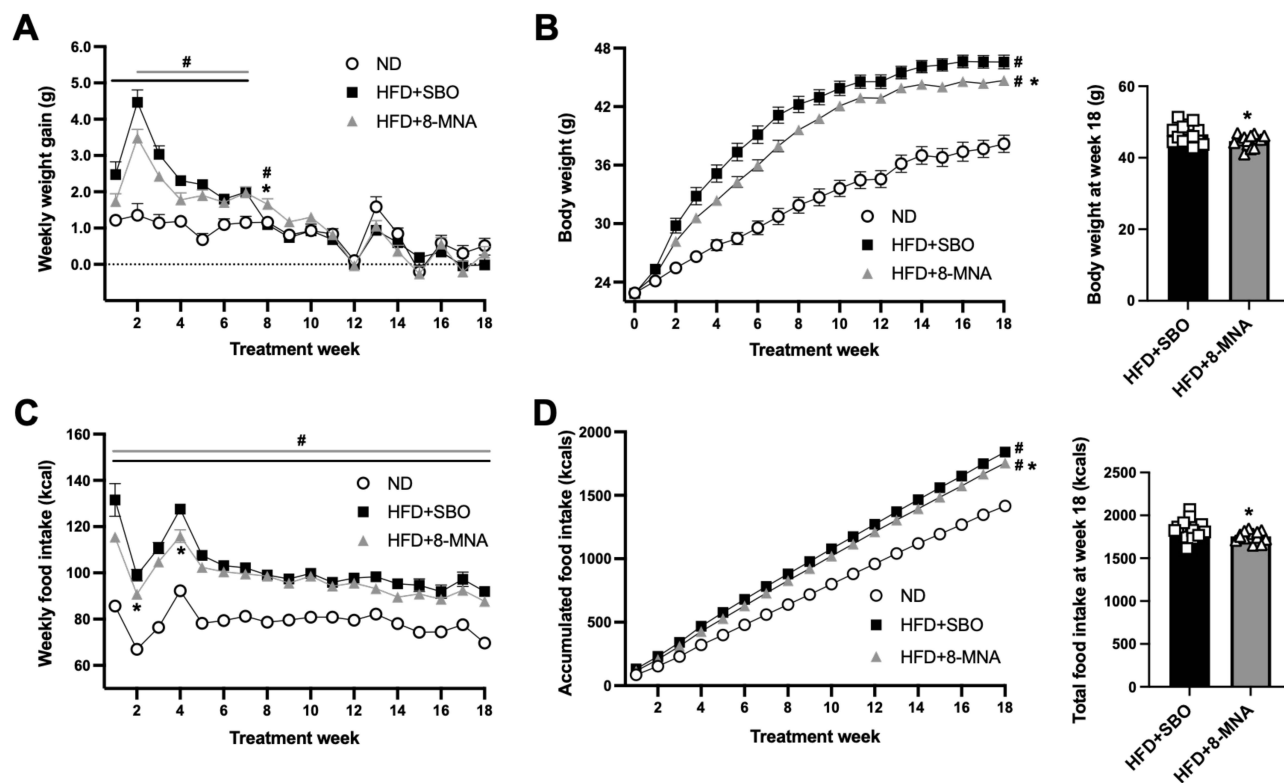


Figure 2 Effect of 8-MNA on the body weight and food intake in HFD-fed mice. Wild-type mice were fed a normal diet (ND) or a high-fat diet (HFD) supplemented with soybean oil (SBO) or triacylglycerol composed of 8-methyl nonanoic acid (8-MNA) for 18 weeks. (A) Weekly weight gain. (B) Body weight. (C) Weekly food intake. (D) Cumulative food intake. Data are presented as the mean $\pm$ SEM and analyzed by a two-way repeated measures ANOVA or Student's *t*-test ($n = 14$). (B and D) A significant treatment $\times$ time interaction was determined by a two-way ANOVA with repeated measures. # $p < 0.05$ vs ND. * $p < 0.05$ vs HFD + SBO. n, sample size.

food intake between the HFD + SBO and HFD + 8-MNA groups, excluding weeks 2 and 4 (Figure 2C); however, 8-MNA consumption produced a slight, but steady anorexigenic effect over the entire duration of treatment. As expected, the cumulative caloric intake of the HFD + 8-MNA group was significantly lower compared with that of the HFD + SBO group (Figure 2D). These results suggest that 8-MNA supplementation partly diminishes HFD-induced BW gain, along with the sustained suppression of caloric intake.

Effect of 8-MNA on Glucose Homeostasis in HFD-Fed Mice

In the same cohort of animals, nonfasting blood glucose (BG) concentrations were measured at weeks 0, 2, 4, 8, 9, 13, and 17. HFD + SBO-fed mice exhibited significant increases in BG levels compared with mice fed an ND or HFD + 8-MNA (Figure 3A). However, by week 17, 8-MNA-mediated glycemic normalization in HFD mice disappeared (Figure 3A). This finding was confirmed in subsequent experiments evaluating glucose disposal and insulin sensitivity using an intraperitoneal glucose tolerance test (IPGTT) and an IP insulin tolerance test (IPITT), respectively (Figure 3B and C). At week 16, a time course and area under the curve (AUC) analysis of IPGTT revealed no significant changes among the three groups (Figure 3B and Supplementary Figure 1A, respectively). Moreover, the IPITT results indicated that the HFD + SBO group was significantly insensitive to insulin compared with the ND group (Figure 3C). Similarly, the HFD + 8-MNA group was less sensitive to insulin than the ND group (Figure 3C). The AUC analysis of IPITT indicated no significant difference in whole-body insulin sensitivity in the HFD groups (Supplementary Figure 1B). Taken together, these results suggest that 8-MNA treatment prevented mice from developing hyperglycemia at the earlier time points of HFD feeding but was not effective until the later period.

Because HFD-induced hyperglycemia is accompanied by IR for which hyperinsulinemia compensates,⁴⁷ insulin concentrations were measured in plasma collected at weeks 9 and 18. Feeding HFD + SBO to mice for nine weeks significantly increased the plasma insulin levels compared with ND feeding (Figure 3D). In contrast, nine-week feeding

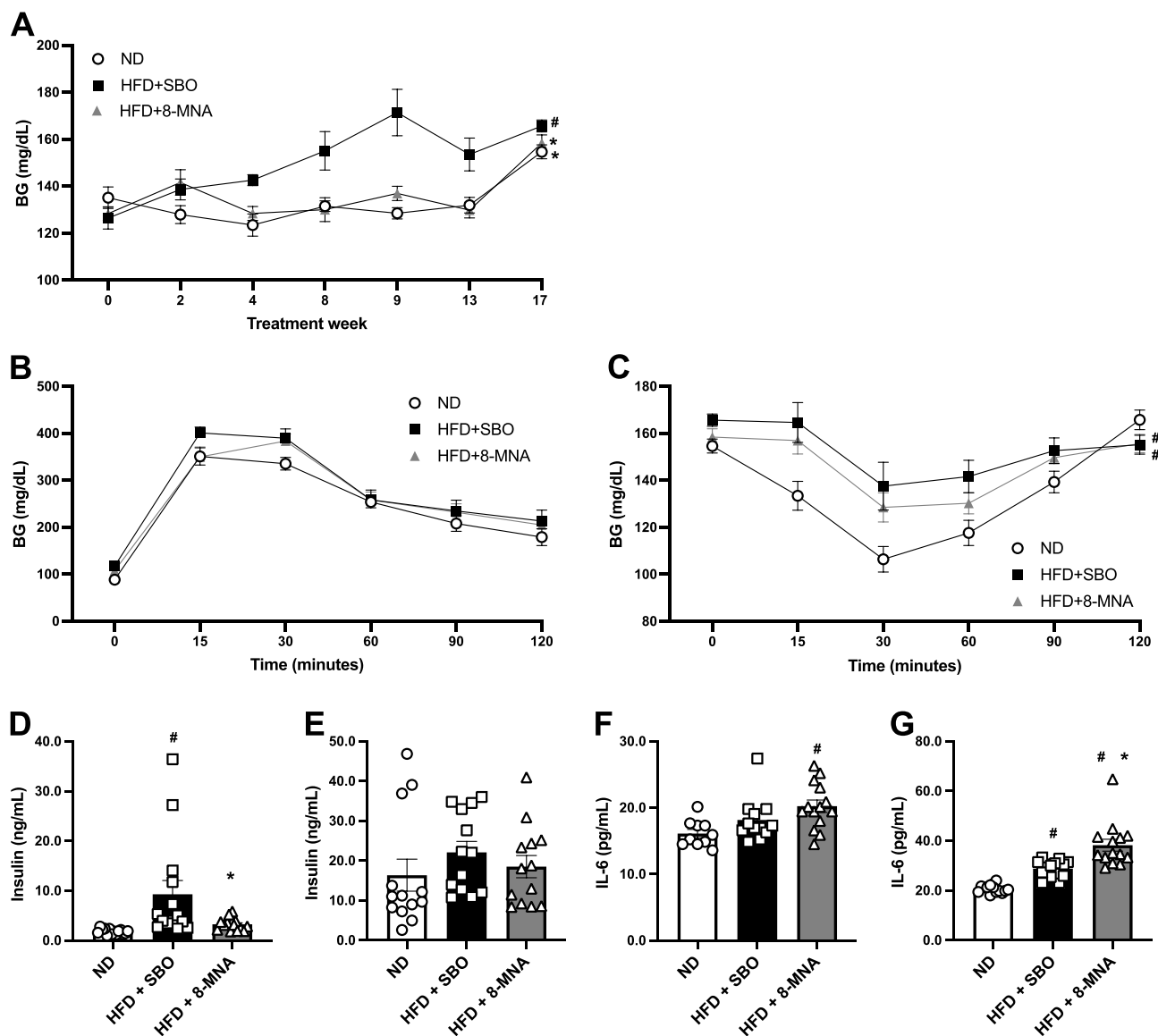


Figure 3 Effect of 8-MNA on glucose homeostasis in HFD-fed mice. **(A)** Nonfasting blood glucose (BG) levels in mice fed an ND or HFD supplemented with SBO or 8-MNA at weeks 0, 2, 4, 8, 9, 13, and 17. **(B)** Intraperitoneal glucose tolerance test (IPGTT) in mice fasted overnight at week 16. **(C)** Intraperitoneal insulin tolerance test (IPITT) in 4-h fasted mice at week 17. **(D and E)** Plasma insulin concentrations at weeks 9 **(D)** and 18 **(E)**. **(F and G)** Plasma interleukin-6 (IL-6) concentrations at weeks 9 **(F)** and 18 **(G)**. Data are presented as the mean $\pm$ SEM analyzed by a one-way ANOVA or a two-way repeated measures ANOVA ($n = 10$ – 14). **(A–C)** A significant treatment $\times$ time interaction was determined by a two-way ANOVA with repeated measures. $^{\#}p < 0.05$ vs ND. $^{*}p < 0.05$ vs HFD + SBO. n , sample size.

with HFD + 8-MNA maintained insulin concentrations as low as that observed for ND (Figure 3D). Based on these data, we can infer that these hyperglycemic mice fed with HFD + SBO developed IR, whereas DIO mice receiving 8-MNA maintained sufficient insulin sensitivity for a normoglycemic condition, similar to ND mice. However, plasma samples collected from the HFD + SBO and HFD + 8-MNA groups at week 18 had comparable amounts of insulin (Figure 3E). The data suggest the disappearance of the insulin-sensitizing effect of 8-MNA by week 18, which is consistent with the ITT results (Figure 3C).

MCFA is implicated in modulating inflammatory responses,¹⁸ which play an important role in the development of obesity-related IR and T2D,⁴⁸ however, the influence of 8-MNA on this pathological process is unknown. Therefore, we measured the plasma concentrations of interleukin-6 (IL-6), which prevents obesity-related glucose intolerance and elicits an antiobesity effect.^{49–51} In plasma samples collected at week 9, there was no significant difference in IL-6 concentrations between mice treated with an ND and HFD + SBO diet (Figure 3F). However, 8-MNA treatment

significantly increased IL-6 concentration compared with the ND group (Figure 3F). Furthermore, the plasma samples of the HFD + 8-MNA group at week 18 exhibited increased IL-6 concentrations compared with those of the other groups (Figure 3G). These data suggest an association of varying IL-6 production with the development of HFD-induced obesity and IR in mice.

Effect of 8-MNA on Tissue and Organ Weights of HFD-Fed Mice

Given that DIO mice supplemented with 8-MNA had significantly less BW compared with SBO mice (Figure 2B), we determined which organs and tissues were responsible for the BW difference. The organ and tissue weights measured at week 18 are listed in Table 1. There were no significant changes in the weights of the heart, lung, and tibialis anterior skeletal muscle among the three groups. The weights of the aorta, liver, spleen, pancreas, inguinal WAT (iWAT), and BAT in HFD + SBO mice were significantly greater compared with those of ND mice. However, no significant increases were observed in the weights of the aorta, liver, and pancreas between ND and HFD + 8-MNA mice. Conversely, both HFD + SBO and HFD + 8-MNA diets reduced the weights of kidney and epididymal WAT (eWAT) compared with those of the ND mice. As seen in patients with MetS who often have enlarged spleens,⁵² the spleen weight of HFD + SBO mice was significantly heavier compared with that of ND mice; however, this pathological change was significantly ameliorated in HFD + 8-MNA mice. Overall, we conclude that the changes in the organ and tissue weights in HFD-fed mice by 8-MNA supplementation were statistically insignificant except for spleen, but each of the subtle reduction, when considered collectively, contributes to the body weight-lowering effect of 8-MNA in HFD mice (Figure 2B).

Effect of 8-MNA on Thermogenic Markers and Triacylglycerols in HFD-Fed Mice

Obesity is caused by an inverse balance of energy homeostasis (ie, decreased EE and increased caloric intake).⁵³ Previous studies have shown that MCFAs increase EE in BATs via thermogenesis,^{21–23} the activation of which is associated with the upregulation of uncoupling protein 1 (UCP1) and phosphorylation of AMP-activated kinase (AMPK).^{23,54} We explored the possibility that 8-MNA supplementation to HFD mice may increase EE, thereby leading to a reduction in BW (Figure 2B). We measured the expression of UCP-1 and phosphorylated AMPK in BATs; however, Western blot analysis revealed no further increase in UCP1 (Figure 4A) or phosphorylated AMPK (Figure 4B) in BATs of HFD + 8-MNA mice compared with those in HFD + SBO mice. The other blots for quantification and raw images are shown in [Supplementary Figures 2](#) and [3](#). These data suggest that the 8-MNA-mediated reduction of BW occurs through a mechanism independent of the thermogenic features in BATs.

In obesity, besides an increase in mass of adipose tissues, ectopic intra- and peri-organ accumulation of triacylglycerols (TAGs) occurs not only from the excessive release of free FAs from dysregulated adipose tissue, but also from

Table 1 Effect of 8-MNA on Organ and Tissue Weights of HFD-Fed Mice

Weight (g)	ND	HFD+SBO	HFD+8-MNA
Heart	0.172 ± 0.003	0.172 ± 0.007	0.161 ± 0.008
Aorta	0.035 ± 0.001	0.052 ± 0.003 [#]	0.042 ± 0.003
Lung	0.185 ± 0.004	0.193 ± 0.005	0.182 ± 0.006
Liver	2.405 ± 0.141	3.030 ± 0.143 [#]	2.664 ± 0.134
Spleen	0.080 ± 0.003	0.127 ± 0.007 [#]	0.107 ± 0.004 ^{#,*}
Kidney	0.460 ± 0.006	0.427 ± 0.009 [#]	0.405 ± 0.007 [#]
Pancreas	0.243 ± 0.014	0.302 ± 0.014 [#]	0.284 ± 0.014
Inguinal white adipose tissue	0.944 ± 0.078	2.203 ± 0.092 [#]	2.014 ± 0.047 [#]
Epididymal white adipose tissue	1.602 ± 0.075	1.301 ± 0.078 [#]	1.317 ± 0.055 [#]
Brown adipose tissue	0.308 ± 0.016	0.499 ± 0.022 [#]	0.464 ± 0.022 [#]
Tibialis anterior skeletal muscle	0.105 ± 0.004	0.107 ± 0.005	0.121 ± 0.006

Notes: Data are presented as the mean ± SEM analyzed by a one-way ANOVA (n = 14). [#]p < 0.05 vs ND. *p < 0.05 vs HFD + SBO. n, sample size.

Abbreviations: ND, normal diet; HFD, high-fat diet; SBO, soybean oil; 8-MNA, triacylglycerols with 8-methyl nonanoic acid.

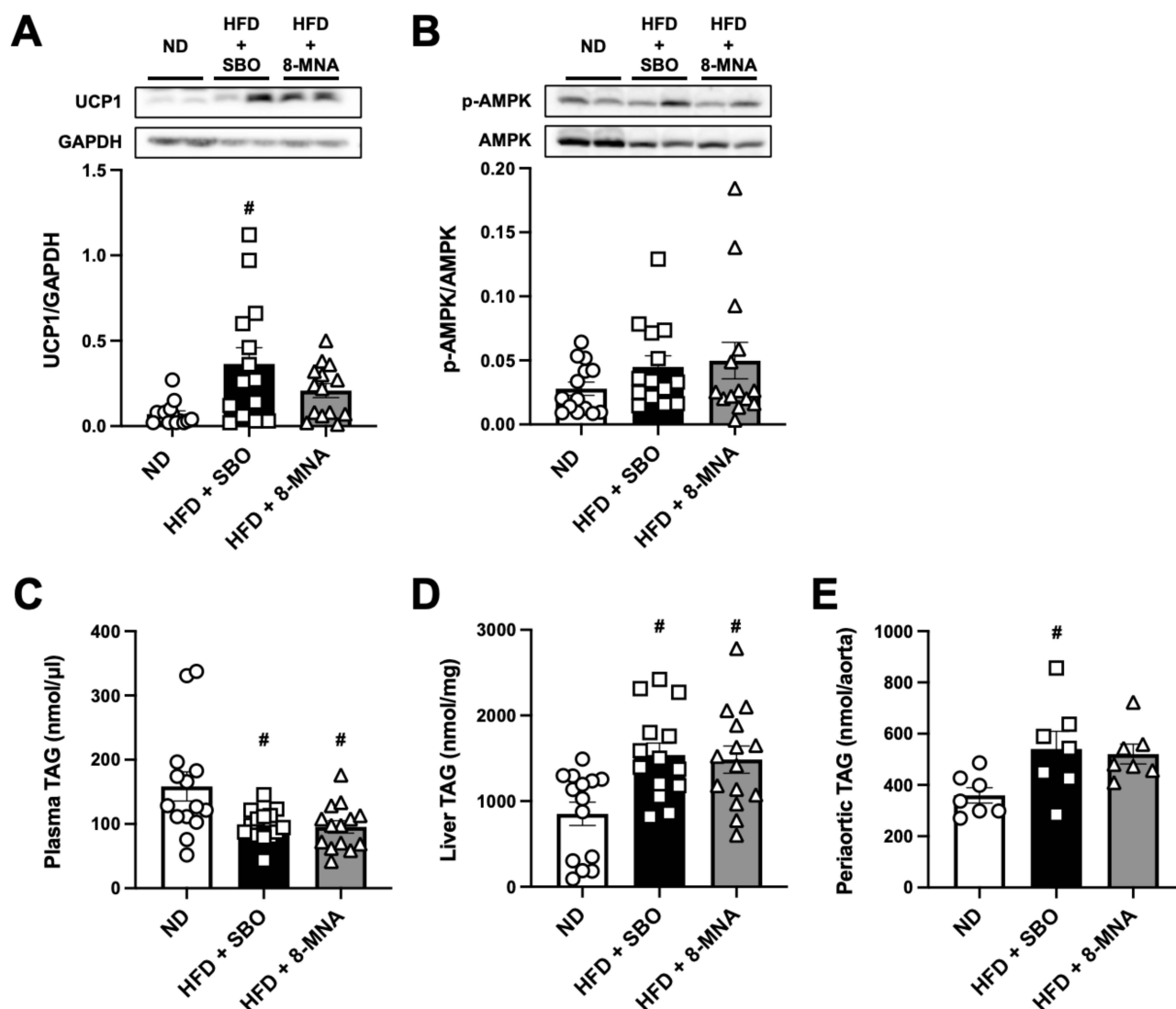


Figure 4 Effects of 8-MNA on thermogenic markers and triacylglycerols in HFD-fed mice. The expression of uncoupling protein I (UCP1) (**A**) and phosphorylated AMP-activated protein kinase (p-AMPK) (**B**) in brown adipose tissues of mice fed with an ND or HFD supplemented with SBO or 8-MNA. (**C–E**) Triacylglycerol (TAG) levels in plasma (**C**), liver (**D**), and periaortic tissues (**E**). Data are presented as the mean $\pm$ SEM analyzed by a one-way ANOVA ($n = 7–14$). [#] $p < 0.05$ vs ND. n, sample size. The other blots are shown in [Supplementary Figure 2](#).

overconsumption of nutritional fat.⁵⁵ Animals fed with an MCFA-rich obesogenic diet have less lipid accumulation in non-adipose tissues compared with their HFD-fed or equicaloric LCFA-fed counterparts,^{26–28} suggesting the potential effect of 8-MNA on these uncontrolled anabolic events. In HFD-treated groups, regardless of the supplements, the plasma concentrations of TAG were significantly lower compared with those in ND mice ([Figure 4C](#)). Consistent with studies in DIO rodents,^{56,57} HFD + SBO significantly increased hepatic and periaortic TAG levels ([Figure 4D](#) and [E](#)); however, the ectopic TAG accumulation was not affected by 8-MNA ([Figure 4D](#) and [E](#)). These results indicate that 8-MNA does not affect HFD-induced changes in plasma, hepatic, and periaortic TAGs.

Effect of 8-MNA on the Expression of Appetite-Related Genes in the Hypothalamus of HFD-Fed Mice

Based on the data in [Figure 4](#), the suppression of BW gain in response to 8-MNA ([Figure 2B](#)) was induced by enhancing satiety (ie, less appetite), rather than EE stimulation. The hypothalamus is the regulatory hub of appetite and EE in the brain.⁵⁸ The arcuate nucleus of the hypothalamus primarily encompasses two neuronal subsets characterized by the

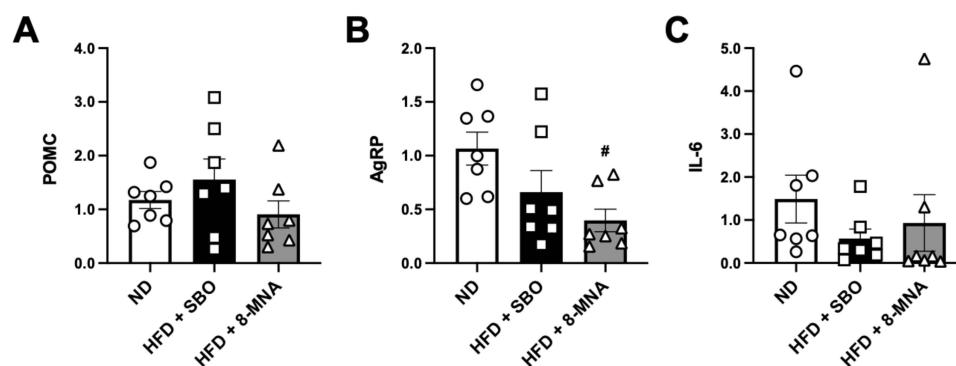


Figure 5 Effect of 8-MNA on the expression of appetite-related genes in the hypothalamus of HFD mice. The mRNA expression levels of proopiomelanocortin (POMC) (A), agouti-related protein (AgRP) (B), and IL-6 (C) relative to β -actin in the hypothalamus of mice fed with an ND or HFD supplemented with SBO or 8-MNA. Data are presented as the mean $\pm$ SEM analyzed by a one-way ANOVA ($n = 7$). # $p < 0.05$ vs ND. n, sample size.

expression of proopiomelanocortin (POMC) and agouti-related protein (AgRP). While releasing alpha-melanocyte-stimulating hormone (α -MSH) derived from the precursor POMC peptide to the secondary neurons in the paraventricular nucleus (PVN) decreases appetite and increases EE, the release of AgRP to the PVN neurons inhibits the α -MSH-mediated outcomes.⁵⁹ Moreover, C8:0 excites POMC neurons²⁴ while lauric triglyceride (C12:0-containing TAG) decreases the expression of AgRP,²⁵ and hypothalamic IL-6 affects eating behavior and the regulation of BW in male rodents.^{51,60} Thus, we hypothesized that the reduction of caloric intake mediated by 8-MNA may result from an appetite-suppressing response evoked by the modulation of hypothalamic POMC, AgRP, and/or IL-6 transcripts. None of these three diet groups showed significant changes in anorexigenic POMC or IL-6 transcripts (Figure 5A and C). Although there was no significant difference in AgRP expression between ND and HFD + SBO mice (Figure 5B), the transcription of this orexigenic peptide was significantly suppressed in HFD + 8-MNA mice compared with the ND group (Figure 5B). The results indicate that the downregulation of hypothalamic AgRP expression may be associated with the reduction of accumulated food intake in the HFD + 8-MNA group (Figure 2D).

Discussion

We evaluated the metabolic improvement of DIO mice after consuming 8-MNA, a degradation by-product of DHC, over 18 weeks. In a MetS mouse model, 8-MNA 1) suppressed caloric intake and BW gain along with a reduction in tissue and organ weight, 2) delayed the HFD-induced increase in BG and insulin, 3) increased plasma IL-6 levels, and 4) downregulated orexigenic AgRP in the hypothalamus without altering thermoregulatory protein markers in BATs as well as plasma, hepatic, and periaortic TAGs.

The health benefits of chili consumption are evident based on epidemiological studies showing that the mortality rate of patients with cardiometabolic diseases who frequently eat chili-containing foods is lower compared with that of nonchili eaters.⁶¹ Chili peppers contain capsaicinoids, including CAP and DHC, which are responsible for its spiciness and the major bioactive ingredients that exert pharmacological effects on energy metabolism.^{8,10} The burning sensation and metabolic effects of capsaicinoids are mediated through transient receptor potential vanilloid 1 (TRPV1). Some of the latter effects may be exerted through a mechanism independent of TRPV1.⁶² Nonetheless, considering capsaicinoids as a therapeutic agent for MetS is challenging as the oral administration of capsaicinoids to animals and humans causes gastrointestinal discomfort,^{10,11} which complicates preclinical and clinical studies. Contradictory evidence that capsaicinoids exhibit both cancer-inhibiting and promoting activities remains controversial.⁶³ In addition, the bioavailability of capsaicinoids is rapidly decreased within 24 h post administration.¹⁰ Collectively, there could be some uncertainty regarding the medicinal use of capsaicinoids, excluding their topical application for FDA-approved pain management.⁶⁴

An in vivo degradation by-product of DHC was identified by radioactive tracer studies. Tritiated DHC was found as 8-MNA in the portal blood of rats,¹³ whereas rat liver homogenates can convert DHC to 8-MNA.¹⁴ These studies demonstrate that DHC is degraded to 8-MNA during and after absorption in animals. Therefore, it is reasonable to assume that the biological responses to 8-MNA correspond, at least in part, to the metabolic consequence of DHC and

chili consumption. Although some MCFAs are metabolic modulators in animals,^{16,65} the role of 8-MNA in energy metabolism has not been known until our recent *in vitro* study.³² The report indicates that in 3T3-L1 adipocytes, 8-MNA treatment reduces lipid droplet accumulation during nutrient starvation in association with AMPK activation, which may promote β -oxidation and suppress *de novo* TAG synthesis.^{66,67} Moreover, 8-MNA elicits an anti-lipolytic effect in the presence of isoproterenol. The suppression of lipolytic response may reduce ectopic fat deposition by decreasing blood concentrations of non-esterified FAs.⁶⁸ 8-MNA treatment also enhances glucose uptake following the treatment of 3T3-L1 adipocytes with insulin.³² Accordingly, to extend our knowledge on the metabolic roles of 8-MNA *in vivo*, we determined whether 8-MNA could normalize metabolic impairments in DIO mice.

In contrast to capsaicinoids, which cause TRPV1-dependent cell death,⁶⁹ 8-MNA lacking the vanillyl head group required for binding to TRPV1 is unlikely to cause such detrimental outcomes. MCFAs at low concentrations are largely nontoxic⁷⁰ compared with LCFAs (ie, major components of SBO and HFD), which exhibit lipotoxicity in various cell types.^{71,72} In fact, in 3T3-L1 cells, 8-MNA treatment for 24 h does not affect viability up to 1 mM.³² Similarly, we showed that treatment with 2% 8-MNA ($n = 14$) for 18 weeks did not cause death or any health issues in HFD-fed mice. Although feeding 10% lauric acid (C12:0) to mice is lethal within a week, probably because of myocardial atrophy and oxidative stress,⁷³ the present study indicates a favorable safety profile for low-dose 8-MNA.

Consistent with the insulin-sensitizing effects of 8-MNA in 3T3-L1 adipocytes³² and of other MCFAs in humans and rodents,^{17–20} our results indicate that adding 8-MNA to HFD slowed the increase in nonfasting BG and insulin levels, which are characteristics of IR in DIO mice.³³ Interestingly, 8-MNA treatment also resulted in an increase in IL-6 concentration in the plasma, but not in the hypothalamus. IL-6 modulates the activity of proinflammatory mediators (eg, TNF- α), which play an important role in MetS development.⁴⁸ More specifically, previous studies in which exogenous IL-6 was administered to IL-6-deficient mice indicated that this cytokine counteracts obesity and glucose intolerance.^{49–51} It also has been reported that C8:0 and C10:0 increase IL-6 production in the presence of an inflammatory stimulant in human peripheral blood mononuclear cells.⁷⁴ Based on these results, we hypothesize that the increased peripheral production of IL-6 by 8-MNA in DIO mice contributes to the regulation of normal glycemia at least up to week 13. However, prolonged 8-MNA treatment did not preserve normal glucose homeostasis until later, despite the further increase of IL-6 at week 18. This is probably due to HFD-induced IL-6 resistance in the liver,⁴⁹ a pivotal organ in maintaining blood glucose homeostasis.⁷⁵ Therefore, this resistance mechanism might limit the capacity of 8-MNA to prevent HFD-fed mice from developing hyperglycemia and IR.

Unexpectedly, some of the anti-MetS activities of MCFAs were not recapitulated in DIO mice after being fed 8-MNA for 18 weeks. For instance, unlike other MCFAs that prevent TAG accumulation in adipose tissues, liver, and aorta of HFD-fed rodents,^{26–28} our study testing 8-MNA on DIO mice did not reproduce such results. Our previous *in vitro* study³² shows that in 3T3-L1 adipocytes, 8-MNA suppresses *de novo* lipogenesis, an anabolic process which synthesizes FAs (a component of TAGs) from excess carbohydrates,⁷⁶ during serum starvation (with no FAs supplement, but with glucose). This finding raises the possibility that in adipose tissues, liver, and aorta of HFD-fed mice, 8-MNA may block anabolic pathways related to *de novo* lipogenesis but not FA influx. In this case, since normoglycemia was maintained in HFD + 8-MNA mice at least until week 13, the inhibitory effect of 8-MNA on *de novo* lipogenesis might not be observed in the tissue samples collected at week 18. To test this hypothesis, a future study needs to be conducted in animals fed carbohydrate-enriched diet known to induce ectopic fat accumulation through *de novo* lipogenesis.³³ Furthermore, given that 8-MNA failed to mimic the MCFA-mediated increase in thermogenesis (and/or the relevant markers) in BATs of rodents,^{21–23,54} 8-MNA is likely to lack sufficient ability to bind to the MCFA-sensing G protein-coupled receptor 84 (GPR84)⁷⁷ which is abundantly expressed in BAT and involved in signal transduction promoting thermogenesis.⁷⁸ This assumption may be explained by 8-MNA's additional methyl group that can influence the molecular interactions between organic compounds and bioreceptors.⁷⁹

In the arcuate nucleus of the hypothalamus, excitation of POMC neurons by endocrine factors results in increased satiety and EE, whereas activation of AgRP neurons inhibits the effects of POMC stimulation on metabolism.⁵⁹ As POMC neurons express TRPV1-like receptors, capsaicinoids that cross the blood–brain barrier in animals⁸⁰ are likely to depolarize POMC neurons to suppress food intake.⁸¹ Alternatively, the anorexigenic effect of capsaicinoids could result from gastrointestinal distress.^{82,83} The canonical mechanism underlying capsaicinoid-stimulated EE involves the release of catecholamines from the adrenal gland in response to their activation of TRPV1.^{84,85} POMC neurons are also activated

by C8:0 to increase satiety and EE.²⁴ However, we found that feeding DIO mice with 8-MNA reduced AgRP expression in the hypothalamus without altering POMC and IL-6 expression. This indicates that 8-MNA affects satiety through an AgRP-dependent mechanism distinct from capsaicinoids and C8:0 in the hypothalamus. In ND and HFD mice, food consumption behavior is also acutely influenced by high concentrations of IL-6 in the periphery that can reach the central nervous system.⁵¹ Because 8-MNA-fed DIO mice exhibited significantly increased plasma concentrations of IL-6 until the end of the experiment, the appetite-reducing effect of 8-MNA may increase from the peripheral IL-6 elevation, but not from IL-6 in the lateral hypothalamus.⁸⁶

Besides GPR84, GPR40 and GPR120 have been identified as medium- and long-chain FA receptors.^{87,88} In mice harboring AgRP neuron-specific deletion of GPR40, AgRP peptide production is elevated in the hypothalamus,⁸⁹ implying the possible involvement of the GPR40 signaling in AgRP suppression by MCFAs.^{25,90} Moreover, IL-6 released from islet macrophages following GPR120 stimulation appears to be associated with enhanced insulin secretion in response to a high concentration of glucose.⁹¹ These previous findings may suggest the potential mechanisms by which the hypothalamic expression levels of AgRP and the plasma levels of IL-6 in DIO mice can be modulated by 8-MNA via GPR40 and GPR120, respectively.

The duration of preclinical and clinical studies in which MCFAs are fed to animals and humans was primarily 2–12 weeks,^{16,18,26,27,73} which is shorter than the time frame of the present study. Interestingly, male DIO mice fed 8-MNA maintained normoglycemia until week 13 with significantly less BW compared with HFD + SBO mice with IR. These earlier responses to 8-MNA are consistent with the results of short-term preclinical and clinical studies of MCFA, although it subsequently became ineffective. Moreover, it is still unknown whether these favored and unfavored results observed in male mice treated with HFD + 8-MNA are also consistent in female mice and human subjects, both of which may show considerable variability in metabolic responses^{92,93} to 8-MNA. Thus, further preclinical and clinical studies of 8-MNA including female groups are warranted to address this limitation.

Conclusion

We conclude that 8-MNA administration is beneficial to MetS-related metabolic derangements, partially similar to capsaicinoids and other MCFAs. The mechanism underlying 8-MNA's beneficial properties against MetS appears to depend on its appetite-suppressing and insulin-sensitizing actions, possibly through AgRP- and IL-6-related pathways, respectively without affecting thermogenesis or lipid metabolism. Although 8-MNA attenuates hyperglycemia and IR earlier in male DIO mice, these metabolic benefits wane over time, warranting further studies that examine the waning mechanism and gender-specific effects of 8-MNA. Moreover, these findings support the idea that the health benefits mediated by chili consumption are, to some extent, attributable to 8-MNA in animals. In addition, because of their nonpungent and nontoxic properties, the results may lead to the development of 8-MNA-based nutraceuticals (eg, fermented chili products which are mildly spicy but rich in 8-MNA) for individuals with MetS, despite the need for clinical studies designed to assess the safety and long-term efficacy of 8-MNA in humans.

Abbreviations

AgRP, Agouti-related protein; α -MSH, Alpha-melanocyte-stimulating hormone; AMPK, AMP-activated protein kinase; AUC, Area under the curve; BAT, Brown adipose tissue; BG, Blood glucose; BW, Body weight; C8:0, Octanoic acid; C9:0, Nonanoic acid; C10:0, Decanoic acid; CAP, Capsaicin; CO₂, Carbon dioxide; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, Dichloromethane; DHC, Dihydrocapsaicin; DMAP, 4-dimethylaminopyridine; DIO, Diet-induced obese; EE, Energy expenditure; FFA, Free fatty acid; eWAT, Epididymal white adipose tissue; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GPR, G protein-coupled receptor; HFD, High-fat diet; IL-6, Interleukin-6; IPGTT, Intraperitoneal glucose tolerance test; IPITT, Intraperitoneal insulin tolerance test; IR, Insulin resistance; iWAT, Inguinal white adipose tissue; LCFA, Long-chain fatty acid; MCFA, Medium-chain fatty acid; MetS, Metabolic syndrome; ND, Normal diet; 8-MNA, 8-methyl nonanoic acid; POMC, Proopiomelanocortin; PPAR γ , Peroxisome proliferator-activated receptor γ ; PVN, Paraventricular nucleus; SEM, Standard error of the mean.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article and its [supplementary files](#).

Ethics Approval and Informed Consent

The use of animals and the animal procedures were approved by the Mahidol University Institutional Animal Care and Use Committee (MU-IACUC, Protocol numbers: MUSC64-016-562/F02-66-010). This study was conducted in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0.⁷⁴

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. More specifically, P. Keawsomnuk and K.M. conceptualized and led the project., C.M., C.B., P.P., and U.W. synthesized and analyzed 8-MNA (Figure 1). P. Keawsomnuk, T.D-U, S.T., N.W., and K.M. performed the experiments (Figures 2–5 and Table 1). P. Keawsomnuk, U.W., and K.M. wrote the manuscript. P. Keawsomnuk, P. Ketsawatsonkron, U.W., and K.M. discussed and edited the manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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