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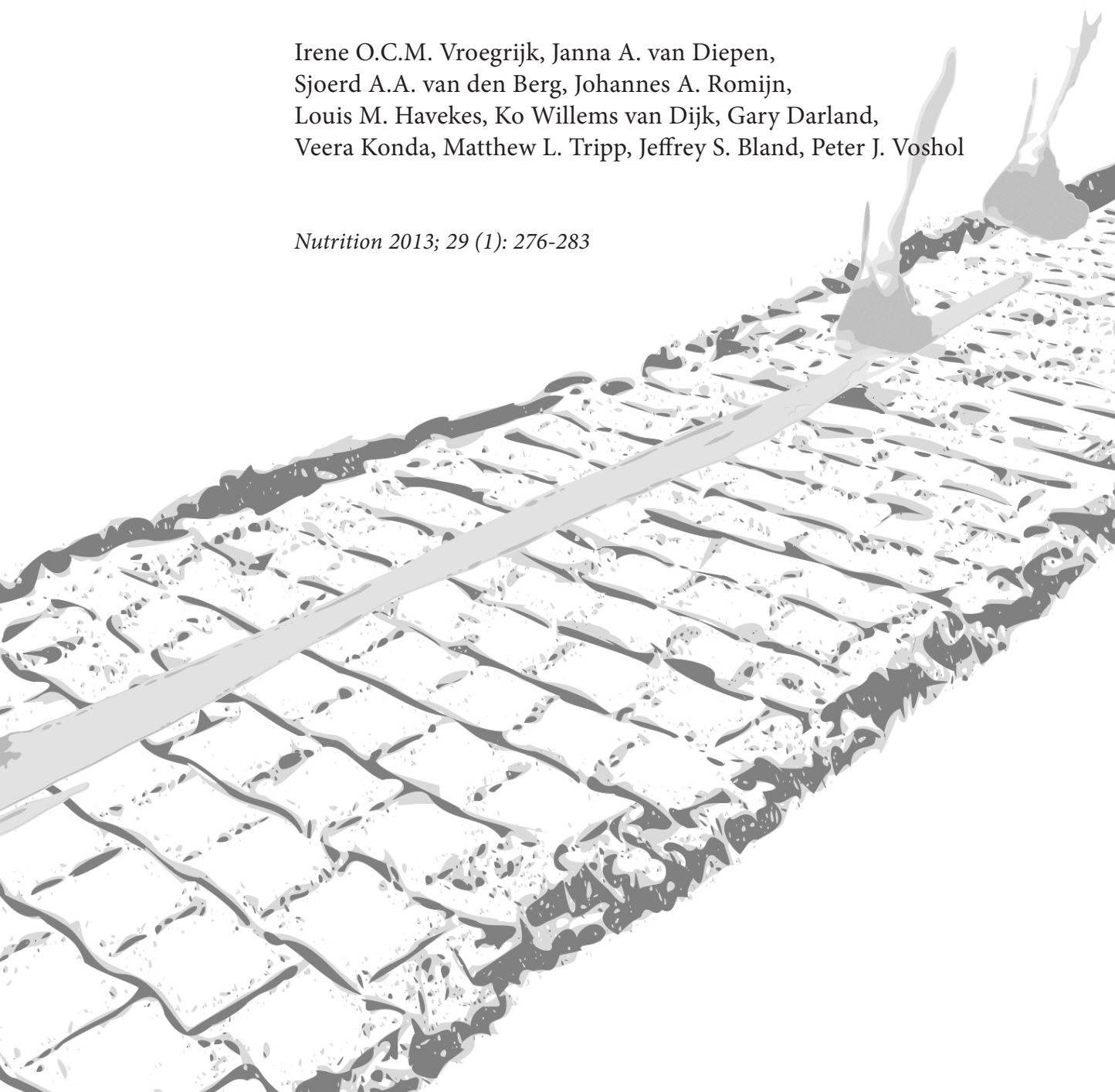
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META060 PROTECTS AGAINST DIET-INDUCED OBESITY AND INSULIN RESISTANCE IN A HIGH-FAT DIET FED MOUSE

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ABSTRACT

We investigated whether a reduced iso-alpha acid derived from an extract of *Humulus lupulus L.*, META060, had an effect on weight gain, body composition and metabolism in a high fat diet (HFD) fed mouse. Weight gain was monitored for up to twenty weeks in mice receiving either a low fat diet, a high fat diet, or a high fat diet supplemented with META060 or rosiglitazone. Body composition was determined using DEXA scan analysis. Indirect calorimetry measurements were performed to investigate energy balance in the mice, and oral glucose tolerance tests were administered to examine the effect of META060 on glycemic response. HFD-fed mice administered META060 for fourteen weeks had a significantly lower mean weight than that of HFD-fed mice (30.58 ± 0.5 g versus 37.88 ± 0.7 g; $P < 0.05$). Indirect calorimetry measurements revealed increased metabolic flexibility in mice supplemented with META060. In addition, glucose tolerance was improved, comparable to the effects of rosiglitazone treatment. We conclude that META060 has potential therapeutic value for managing obesity and insulin resistance, and further research into the mechanism of action is warranted.

4.1 INTRODUCTION

Management of obesity has become a primary goal for healthcare practitioners in response to the rising epidemic of obesity related chronic diseases, including type 2 diabetes mellitus (T2DM) and cardiovascular disease. Pharmaceutical approaches that alter appetite, metabolism, or fat absorption include antidepressants, CNS stimulants, or peripherally acting antiobesity drugs, and all have been associated with adverse effects (reviewed in ¹). Many people seek natural therapies as an alternative to pharmaceuticals for weight management. Yerba mate, yohimbe, aloe, pyruvate, St. John's Wort, dandelion, and herbal diuretics have been used for weight loss, although significant clinical studies supporting their efficacy are lacking (reviewed in ²).

Iso-alpha acids derived from the hop plant (*Humulus lupulus L.*) reduced plasma triglyceride and free fatty acid levels in mice ^{3,4}. C57BL/6N mice fed a high fat diet (HFD) exhibited improved glucose tolerance after 14 days and reduced insulin resistance after 10 days of administration of iso-alpha acids. Furthermore, in a double-blind, placebo-controlled pilot study, diabetic subjects receiving iso-alpha acids for 8 weeks had an average 10.1% reduction in blood glucose levels and 6.4% reduction in glycated hemoglobin levels ⁴.

Iso-alpha acids are not particularly stable compounds, although the reduced derivatives have been found to exhibit greater stability ⁵. Furthermore, reduced iso-alpha acids have recently demonstrated greater bioavailability than iso-alpha acids in humans ⁶.

Previous work in our lab to screen various botanical extracts for lipogenic activity resulted in the identification of a family of reduced iso-alpha acids ⁷. One of the reduced iso-alpha acids, META060, has demonstrated anti-inflammatory activity *in vitro*, mediated by inhibition of NF- κ B pathways ^{8,9}. Several reports have suggested a link between obesity-induced inflammation and related metabolic disorders such as insulin resistance (reviewed in ^{10,11}). Objectives of the current study were to determine the effects of META060 compared with rosiglitazone, a commonly used drug in the treatment of T2DM, on body weight, energy metabolism, glucose tolerance and insulin sensitivity in HFD-induced obese mice.

4.2 MATERIALS AND METHODS

Animals and diet intervention

Wild-type (WT) C57Bl/6J male mice were purchased from Charles River (Maastricht, The Netherlands). Mice were housed under standard conditions with access to water and food ad libitum. For the 14-week diet intervention, the study was started when the animals were 19 weeks of age. Mice were fed either a low fat diet (LFD) (10% energy derived from lard fat; D12450, Research Diet Services, Wijk bij Duurstede, The Netherlands), with a caloric content of 3.85 kcal/g, a high fat diet (HFD) (45% energy derived from lard fat; D12451,

Research Diet Services, Wijk bij Duurstede, The Netherlands), with a caloric content of 4.73 kcal/g, or a HFD supplemented with META060 (100 mg/kg/day) or rosiglitazone (1 mg/kg/day) (SmithKline Beecham Farma, Rijswijk, The Netherlands). META060 was supplied by Hopsteiner, and standards were purchased from ASBC. The chemical composition of META060 has been described previously ¹². META060 or Rosiglitazone were added to self-made HFD. Briefly, rosiglitazone tablets (Avandia® = rosiglitazone maleate, SmithKline Beecham Farma, Rijswijk, The Netherlands) or META060 powder were crushed in a mortar with a pestle. Subsequently, the powder was mixed with the 45% lard HFD powder diet from Research Diets Service (45% energy derived from lard fat; D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands). For HFD + META060, 1.875 g of META060 per kg of HFD powder was used. For rosiglitazone, 12 mg powder was added to 1 kg of HFD powder. Pellets were made by adding 2% agar (Sigma), and were then freeze-dried to remove water and stored at -20°C. A fixed dosage was used throughout the diet intervention. Based on previously assessed food intake data we know that C57Bl/J6 mice on a HFD+META060 diet (D12451, Research Diet Services diet) eat ~2.5 g of diet per day. Each treatment group in the 14-week intervention included 12 mice, and mice were weighed weekly. Food intake was monitored weekly by weighing the food in the cages manually. After 14 weeks, animals in the LFD or HFD plus rosiglitazone groups were sacrificed, following 4 hours of fasting. Mice from the HFD group were randomly divided into 2 groups: six were shifted to HFD plus META060 (100 mg/kg/day), and the remaining six mice continued receiving a HFD for six weeks. Likewise, mice supplemented with HFD plus META060 were divided into 2 groups: six were shifted to HFD, and the remaining six mice continued receiving HFD plus META060.

For the 5-week diet intervention, 12-week-old mice were fed a HFD, a HFD supplemented with META060 (100 mg/kg/day), or a HFD supplemented with rosiglitazone (1 mg/kg/day). Each dietary group consisted of 9 animals. Body weight was measured weekly during the diet intervention. All experiments were approved by the animal ethics committee of Leiden University Medical Center.

DEXA Scan

Animals were subjected to DEXA scan analysis after 4 hours of fast. Animals were weighed and sedated by a single intraperitoneal injection of a mixture of acepromazine (6.25 mg/kg Neurotranq, Alfasan International BV, Weesp, The Netherlands), midazolam (6.25 mg/kg Dormicum, Roche Diagnostics, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg Janssen Pharmaceuticals, Tilburg, The Netherlands). Sedated animals were scanned in toto using a small animal DEXA scanner (pDEXA, Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany) and data were analyzed by the software supplied by the manufacturer. Fat mass and lean body mass were determined.

Indirect calorimetry

Groups of 8 mice were subjected to individual indirect calorimetry measurements for a period of 4 consecutive days using a Comprehensive Laboratory Animal Monitoring

System (Columbus Instruments, Columbus Ohio, US). Cages were made of clear plexiglass (30 x 10 x 9 cm (l x b x h)). Prior to the start of the experiment, animals were acclimated to the cages and the single housing for a period of 24 h. Experimental analysis started at 09:00 h and continued for 36 hours. In the next 36 hours of monitoring, animals were fasted overnight, and then food was replaced to assess metabolic flexibility. Analyzed parameters included real time food and water intake, as well as meal size, frequency and duration. Oxygen consumption (VO₂) and carbon dioxide production rates (VCO₂) were measured at intervals of 7 minutes. Respiratory exchange ratio (RER) as a measure for metabolic substrate choice was calculated as the ratio between VCO₂ and VO₂. Carbohydrate (CHO) and fat (FA) oxidation rates were calculated using the following formulas ¹³:

$$\text{CHO} = ((4.585 * \text{VCO}_2) - (3.226 * \text{VO}_2)) * 4 / 1000$$

$$\text{FA} = ((1.695 * \text{VO}_2) - (1.701 * \text{VCO}_2)) * 9 / 1000$$

Total energy expenditure was calculated from the sum of CHO and FA oxidation. Activity was monitored as 2-dimensional infrared beam breaks.

Fecal fatty acid composition and concentration

Feces were collected over 4 days during week 4 of the 5-week diet intervention. Feces were weighed, freeze-dried, and ground, and fecal fatty acids (FA) were subsequently derivatized by methyl esterification. Therefore, 2 mL methanol/hexane (4:1 v/v) containing 80 µg pentadecanoic acid (C15:0) as an internal standard (Fluka) was added to 15 mg feces. Then, 200 µL acetyl chloride (Merck) was added, and samples were incubated at 95°C. After subsequent cooling to 4°C, 5 mL 6% K₂CO₃ (Sigma) was added and samples were centrifuged (10 min, 4000 rpm, 4°C). The upper hexane layer was isolated and used for GC analysis of FA methyl esters (FAME). FAME were separated on a 50 m x 0.25 mm capillary GC column (CP Sil 88, Agilent technologies) in a 3800 GC gas chromatograph (Varian) equipped with a flame ionization detector. The injector and flame ionization detector were kept at 270°C. The column temperature was programmed from 170°C to 210°C. FAME were introduced by split injection (split ratio 20:1). Quantification was based on the area ratio of the individual FA to the internal standard.

Oral glucose tolerance test

Glucose and insulin levels were determined after overnight fasting during week 5 of the 5-week diet intervention, and also after 14 weeks of the 14-week diet intervention. Blood was obtained via tail bleeding, and glucose and insulin concentrations were determined. Subsequently, mice received an intragastric load of D-glucose (2 g/kg) provided as a 20% solution in PBS. Additional blood samples (30 µl) were collected via tail bleeding at 5, 15, 30, 60, 90 and 120 min after glucose loading for measurement of plasma insulin and glucose concentrations. Glucose concentration was determined with a glucose analyzer (Accu-

Check, Sensor Comfort, Roche Diagnostics GmbH, Germany) and insulin concentration was determined by immunoassay (Chrystal Chem Inc).

Statistical Analysis

Data are presented as means \pm SE. Statistical differences were calculated using the unpaired *t*-test (SPSS 17, SPSS Inc, Chicago, IL) or 2-way Analysis of Variance with Bonferroni posttest (GraphPad Prism, San Diego, CA). A P-value <0.05 was regarded as statistically significant.

4.3 RESULTS

Supplementation with META060 for 14 weeks prevents HFD-induced obesity

To determine the effect of META060 on high fat diet (HFD)-induced obesity, mice were fed a low fat diet (LFD), a HFD, or a HFD supplemented with either 100 mg/kg/day META060 or 1 mg/kg/day rosiglitazone for 14 weeks. Previous studies in a mouse model of collagen-induced arthritis demonstrated an effect of META060 with 50 mg/kg/day for reducing cartilage degradation and bone erosion, and doses up to 250 mg/kg/day were well tolerated⁸. Therefore, a dose of 100 mg/kg/day was selected for the current study investigating the effect of META060 on body weight and metabolism in HFD fed mice. Rosiglitazone is an antidiabetic agent from the thiazolidinedione class of drugs. Its mechanism of action is well-known, involving activation of PPAR γ , and studies in HFD fed mice demonstrated reduction of insulin levels with a dose of 1 mg/kg/day rosiglitazone¹⁴. Mice receiving HFD supplemented with META060 maintained similar body weights to those on a LFD over 14 weeks, and were significantly lower in weight than HFD-fed mice at week 3 and every subsequent time point up to week 14 (Fig. 1A). After 14 weeks, META060 supplemented mice weighed 19% less than HFD-fed control mice (30.58 ± 0.5 g versus 37.88 ± 0.7 g; $P<0.05$), and were comparable in weight to mice fed a LFD for 14 weeks (29.71 ± 0.7 g). Mice supplemented with rosiglitazone did not gain as much weight as those without supplementation, although they gained significantly more weight than HFD plus META060 or LFD fed mice (Fig. 1A). During the 14 week diet intervention no differences in food intake were observed.

To determine whether reduced weight gain in META060 supplemented mice reflected lower fat accumulation compared with HFD-only fed mice, body composition of these mice was determined by DEXA scan analysis. Total body fat of mice supplemented with META060 was significantly lower than that of HFD-fed mice (3.29 ± 1.0 g versus 12.12 ± 1.1 g; $P<0.001$) (Fig. 1B). At the end of the experiment, organs were dissected and weighed. META060 supplementation reduced gonadal (1.17 ± 0.2 g versus 2.40 ± 0.09 g; $P<0.001$) and subcutaneous (0.47 ± 0.09 g versus 1.53 ± 0.2 g; $P<0.001$) white adipose tissue (WAT) mass in HFD-fed mice compared to no-supplement controls (Fig. 1C).

At 15 weeks, half of the mice in the HFD group were shifted to HFD plus META060, and half of the mice in the HFD plus META060 group were shifted to HFD only. Body weight

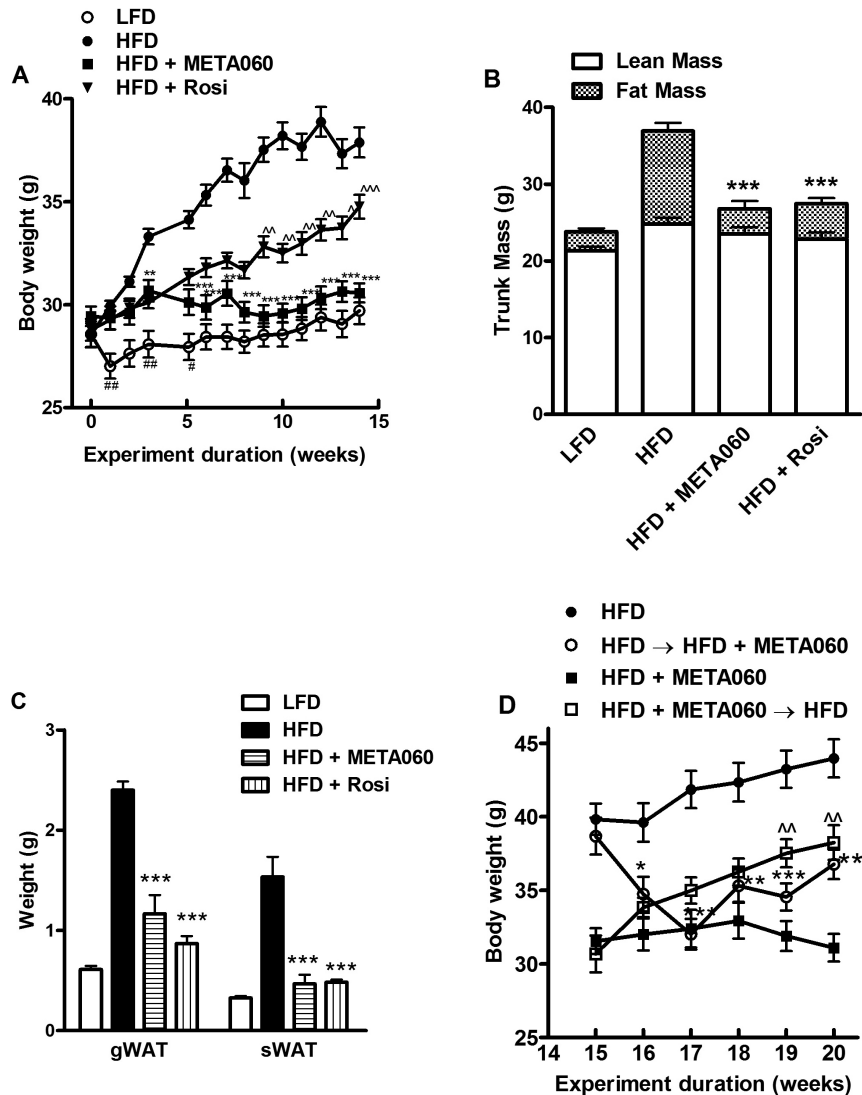


Figure 1. META060 prevents high fat diet (HFD)-induced obesity. Mice were fed a low-fat diet (LFD), a HFD, or a HFD supplemented with either 100 mg/kg/day META060 or 1 mg/kg/day rosiglitazone (Rosi) for 14 weeks. Mouse body weights were recorded every week ($n = 12$ per group); $**P < 0.01$, $***P < 0.001$ HFD vs. HFD + META060, $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ HFD + META060 vs. HFD + Rosi, $\#P < 0.05$, $\#\#P < 0.01$ HFD + META060 vs. LFD (A). After a 4 h fast, lean body mass and fat mass were determined by DEXA scan ($n = 6$ per group for HFD and META060 and $n = 12$ per group for Rosi); $***P < 0.001$ HFD vs. HFD + META060 and HFD vs. HFD + Rosi (B). After mice were sacrificed, mass of gonadal White Adipose Tissue (gWAT) and subcutaneous WAT (sWAT) was determined ($n = 6$ per group for HFD and META060 and $n = 12$ per group for Rosi); $***P < 0.001$ HFD vs. HFD + META060 and HFD vs. HFD + Rosi (C). At 14 weeks, half of the mice in the HFD group were shifted to HFD + META060, and half of the mice in the HFD + META060 group were shifted to HFD only. Body weights were recorded weekly ($n = 6$ per group); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ HFD vs. HFD → HFD + META060, $^{\wedge\wedge}P < 0.01$ HFD + META060 vs. HFD + META060 → HFD (D). Data are presented as mean \pm SE.

was monitored weekly for 5 weeks in these 4 treatment groups. While animals maintained on HFD for the entirety of the experiment continued to gain weight, those shifted to HFD plus META060 lost a significant amount of weight during weeks 16 and 17, after which they began to gain weight again (Fig. 1D). A concomitant reduction in food intake was observed in the first two weeks after switching diets, followed by a rebound to even greater levels than food intake in the HFD plus META060 group that did not switch diets (data not shown), perhaps a reflection of adjustment to palatability differences between the distinct diets.

META060 increases RER and metabolic flexibility in mice fed a HFD

To investigate how META060 protects against HFD-induced obesity, an independent, 5-week study was initiated with three treatment groups: HFD; HFD supplemented with META060 (100 mg/kg/d); or HFD supplemented with rosiglitazone (1 mg/kg/d). In the first five weeks of the 14-weeks intervention study, average weight gained in the HFD group was 5.61 ± 0.7 g, while mice supplemented with META060 gained 0.68 ± 0.3 g. In the 5-weeks study, average weight gained in the HFD group was 2.58 ± 0.4 g, and mice supplemented with META060 gained 0.54 ± 0.9 g. (Fig. 2). Despite differences in absolute weight gained, which was likely due to the difference in age of mice at the start of each study, META060 supplementation reproducibly reduced relative HFD-induced body weight gain in both experiments.

Whole body substrate utilization was examined for approximately 36 h during week 4 of the diet intervention. Four weeks of diet intervention was chosen because at this time point in the 14-week study, body weight was still increasing and a new set point had not yet been reached.

Although we did not directly compare a LFD group during the 5-week study, we know from published and experimental data that 5 weeks of HFD feeding in mice results in an

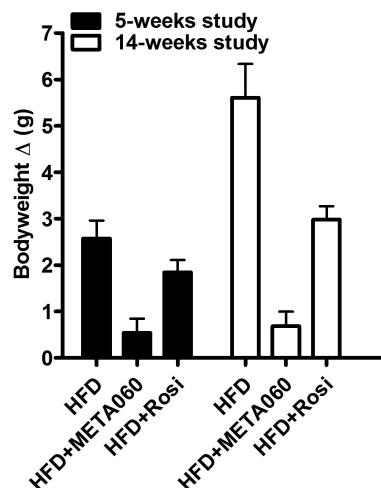


Figure 2. Comparison of 5-weeks' bodyweight increase in 5-weeks and 14-weeks studies. Mice were fed a high fat diet (HFD), HFD supplemented with META060, or HFD supplemented with rosiglitazone (Rosi) in two independent studies of varying durations, 5 weeks or 14 weeks. In both studies, mice were weighed at 5 weeks. Mean body weight difference from baseline is represented for each group \pm SE (N=9-12 per group).

unaltered total energy expenditure (EE) (kcal/h), but changes respiratory exchange rate (RER) and fat (FA) and CHO oxidation. Daytime RER was 0.84 ± 0.04 versus 0.94 ± 0.04 , and night time RER was 0.84 ± 0.03 versus 0.93 ± 0.04 , for HFD versus LFD, respectively ($p < 0.05$). Daytime FA oxidation was 0.17 ± 0.05 versus 0.07 ± 0.04 , and night time FA-oxidation was 0.19 ± 0.05 versus 0.08 ± 0.06 , for HFD versus LFD, respectively ($p < 0.05$). META060 or Rosi intervention started from Day 0, when the animals were switched from chow to HFD as described in Materials and Methods.

Nocturnal and diurnal data were analyzed separately to distinguish between periods of low (diurnal) and high (nocturnal) physical activity. Total EE was similar across all dietary intervention groups (Fig. 3A). For food intake (FI), no significant differences were observed between the groups (data not shown). Despite similarities in total EE and FI, HFD-fed mice supplemented with META060 or rosiglitazone exhibited a significantly lower mean nocturnal FA oxidation rate than HFD only (0.16 ± 0.01 kcal/h and 0.18 ± 0.01 kcal/h versus 0.22 ± 0.01 kcal/h; $P < 0.001$, $P < 0.01$, respectively), and rosiglitazone had a lower mean diurnal FA oxidation rate compared to that of control (0.12 ± 0.01 kcal/h versus 0.15 ± 0.01 ; kcal/h $P < 0.05$) (Fig. 3B). In addition, nocturnal CHO oxidation levels were increased in HFD fed mice that received META060 or rosiglitazone, compared with controls (0.36 ± 0.02 kcal/h and 0.35 ± 0.01 kcal/h versus 0.31 ± 0.01 kcal/h; $P < 0.01$) (Fig. 3C). This increased carbohydrate-to-fat oxidation ratio was reflected in the RER. META060 and rosiglitazone both significantly increased RER in HFD-fed mice during the diurnal period compared to HFD-only fed mice (0.88 ± 0.00 and 0.89 ± 0.01 versus 0.86 ± 0.01 ; $P < 0.05$, $P < 0.01$ respectively), and also during the nocturnal period (0.87 ± 0.01 and 0.86 ± 0.00 versus 0.83 ± 0.01 ; $P < 0.001$, respectively) (Fig. 4A-B). To test the ability of the animals to adjust fuel oxidation to fuel availability (metabolic flexibility), animals were fasted overnight; subsequently, the food was replaced and RER was monitored. META060 and rosiglitazone supplemented mice had a significantly higher RER when the food was replaced compared to HFD-only treated mice (0.94 ± 0.00 and 0.94 ± 0.00 versus 0.92 ± 0.00 ; $P < 0.001$), indicating greater metabolic flexibility in META060 or rosiglitazone fed animals (Fig. 4C). Physical activity measurements did not show differences in either treatment group compared with HFD-only fed mice (data not shown).

META060 has no effect on fecal fatty acid composition and concentration

Since META060 reduced fat oxidation, we investigated whether fat absorption was impaired in META060 supplemented mice. Fecal fatty acid composition and concentration were determined in samples collected during metabolic cage experiments (data not shown). No difference was found in total fecal weight. Furthermore, quantitative gas chromatography analysis revealed equal fecal fatty acid composition and fecal fatty acid content in all treatment groups. Together with the equivalent food intake, this implies similar intestinal absorption of lipids.

META060 improves glucose tolerance in HFD fed mice

Since increased carbohydrate-to-fat oxidation ratio and increased metabolic flexibility suggest protection against HFD-induced insulin resistance, oral glucose tolerance tests

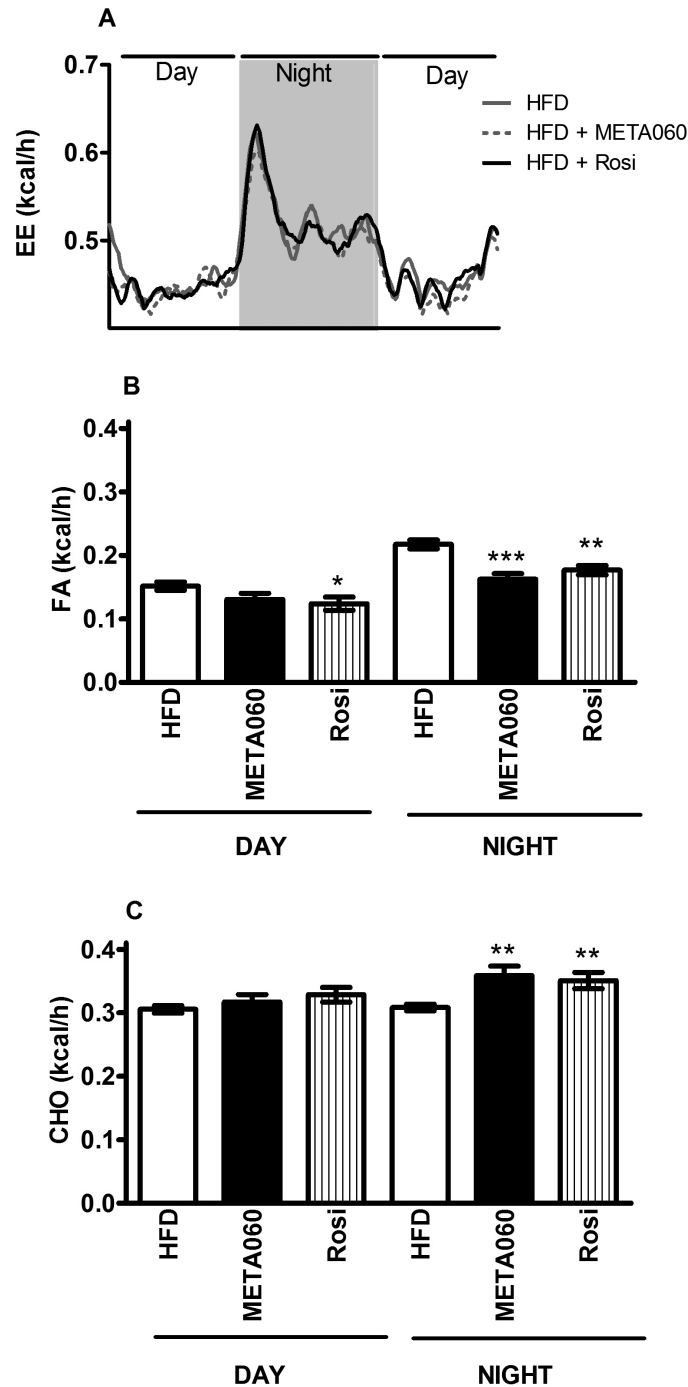


Figure 3. METAO60 affects substrate utilization. During week 4 of the diet intervention, O_2 consumption and CO_2 production were recorded for 36 h in HFD fed mice without supplementation or supplemented with 100 mg/kg/d METAO60 or 1 mg/kg/d rosiglitazone (Rosi). The night period is represented by the shaded area on the graph. EE was calculated as described in Methods (A). Fatty acid (FA) (B) and carbohydrate oxidation (CHO) (C) for day phase or night phase were calculated for HFD fed mice without supplementation or in those supplemented with METAO60 or Rosi. Data are presented as mean \pm SE (n=8 per group); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

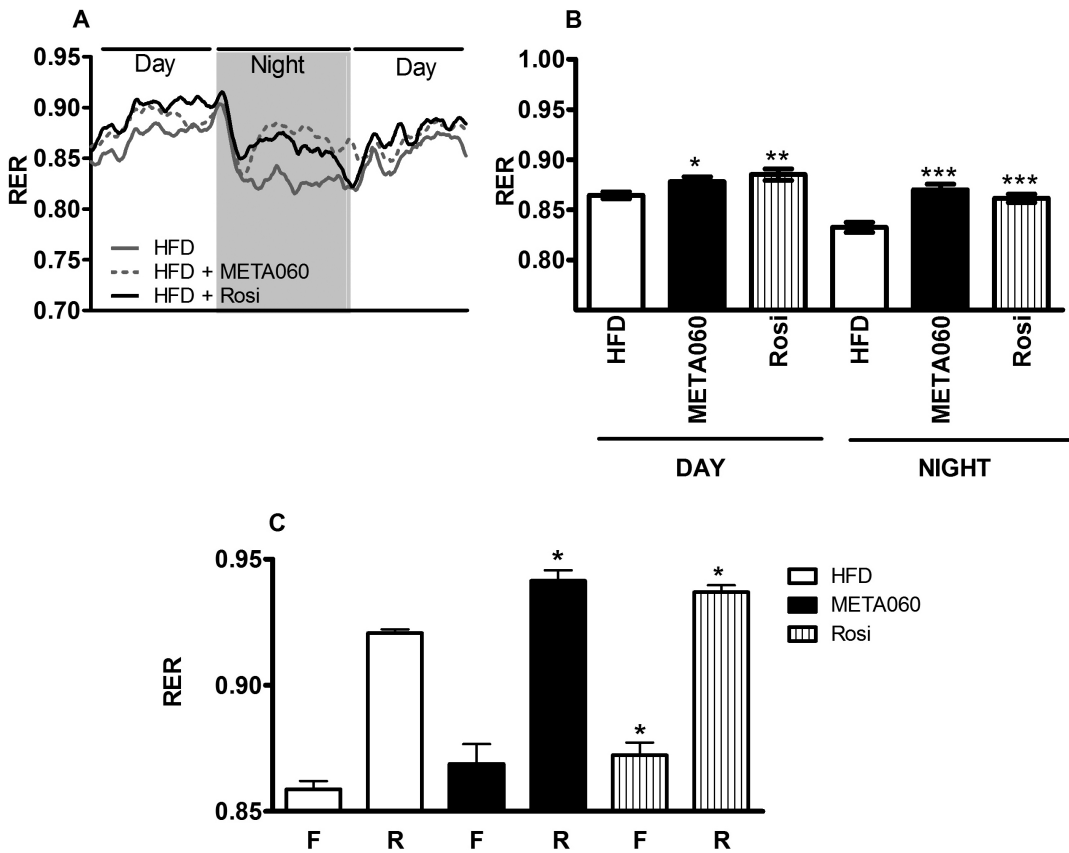


Figure 4. META060 increases respiratory exchange rate (RER) and metabolic flexibility. RER was monitored for 36 h in HFD fed mice without supplementation or supplemented with 100 mg/kg/d META060 or 1 mg/kg/d rosiglitazone (Rosi) (A). RER for day phase or night phase was calculated for each treatment group (n=8 per group); *P<0.05, **P<0.01, ***P<0.001 (B). HFD fed mice with or without supplementation with META060 or Rosi for 4 weeks were fasted overnight and then feeding was resumed. RER was calculated after fasting (F) and after refeeding (R) (n=8 per group; *P<0.05) (C). Data are presented as mean \pm SE.

(OGTT) were performed during week 5 of the diet intervention. After an overnight fast, blood glucose concentration was lower in META060 supplemented mice compared to that of HFD-only treated mice (4.66 ± 0.2 versus 5.34 ± 0.3 ; $P<0.05$) (Fig. 5A), while fasting insulin levels were not significantly different among treatment groups (Fig. 5D). Following glucose challenge, plasma glucose and insulin levels were determined at time intervals up to 120 min, and areas under the curve (AUC) were calculated. Glucose concentrations were significantly decreased for mice supplemented with META060 compared to HFD-fed mice at 15 min, 30 min, 90 min, and 120 min after glucose challenge, and mean AUC was 20% lower than in HFD-fed mice ($P<0.05$) (Fig. 5B-C). Rosiglitazone also significantly decreased plasma glucose levels at 5, 30, 60, and 90 min after glucose challenge, and mean AUC was 15% lower than in HFD-fed mice ($P<0.05$). These observations demonstrate that META060 and rosiglitazone improved glucose tolerance in mice fed a HFD for 5 weeks.

This may be due to increased insulin sensitivity in response to an oral glucose load, since time course and AUC for plasma insulin levels were comparable in all groups (Fig. 5E-F).

After 14 weeks of diet-intervention, fasting blood glucose concentration in META060 supplemented mice was significantly lower than that of HFD fed mice (4.5 ± 0.3 mM versus 5.9 ± 0.3 mM; $P < 0.05$) (Fig. 6A). Moreover, fasting insulin concentration was also significantly reduced in META060-supplemented mice compared to that of HFD-fed mice (0.14 ± 0.05 ng/mL versus 0.42 ± 0.09 ng/mL; $P < 0.001$) (Fig. 6C). This implies that after long-term META060 supplementation, insulin sensitivity in HFD-fed mice was increased. OGTT were performed on mice and blood glucose and insulin concentrations were recorded at several time points up to 120 min post-challenge. Area under the curve (AUC) for glucose was similar among all groups (Fig. 6B). However, AUC for insulin was increased in the HFD group, and only rosiglitazone supplementation had a statistically significant effect on reducing the insulin response compared to HFD (40%; $P < 0.05$) (Fig. 6D).

4.4 DISCUSSION

In the current study we investigated the effects of META060 on HFD-induced obesity and insulin resistance. Supplementation with META060 reduced weight gain in HFD fed mice. This effect was significant after 3 weeks, and was sustained for up to 20 weeks. Furthermore, when META060 feeding was terminated, mice began to gain weight rapidly. META060 inhibited fat accumulation in HFD fed mice as evidenced by a reduction in adipose tissue mass in mice supplemented with META060 compared with HFD fed control mice. In addition, META060 improved glucose tolerance after 5 weeks of supplementation. Moreover, long-term META060 supplementation in HFD-fed mice clearly reduced fasting blood glucose and insulin levels. These data suggest that META060 improves glucose homeostasis similarly to rosiglitazone and prevents HFD-induced obesity and insulin resistance.

Rosiglitazone, an antidiabetic drug from the class of thiazolidinediones, increases insulin sensitivity through its action on peroxisome proliferator activated receptor gamma (PPAR γ), and also has demonstrated anti-inflammatory activity through a mechanism involving nuclear factor kappa-B (NF- κ B)^{15,16}. While the mechanistic target(s) of META060 has not been identified, previous studies indicate that META060 has potent inhibitory effects on several kinases regulating the NF- κ B pathway, including glycogen synthase kinase 3 (GSK-3) and phosphatidylinositol-3 kinase (PI3K)¹². In this study, META060 demonstrated effects on insulin sensitivity similar to that of rosiglitazone, prompting us to speculate whether improvement of glucose tolerance in META060 treated mice is mediated through a PPAR γ dependent mechanism. However, rosiglitazone was not as effective at preventing weight gain in HFD fed mice as was META060, suggesting an alternative or additional mechanism of action for META060.

Results from metabolic experiments indicated that supplementation with META060 increased RER, metabolic flexibility, and carbohydrate-to-fat oxidation ratio in HFD fed mice.

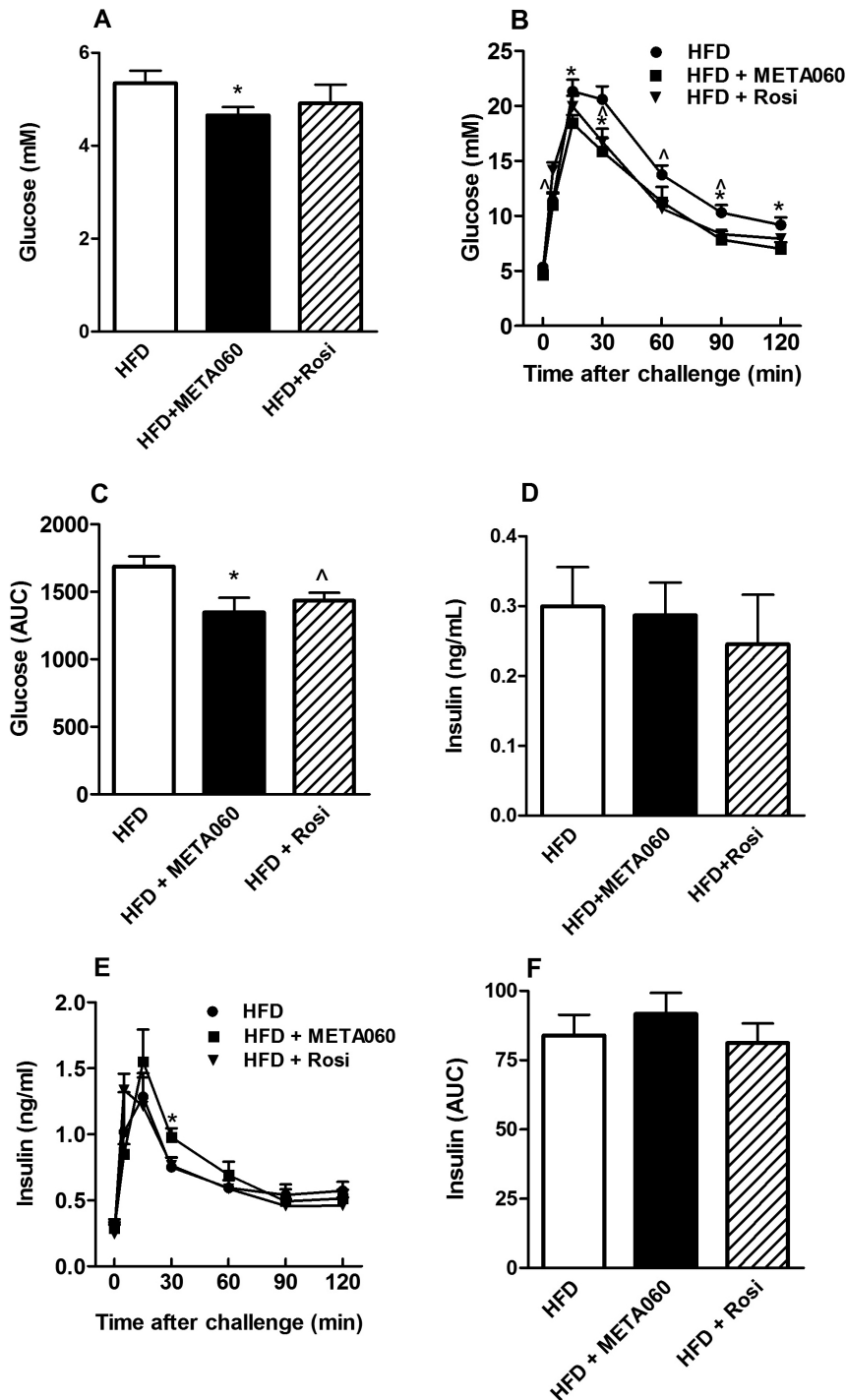


Figure 5. META060 improves glucose tolerance. Mice were fed a HFD or a HFD supplemented with 100 mg/kg/day META060 or 1 mg/kg/day rosiglitazone (Rosi). During week 5 of the diet intervention and after overnight fast, blood samples were collected, and glucose (A) and insulin (D) concentrations were determined. Oral glucose tolerance tests (OGTTs) were performed on fasted mice. Glucose (B) and insulin (E) concentrations were recorded at time points up to 120 min and areas under the curve (AUC) for glucose (C) and insulin (F) were calculated. Values are mean \pm SE (n=7-9 per group). * P <0.05 HFD vs. HFD + META060, [^] P <0.05 HFD vs. HFD + Rosi. 1

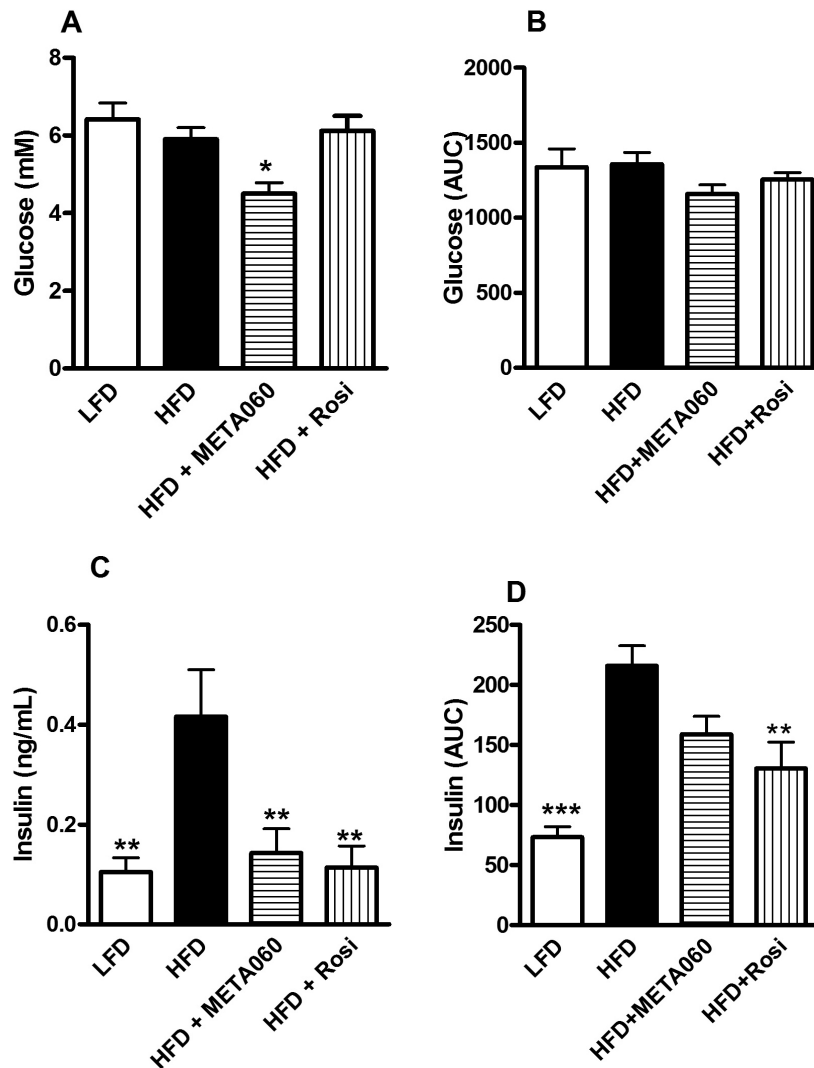


Figure 6. Long-term META060 supplementation reduces plasma glucose and insulin concentrations. Fasting plasma glucose (A) and fasting insulin concentrations (C) were determined in mice fed a LFD, a HFD, or a HFD supplemented with 100 mg/kg/day META060 for 14 weeks as described in Methods. OGTT was performed on fasted mice, and blood concentrations of glucose and insulin were determined at time points up to 120 min. Area under the curve (AUC) is presented for glucose (B) and insulin (D). Values are presented as mean \pm SE (n= 5-6 per group). *P<0.05, **P<0.01, ***P<0.001.

These observations are congruent with increased insulin sensitivity and improved carbohydrate handling induced by META060. Differences in metabolism and weight may also be observed if fat intake or absorption was not consistent between treatment groups. However, the metabolic experiments also indicated that META060 did not affect total energy expenditure, food intake, nor fatty acid secretion into the feces, and thus, do not explain the reduction in weight gain of META060 supplemented mice. Therefore, metabolic measurements may not be sufficient to resolve a mechanism for global effects of META060 on mouse metabolism.

Mice used in the 5-week study were slightly younger than those in the longer term experiment, and age may have a potential impact on physical activity, food intake, energy expenditure, or other metabolic processes. While it is possible that mice of different ages may have distinct metabolic characteristics contributing to the results we observed, the effects of META060 on weight gain and glucose homeostasis were consistent in both the short term and long term experiments.

Results from *in vitro* studies in a human cecal cell line demonstrated that META060 increases GLP-1 secretion (data not shown). Since GLP-1 is an insulin sensitizing hormone, this *in vitro* effect of META060 is consistent with *in vivo* effects on glucose homeostasis. Activation of GPR120, a G protein-coupled receptor that regulates GLP-1 secretion¹⁷⁻¹⁹, may function as a mechanistic target for META060 dependent GLP-1 secretion, although further studies will be required to investigate this possibility.

Future studies will focus on elucidating the mechanism of action underlying the effects of META060 on preventing weight gain in HFD fed mice and investigating whether META060 is effective in reducing weight in obese animals. META060 reduces fasting plasma glucose and insulin concentrations, and further research into its activity on insulin signaling and hepatocyte metabolism is needed. Data presented here suggest that META060 may have therapeutic value as an antidiabetic or antiobesity agent, and future investigations will evaluate its potential clinical use.

META060 supplementation significantly reduced the amount of weight gained in mice on a HFD. Indirect calorimetry measurements revealed increased metabolic flexibility in mice, and mice demonstrated improved glucose tolerance, comparable to the effects of rosiglitazone treatment. We conclude that META060 has potential therapeutic value for managing obesity and insulin resistance.

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