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Treatment with a Cholesterol Absorption Inhibitor (FM-VP4) Reduces Body Mass and Adipose Accumulation in Developing and Pre-Obese Mice

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Objective: **Disodium ascorbyl phytostanol phosphate (FM-VP4) is a cholesterol absorption inhibitor, a new class in cholesterol-lowering drug. Previous research on the lipid-lowering and anti-atherosclerotic effects of this drug has reported that administration of FM-VP4 results in a decrease in body mass. This study examined the FM-VP4 dose-dependent mass loss in mice and investigated some potential mechanisms by which decreased mass accumulation may have occurred. The effect of FM-VP4 administration on pre-obese mice was also tested.**

Research Methods and Procedures: **We conducted a dose-dependent study on mouse growth, food and water intake, organ mass, femur length, resting metabolic rate (RMR), maximal oxygen consumption under various conditions (VO_{2swim} and $VO_{2heliox}$), and fecal fat and plasma assessment for cholesterol and non-esterified fatty acids (NEFA) in mice fed a low fat (LF) or high fat (HF) diet, with or without FM-VP4. The ratio of lean to fat body mass of each animal was also assessed using magnetic resonance spectroscopy. To establish the effect of FM-VP4 on pre-existing obesity, mice were fed a high fat diet for 57 days, followed by administration of a diet containing 2% (w/w) FM-VP4 for 93 days.**

Results: **Animals exhibit a dose-dependent decline in body mass without a concomitant decrease in food intake, water intake, spleen, heart, or kidney mass, femur length or lean body mass. A dose-dependent trend toward a reduction in fat mass was observed in both high fat and low fat diet groups, becoming significant at a 1 and 2% FM-VP4 dosage (w/w). No FM-VP4 induced change in food or water intake, or resting metabolic rate was observed; however, an increase in VO_{2swim} was observed in the 2% FM-VP4 group over HF control. These findings were also observed in the pre-obese group treated with 2% FM-VP4.**

Discussion: **We found a dose dependent reduction in mass accumulation in mice treated with FM-VP4. This loss of mass is not due to an increase in resting metabolic rate or decreased food or water intake. The only tissues exhibiting a decrease in mass with FM-VP4 treatment are liver and body fat. Fecal fat content increased significantly with FM-VP4 treatment in a dose-dependent manner, suggesting that the treatment reduces mass accumulation through decreased absorption or increased excretion of lipids.**

Keywords phytosterol analog; FM-VP4; obesity; mice; adipose accumulation

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INTRODUCTION

Obesity has reached epidemic proportions in industrialized nations, leading to a significant and potentially lethal rise in the incidence of Type II diabetes, atherosclerosis, coronary artery disease, and systemic inflammation (Das, 2001). However, the development of efficacious pharmacological treatment options remains elusive. Current pharmacotherapies target three main areas of physiology: satiety, metabolism, and fat absorption, all with significant side effects (Wasan & Looije, 2005).

Our previous research investigating the lipid lowering and anti-atherosclerotic effects of a novel cholesterol absorption inhibitor, FM-VP4 (disodium ascorbyl phytostanyl phosphates) indicated that the drug might also have weight loss properties (Looije et al., 2005; Wasan et al., 2001). In Looije et al. (2005), animals fed high fat (45% kcal from fat) and low fat (10% kcal from fat) with or without FM-VP4 (2% w/w) for 12 weeks showed a significant difference in weight gain over the course of the study. No difference in food or water intake was observed. To further examine the weight loss properties and potential mechanisms of action of FM-VP4, we conducted a dose-dependent study on growth, food and water intake,

organ mass, femur length, resting metabolic rate (RMR), maximal oxygen consumption under various conditions (VO_{2swim} and $VO_{2heliox}$), and fecal fat and plasma assessment for cholesterol and non-esterified fatty acids (NEFA) in mice fed a low fat or high fat diet, with or without FM-VP4. The ratio of lean to fat body mass of each animal was also assessed using magnetic resonance spectroscopy. To establish the effect of FM-VP4 on pre-existing obesity, mice were fed a high fat diet for 57 days, then administered a diet containing 2% (w/w) FM-VP4 for 93 days. Animals were sacrificed on Day 150 of the study and assessed for the above criteria.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (4 weeks old) were purchased from Charles River laboratories (St. Constant, Quebec, Canada) and housed individually with wood shaving bedding. The mice were kept at a constant temperature of 21°C +/–2°C in a 12 hr light/dark cycle (lights on at 7:00 am) and had unrestricted access to food and water throughout the period of the study. Animal mass and food intake were recorded daily for the first two weeks and then twice per week for all groups of mice. Water intake was recorded twice weekly for 7 weeks.

FM-VP4 Dose Response Studies

Following an acclimatization period of eight days where the mice were fed regular mouse chow, the mice were randomly assigned into 10 groups (n = 8; mass = 23.1 ± 1.13 g). Five groups of mice were fed a low fat (LF) diet containing 10 kcal% fat with low cholesterol (0.0018% cholesterol; Research Diets Inc., New Brunswick, NJ, product # D12450B), and five groups of mice received a high fat (HF) diet containing 45 kcal% fat with normal cholesterol content (0.0195% cholesterol; product # D12451). The five LF groups and the five HF groups received increasing concentrations of FM-VP4: LF and HF control without added drug, 0.25, 0.5, 1.0, and 2.0% (w/w) FM-VP4 (FM-VP4 lot # 006 by Forbes-Medi Tech, Vancouver, BC, Canada). Diets were milled by Research Diets Inc (New Brunswick, NJ). Diet composition has been reported previously (Looije et al., 2005).

Morphometrics

Mice were weighed immediately prior to euthanasia at Day 113 (HF/LF trials) or Day 150 (previously obese trials). Animals were anesthetized by inhalation of halothane (MTC Pharmaceuticals, Cambridge, ON, Canada) provided by a Narkomed-2 North American Draeger anesthesia apparatus (3% halothane to initialize, 1.5% maintenance). Once anaesthetized, venous blood samples were obtained from each mouse via cardiac puncture, followed by euthanasia via IC administration of 0.3 ml Euthanyl (Sodium-Pentobarbital, MTC Pharmaceuticals). Immediately after death, the spleen, liver, heart and right kidney were harvested and weighed to an accuracy of 0.01g. Femur length was measured to an accuracy of 0.01 mm using digital calipers (Digipa SR44).

Body Composition

Whole body fat measurements were carried out on a 7T animal MRI scanner (Bruker, Germany). Unanesthetised mice were placed inside a Plexiglas restrainer, and the restrainer was positioned inside the bore of the magnet. NMR signal from the entire body was acquired with a quadrature volume RF coil tuned to 300MHz. Standard CPMG sequence (TE = 2.377 ms, TR = 10 s) was used to acquire 256 echoes from which the T_2 decay curve was extracted. The decay curves were fit to a double exponential function using software procedure developed in house with Igor (WaveMetrics, OR). The component corresponding to $T_2 \approx 40$ ms was identified as water in lean tissue, and the one with $T_2 \approx 200$ ms as body fat (Kunnecke et al., 2004). The dc shift of the double exponential function was identified as a "free" water component corresponding to body fluids, e.g., urine and CSF, with the typical amounts of less than 5% of the total signal. The ratio of lean tissue/body fat expressed as weight/weight was calculated from the NMR data as described in Kunnecke et al. (2004).

Oxygen Consumption

Measurement of oxygen consumption at rest (resting metabolic rate; RMR), swimming (VO_{2swim}); and in a thermogenic environment of 79% helium: 21% oxygen ($VO_{2heliox}$) was conducted on four groups: HF; HF FM-VP4 2%; LF and LF FM-VP4 2%. For RMR assessment, animals were placed in a sealed black Plexiglas 1075 ml chamber immersed in a 21 ± 0.2°C water bath. Outside atmospheric air was pushed through the chamber at a rate of 800 ml min^{-1} (0–1.5 l air pump, Rena Air 400A; Aalborg Mass Flow controller (0–5 l)). A subsample of excurrent air was dried and scrubbed of CO_2 and passed through an oxygen analyser (Beckman OM-11 polarographic oxygen analyzer) at a rate of 300 ml min^{-1}. Oxygen measurements were recorded each second via a DI-710 A/D converter and the lowest 5 min was averaged to estimate RMR (Windaq DATAQ software), and corrected for pressure and temperature. Immediately prior to all metabolic measurements, body mass was recorded to ± 0.1 g. Resting metabolic rate was defined as the lowest average oxygen consumption at 21 ± 0.2°C over a 5 min period during the light phase (between 1000 and 1800 h) using an open flow respirometry system. Mice were not denied food or water before respirometry measurements; however, most food intake occurs nocturnally and it is an accepted practice to assume the animal is approaching a post-prandial state near the end of a RMR assessment period (Johnson et al., 2001; Krol et al., 2003). Each animal was in the chamber for a minimum of 2 hr, with some individuals remaining in the chamber for up to 5 hr to ensure that RMR had been achieved.

To measure VO_{2swim}, we suspended a glass funnel over a water bath maintained at 20 ± 0.2°C. The animals were introduced into the water bath and the funnel was immediately lowered over the swimming mouse. The funnel was submerged to a predetermined height, leaving an air volume of 250 ml above the water level. Room air was introduced into the funnel at a rate of 800 ml/min via a submerged air stone. The air stone was positioned directly beneath the animal to encourage active swimming. VO_{2swim} was defined as the highest oxygen consumption averaged over 2 min of 5 min swimming. A subsample of excurrent air was evaluated as described above.

For $VO_{2heliox}$ measurements, the chamber containing the animal was submerged in a glycol-based coolant maintained at −2.5 ± 0.2°C. A mixture of 79% helium/ 21% oxygen (heliox) was pushed through the chamber at a rate of 1000 ml/min and a subsample of excurrent gas was extracted and measured as described above. $VO_{2heliox}$ was defined as the highest O_2 consumption averaged over 2 min of the last 5 min of 15 min heliox exposure.

Fecal and Plasma Lipids

Mouse feces were collected over a period of 72 hr and stored at −20°C. Fecal lipids were extracted using the method of Folch et al. (1957). Briefly, 100 mg per sample were resuspended in 2 ml of 2:1 chloroform: methanol mixture (both by Fisher Scientific, HPLC grade) by 10 strokes with a polypropylene pestle (Kimble-Kontes, Vineland, NJ), followed by homogenization using a Kinematica homogenizer (Littau, Switzerland). The samples were centrifuged for 15 min at 1050 g to separate the liquid phase from the solid material. The liquid phase was subsequently washed in 200 ml 0.9% NaCl, followed by a wash of the interphase with a mixture of methanol: water (1:1). Following evaporation under nitrogen gas (TurboVap LV evaporator, Zymark, Hopkinton, MA), lipids were reconstituted in isopropanol, isopropanol with 10% Triton-X100 or NEFA-C buffer (1.34 mM EDTA-2Na, 7.69 mM sodium azide, 0.2% Triton-X100, 0.76 mM NaOH, pH 6.5) for triglyceride, cholesterol or NEFA analysis, respectively.

For both plasma and fecal lipid analysis, cholesterol was quantified by spectrophotometric assay at 540 nm using the Infinity™ Cholesterol Liquid Stable Reagent (Thermo Electron, Louisville, CO) according to manufacturer's instructions. Non-esterified fatty acids were determined with the NEFA C kit (Wako Chemicals GmbH, Neuss, Germany), modified for high throughput use according to the method published by Miksa et al., 2004. Triglyceride analysis was conducted using a spectrophotometric at 540 nm using the Wako L-Type TG H kit (Wako Chemicals, Richmond, VA).

Extraction efficiencies for cholesterol, triglyceride and NEFA were determined by the addition and subsequent extraction of ^{3}H cholesterol, Glycerol-tri [^{14}C] oleate, and 9,10 (n)-[^{3}H] oleic acid.

Previously Obese Studies

Two groups of mice (n = 8; mass = 22.4 ± 1.45 g) were fed regular mouse chow for the first eight days, after which all 16 mice received the HF diet for 57 days until a state of obesity was reached (body mass of greater than 20% above average weight). Starting on day 58, one group (n = 8) continued to receive the HF diet, whereas the other group of mice received the HF diet supplemented with 2% FM-VP (w/w). Animals were sacrificed on Day 150 of the study.

Statistical Analyses

Statistical analysis was conducted using JMP software V. 5.1 for Windows (SAS Institute Inc, Carey, NC). To test effects of diet and FM-VP4 dosage, we used a one-way ANOVA; significantly different group means were then separated by Tukey-Kramer test for Honestly Significant Difference (HSD). For the pre-obese group, statistical significance of the differences between means was determined using Student t test; differences were considered statistically significant when $p < 0.05$. Data are expressed as the mean ± SD (n = 8 mice/group) unless otherwise stated.

RESULTS

Animals

Growth Curves

Animals fed a HF diet containing 1 or 2% FM-VP4 exhibited a significant dose-dependent decline in mass accumulation over the period of the study ($F_{4, 205} = 9.35$, $p < 0.0001$; Figure 1a). This decline was observed immediately following administration and became significant after day 20 of the study. In LF animals, a significant decline in mass accumulation was noted only in the 2% FM-VP4 group ($F_{4.205} = 5.68$, $p = 0.0002$; Figure 1b).

Food and Water Intake

After an initial adjustment period to the new diets, animals maintained a relatively constant daily food intake with no significant difference in caloric intake between treatment groups (Tukey-Kramer HSD, $p = 0.05$, Figure 2a, b). Water intake was assessed in the LF, LF 2% FM-VP4, HF and HF 2% FM-VP4 groups from day 35 to day 85; there is no observable difference between groups ($F_{3,56} = 0.82$, $p = 0.49$; Figure 2c).

Morphometrics

The organ mass to body mass ratio was corrected for organ scaling factors (M^{p}, where M = body mass, and the exponent "p" is the organ-specific scaling factor). Values for p are: kidney 0.85, liver 0.886, spleen 0.9, and heart 0.99). For brevity, graphs in Figure 3 depict the scaled organ mass of control, 1 and 2% groups only. In the HF group, 2% FM-VP4 administration resulted in a significant elevation of spleen, kidney and heart mass. In the LF group, spleen mass was increased in the

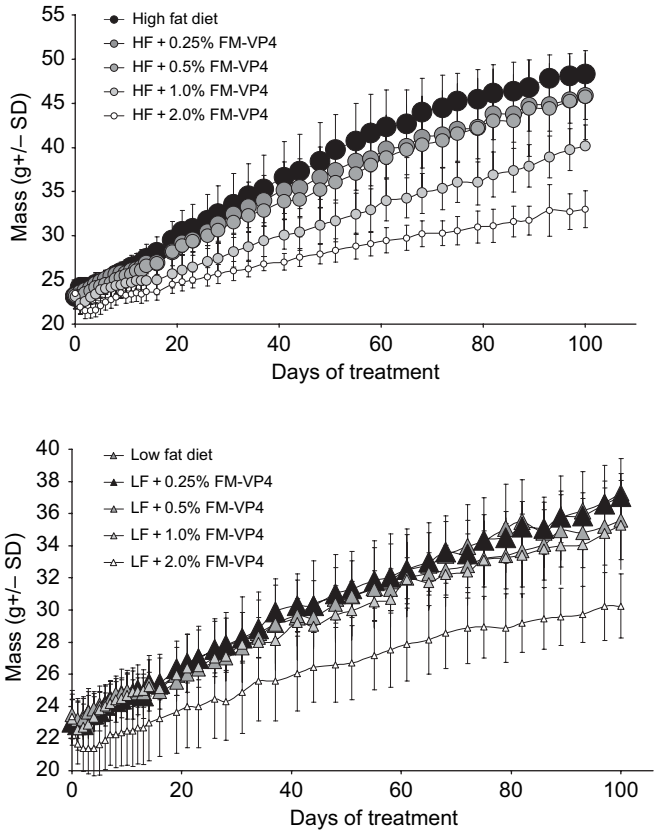

FIGURE 1. Effect of various FM-VP4 dosages on mass accumulation and growth of animals fed HF (Figure 1a) and LF (Figure 1b) diets. Animals fed a HF diet containing 1% or 2% FM-VP4 exhibit a significant dose-dependent decline in mass accumulation over the course of the study ($n = 8$, $F_{4,205} = 9.35$, $p < 0.0001$). In LF animals, a significant decline in mass was noted only in the 2% FM-VP4 group ($n = 8$, $F_{4,205} = 5.68$, $p = 0.0002$).

2% group, while kidney mass increased in both the 1 and 2% groups. The 2% dose was also accompanied by a significant decrease in liver mass in both the HF and LF groups (on post-mortem analysis, the 2% FM-VP4 groups had a visible decrease in "fatty liver" appearance when compared to the HF control group; $n = 8$, $p < 0.05$).

The length of the femur was calculated as the distance between the greater trochanter and the distal lateral condyle, measured in situ during dissection. We found no significant difference in femur length between the groups ($F_{4,34} = 2.06$, $p = 0.11$; Figure 4).

Body Composition

Nuclear magnetic resonance analysis of body composition provides data on the ratio of lean to fat tissue. Data are expressed as grams of lean or fat tissue; no significant difference in lean body mass was observed in the HF group with administration of FM-VP4 (Figure 5). A significant rise in lean body mass was observed in the LF 1 and 2% FM-VP4 groups

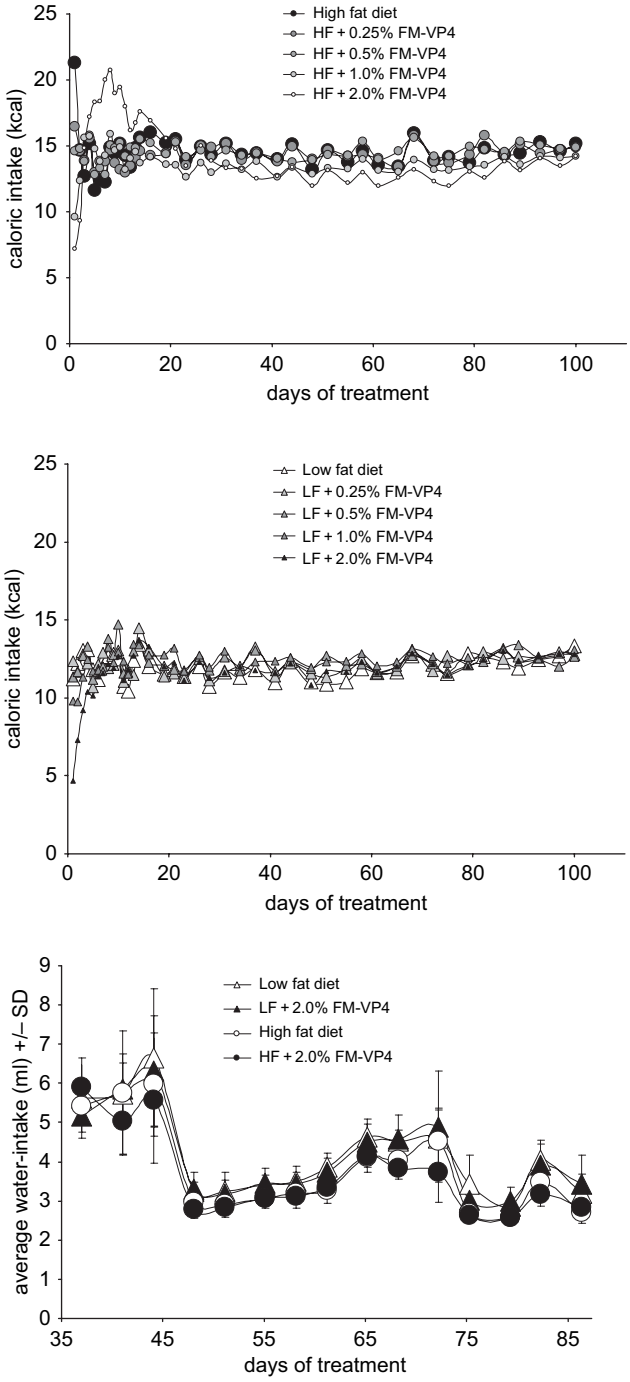

FIGURE 2. Daily caloric and water intake for mice on HF (Figure 2a) and LF (Figure 2b) diets with oral daily dosage of FM-VP4 at concentrations of 0.25, 0.5, 1.0, and 2%. Water intake for LF, LF 2%, HF, and HF2% is depicted in Figure 2c. There is no observable difference in caloric ingestion or water intake between groups ($n = 8$, $p > 0.05$).

($F_{4,34} = 5.11$, $p = 0.0024$) when compared to control and other treatment dosages.

A significant decline in fat mass was observed with FM-VP4 administration in the HF group ($F_{4,30} = 65.55$, $p < 0.0001$). A

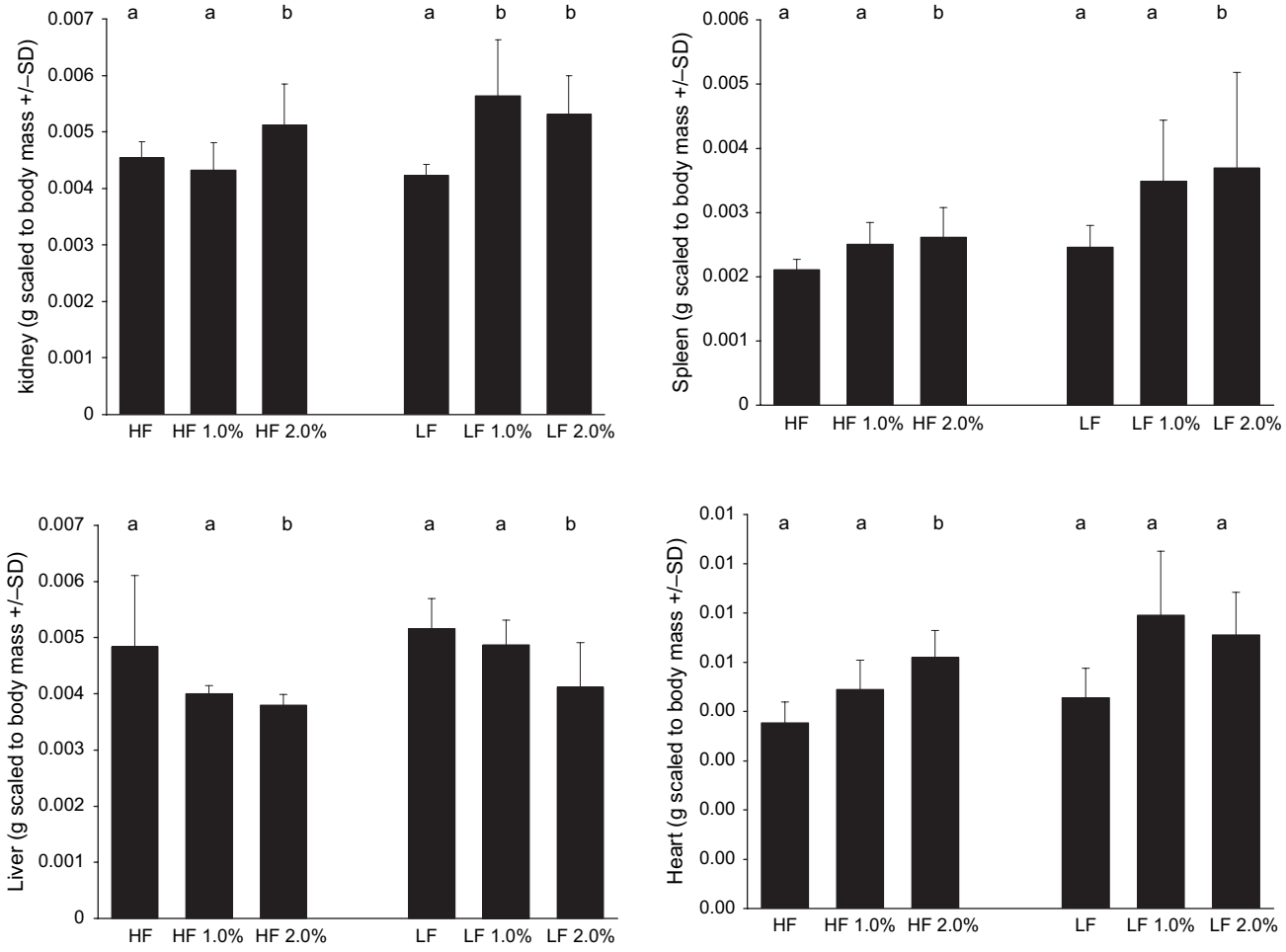

FIGURE 3. Mass of kidney, liver, spleen and heart from animals on a HF and LF diet given 1 and 2% doses of FM-VP4. In the HF group, 2% FM-VP4 administration resulted in significant elevation of spleen, kidney and heart mass. In the LF group, spleen mass was increased in the 2% FM-VP4 group, and kidney mass increased in both the 1% and 2% FM-VP4 groups. Liver mass of HF and LF 2% FM-VP4 is significantly lower than control. Within each dietary group, levels not connected by the same letter are significantly different ($n = 8$, $p < 0.05$).

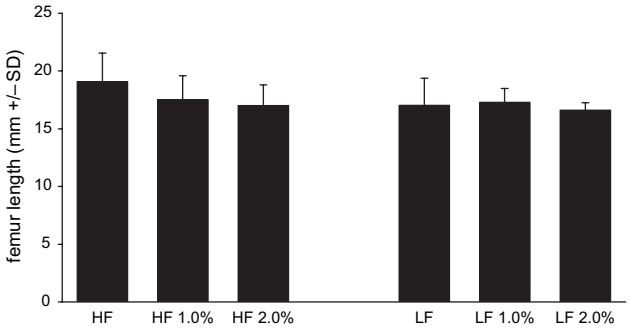

FIGURE 4. Femur length from mice on HF and LF diets administered a daily oral dose FM-VP4 at 1% and 2% w/w. No significant difference in femur length was observed ($n = 8$, p = n/s).

trend toward decreasing fat mass was also observed in the LF group, with the decrease becoming significant in the FM-VP4 1 and 2% groups ($F_{4,34} = 20.9$, $p < 0.0001$). Although all animals were assessed statistically, Figure 5 shows only control, 1 and 2% FM-VP4 groups.

Oxygen Consumption

Metabolic assessment was conducted on HF, HF 2%, LF, and LF 2% groups. No significant differences in RMR ($F_{3,19} = 0.94$, $p = 0.44$) or $VO_{2heliox}$ ($F_{3,19} = 019$, $p = 0.9$) were observed between the groups or with FM-VP4 administration (Figure 6a, b). However, a significant increase in VO_{2swim} was observed in animals receiving 2% FM-VP4 when compared to HF control (Figure 6c; $F_{3,23} = 12.05$, $p < 0.0001$). The range from resting metabolic rate (RMR) to maximal metabolic rate (MMR) to is referred to as the metabolic scope of the animal and is an indication of aerobic fitness. We compared VO_{2swim}-RMR values (measured at 21°C) to provide an estimate of variation in metabolic scope. Figure 6d illustrates the higher oxygen consumption of animals in the 2% FM-VP4 group and the observed increase in scope.

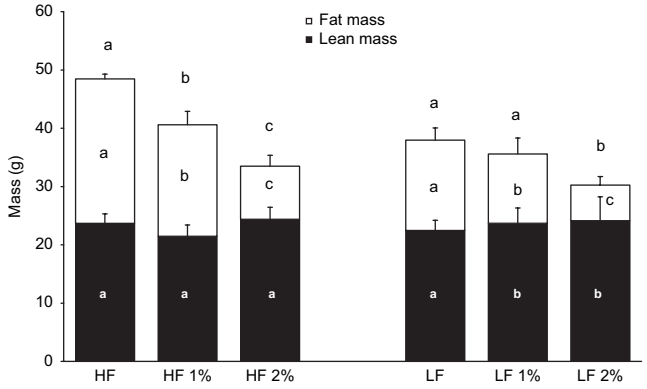

FIGURE 5. NMR assessed body composition of mice on HF and LF diets given daily oral doses of FM-VP4 at a 1 and 2% w/w. Lean body mass (black bars) was not significantly different with treatment in the HF group, but showed a dose-dependent increase in the LF group (n = 7–8, $p > 0.05$). Body fat (white bars) showed a significant dose-dependent decline with FM-VP4 administration in both HF and LF groups (n = 6–8, $p < 0.0001$). Total body mass (total bar) declined in a dose-dependent manner in both groups (n = 7–8; $p < 0.0001$). Within each parameter, levels not connected by the same letter are significantly different.

Fecal and Plasma Lipids

The minimal extraction efficiency of ^{3}H cholesterol, glyceryl-tri [^{14}C] oleate, and 9,10 (n)-[^{3}H] oleic acid was 85%, representing cholesterol, triglyceride, and NEFA extraction from feces. Fecal cholesterol levels showed a significant dose-dependent increase with FM-VP4 in both the LF ($F_{4,35} = 62.4$, $p < 0.0001$) and HF diet group ($F_{4,35} = 128.5$, $p < 0.0001$). There were no differences in fecal cholesterol content between the LF and HF groups. Fecal NEFA levels showed a dose-dependent increase with FM-VP4 in both the LF and HF groups (LF $F_{4,34} = 55.56$, $p < 0.0001$; HF $F_{4,35} = 15.7$, $p < 0.0001$); however, animals on the HF diet had significantly greater NEFA content in the fecal matter than LF at all FM-VP4 doses (Figure 7a, b).

Plasma cholesterol levels showed a dose-dependent decrease with FM-VP4 administration, with 2% FM-VP4 administration resulting in a significant decline over control levels (HF $F_{4,35} = 4.36$, $p = 0.0057$; LF $F_{4,33} = 6.33$, $p = 0.0007$; Figure 8a). No significant differences in plasma NEFA or triglyceride levels were observed, either between diets or with FM-VP4 administration (Figure 8b, c).

Previously Obese Animals

In a group of mice maintained on a HF diet, animals reached and maintained a body mass of greater than 20% above average weight by Day 57 and were defined as obese. Starting on Day 58, one group (n = 8) of animals received the HF diet supplemented with 2% FM-VP4 (w/w). After 93 days on their respective diets, the HF and HF 2% FM-VP4 groups did not exhibit any significant differences in food intake, organ mass, femur length, RMR or $VO_{2heliox}$ (data not shown).

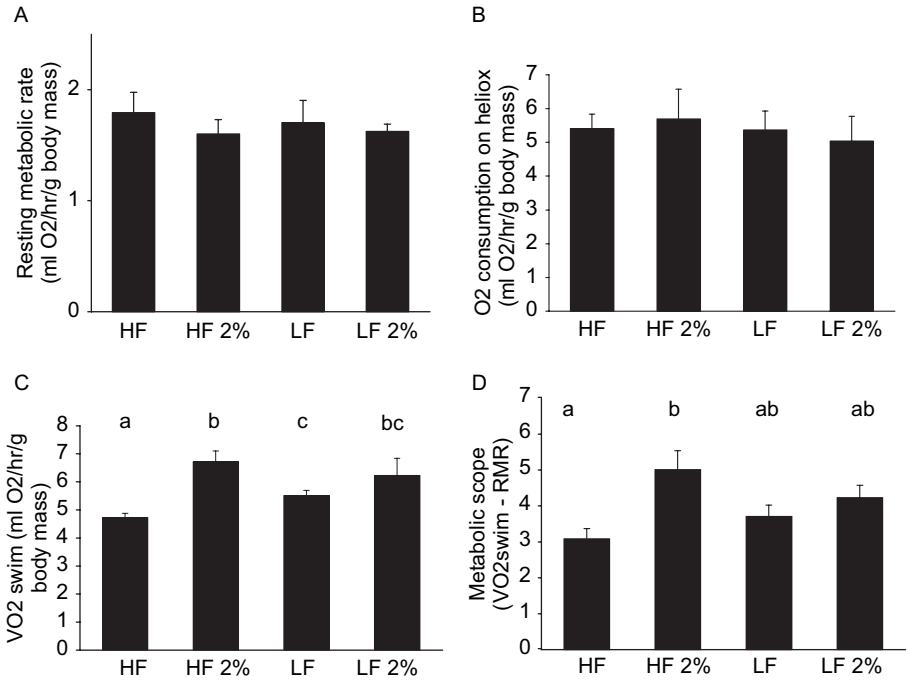

FIGURE 6. Oxygen consumption in mice fed a HF and LF diet with daily oral dose of 2% FM-VP4. Oxygen consumption at rest (Figure 6a) and under thermal challenge in a 79% helium / 21% oxygen environment (Figure 6b) showed no significant difference between groups (n = 5–7, $p > 0.05$). Maximum oxygen consumption during aerobic swim challenge (VO_{2swim}) is significantly higher in the 2% FM-VP4 groups of both diets (n = 6–7, $p < 0.0001$), resulting in an elevated aerobic scope (VO_{2swim} – RMR; n = 5–6, $p = 0.0081$). Levels not connected by the same letter are significantly different.

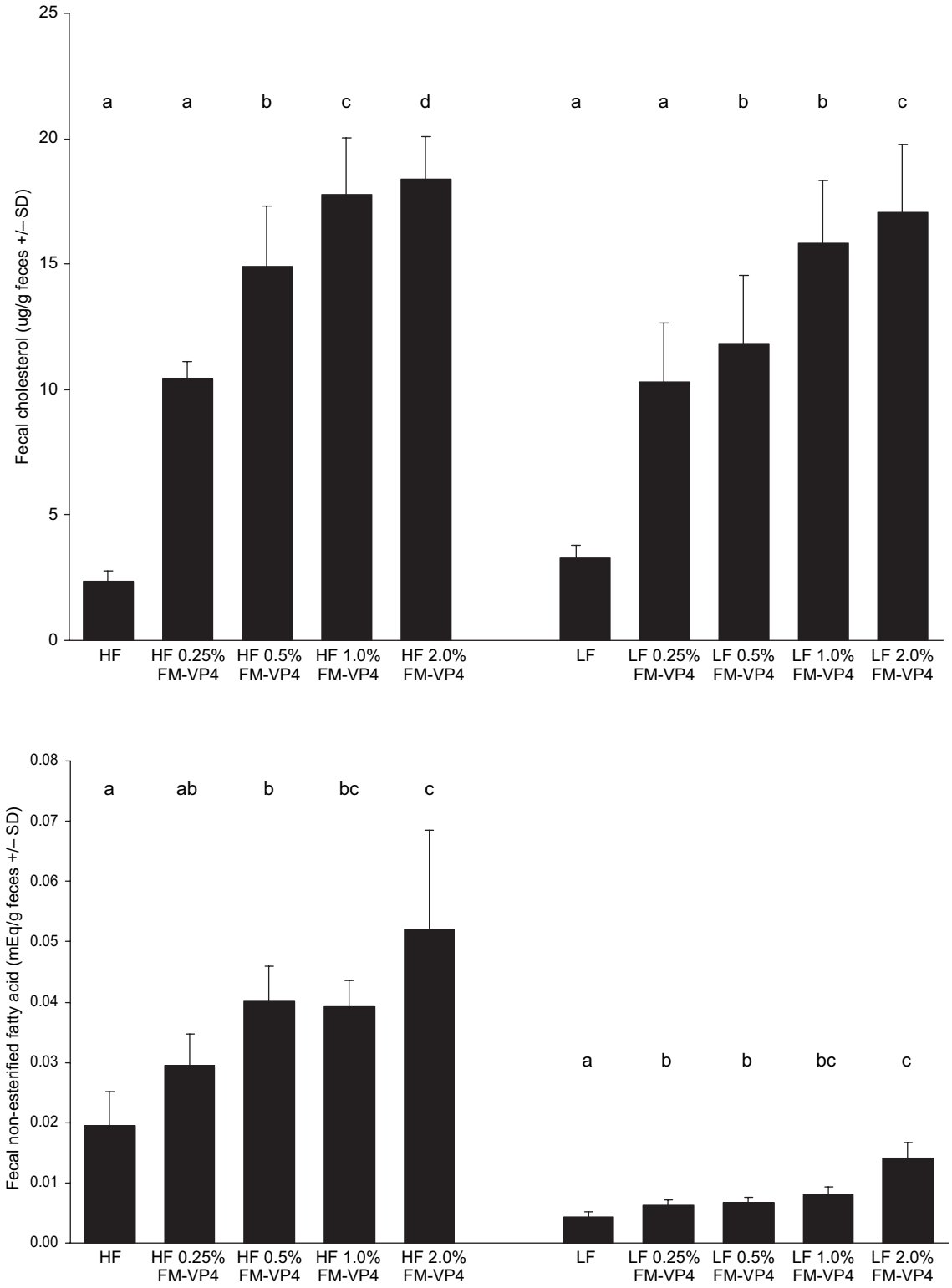

FIGURE 7. Fecal fat content from mice on a HF and LF diet varies with increasing oral daily dose of FM-VP4. Fecal cholesterol (7a) does not vary with diet but shows a significant dose-dependent increase with FM-VP4 administration ($n = 8$; $p < 0.0001$). Non-esterified fatty acids (NEFA; Figure 7b) vary significantly with both diet and FM-VP4 administration ($n = 8$; $p < 0.0001$). Animals in the LF group have significantly lower NEFA than those in the HF group. Within each dietary group, levels not connected by the same letter are significantly different.

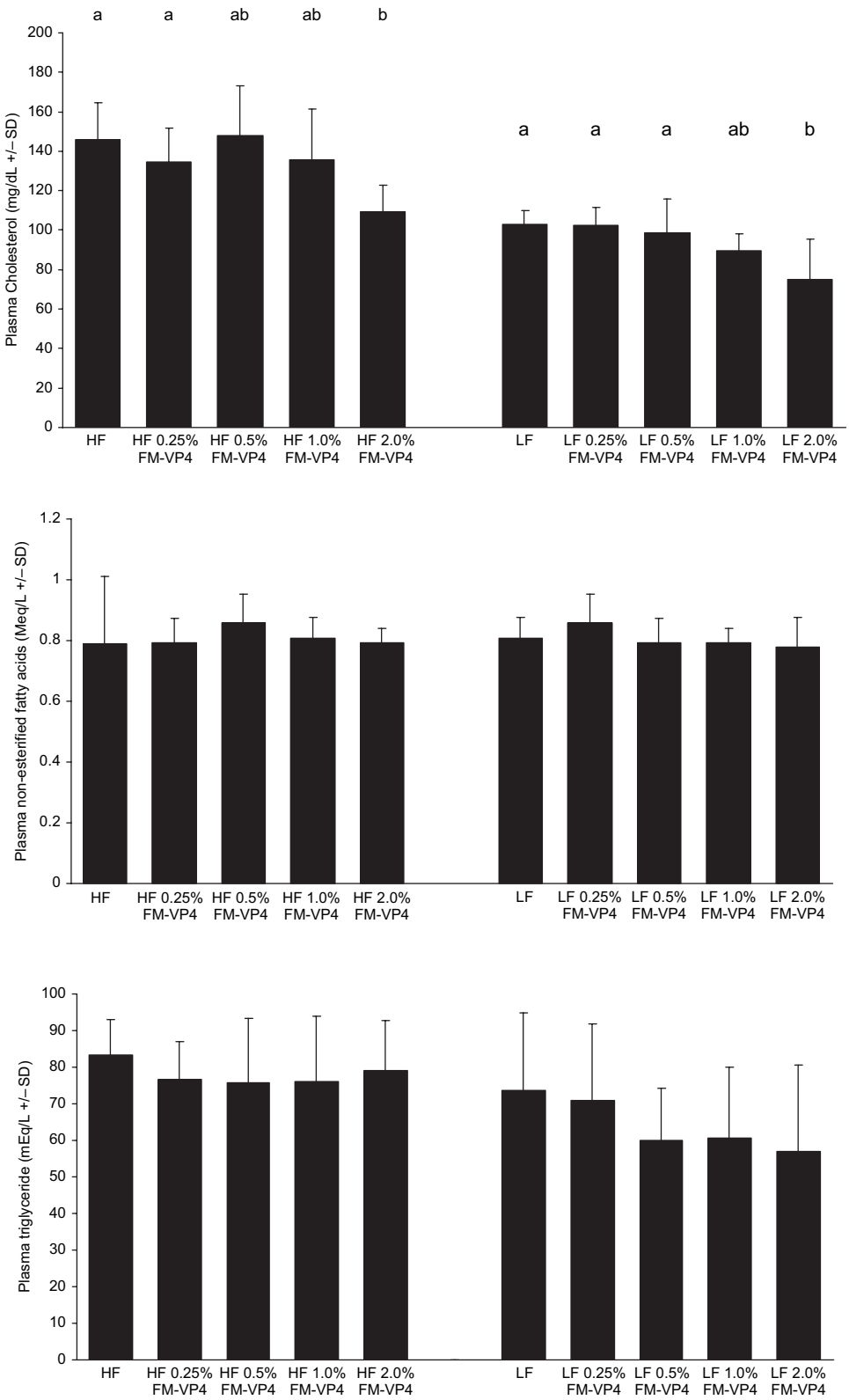

FIGURE 8. Plasma cholesterol, NEFA and triglyceride content from mice on a HF and LF diet varies with increasing oral daily dose of FM-VP4. Plasma cholesterol (8a) varies with diet and FM-VP4 administration ($n = 8$; $p < 0.0001$). Non-esterified fatty acids and triglyceride levels do not vary significantly with diet or FM-VP4 dosage (Figures 8b, 8c). Within each dietary group, levels not connected by the same letter are significantly different.

Growth Curve

Animals receiving the HF diet supplemented with 2% FM-VP4 showed a rapid decline in body mass, becoming and remaining significantly lower than the HF group after day 64 ($p < 0.05$). Within 14 days of administration, the HF FM-VP4 2% group's body mass had dropped to the level observed in untreated animals maintained on a LF diet (LF growth curve is presented for comparison; Figure 9).

Oxygen Consumption

Animals in the HF group and the HF 2% FM-VP4 did not exhibit any difference in RMR or $VO_{2heliox}$ (data not shown); however, a significant increase in the VO_{2swim} was observed in the HF 2% FM-VP4 group ($n = 8$, $p < 0.0001$; Figure 10). This increase in VO_{2swim} results in a greater range of metabolic rate (VO_{2swim} – RMR), indicating an elevation of aerobic scope.

Body Composition

Data are expressed as grams of lean or fat tissue; no significant difference in lean body mass was observed between HF and the HF 2% group. However, significant declines in both body mass and fat mass were observed with 2% FM-VP4 administration ($n = 8$; $p < 0.0001$; Figure 11).

Fecal and Plasma Lipids

Fecal cholesterol ($n = 8$, $p < 0.0001$), NEFA ($n = 8$, $p < 0.0001$) and triglyceride ($n = 4$, $p < 0.0001$) content were all significantly higher in the 2% FM-VP4 group than in HF controls (Figure 12). In pre-obese animals, plasma cholesterol ($n = 8$, $p < 0.0001$) and triglycerides ($n = 8$, $p = 0.0064$) declined significantly with 2% FM-VP4 administration (Figure 13a, c). Plasma NEFA levels were not different between treatment and control (Figure 13b).

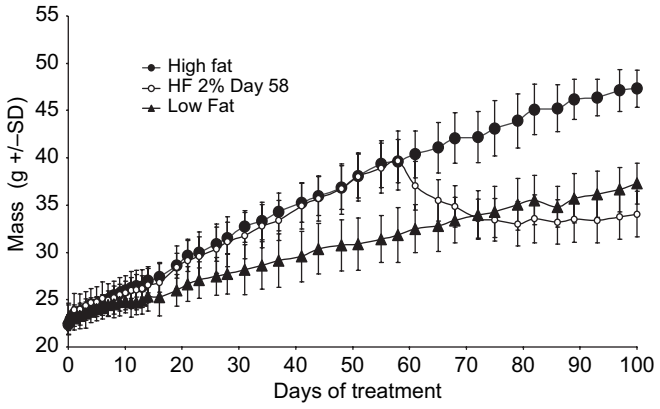

FIGURE 9. Mass accumulation in previously obese animals administered FM-VP4 at Day 58 is significantly lower than control ($n = 8$, $p < 0.05$). Mass accumulation in animals fed a LF diet (σ) is provided for comparison. After Day 64, the mass of animals in the HF 2% FM-VP4 group is not significantly different than LF control ($n = 8$, $p > 0.05$).

DISCUSSION

Oral administration of FM-VP4 has been shown to decrease the concentration of plasma cholesterol and LDL cholesterol when compared to controls (Wasan et al., 2001a, b). These studies both reported an unexpected finding of a reduction in body mass associated with FM-VP4 treatment. Looije et al. (2005) investigated the weight loss properties of FM-VP4 using a dietary-induced obese mouse model, and confirmed that administration of 2% FM-VP4 is accompanied by a significant reduction in body mass. Our data demonstrate that FM-VP4 causes a dose-dependent decrease in mass accumulation in mice on both LF and HF diets. In the HF fed group, this decrease is significant at both 1 and 2% FM-VP4 administration. Animals on the LF diet exhibited a less profound dose-dependent reduction in mass accumulation, with a significant

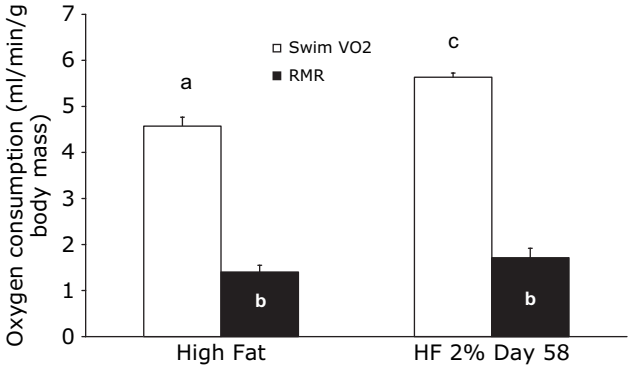

FIGURE 10. Oxygen consumption in mice fed a HF diet with daily oral dose of 2% FM-VP4. Oxygen consumption at rest (black bars) showed no significant difference between groups ($n = 8$; $p > 0.05$). Maximum oxygen consumption during aerobic swim challenge (VO_{2swim}) is significantly higher in the 2% FM-VP4 group ($n = 8$, $p < 0.0001$). Within each parameter, levels not connected by the same letter are significantly different.

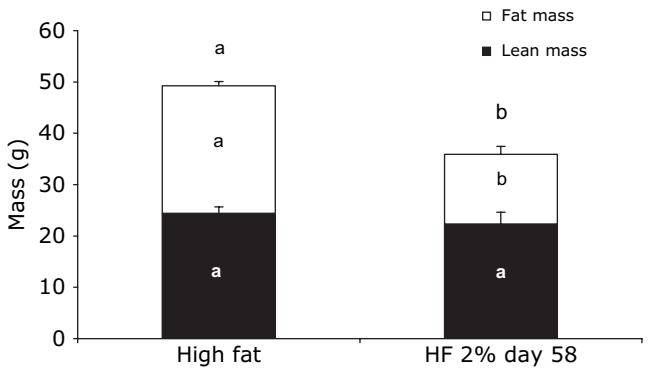

FIGURE 11. NMR assessed body composition of previously obese mice on HF diet given daily oral dose of 2% FM-VP4. Lean body mass (black bars) did not significantly differ between groups. Body fat (white bars) and total body mass (total bar) showed a significant decline with FM-VP4 administration ($n = 8$, $p < 0.0001$).

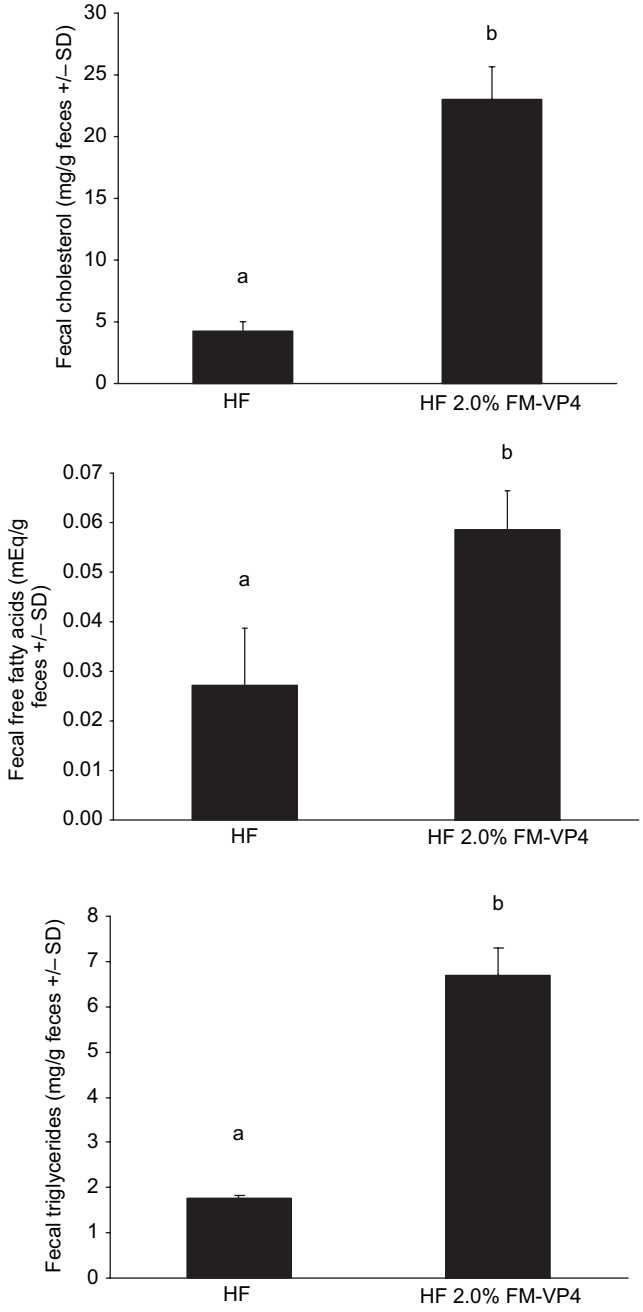

FIGURE 12. Fecal fat content of previously obese animals given a daily dose of 2% FM-VP4. Cholesterol (Figure 12a, $n = 8$, $p < 0.0001$), NEFA (Figure 12b, $n = 8$, $p < 0.0001$) and triglyceride (Figure 12c, $n = 4$, $p < 0.0001$) levels in fecal fat are significantly elevated with FM-VP4 administration. Levels not connected by the same letter are significantly different.

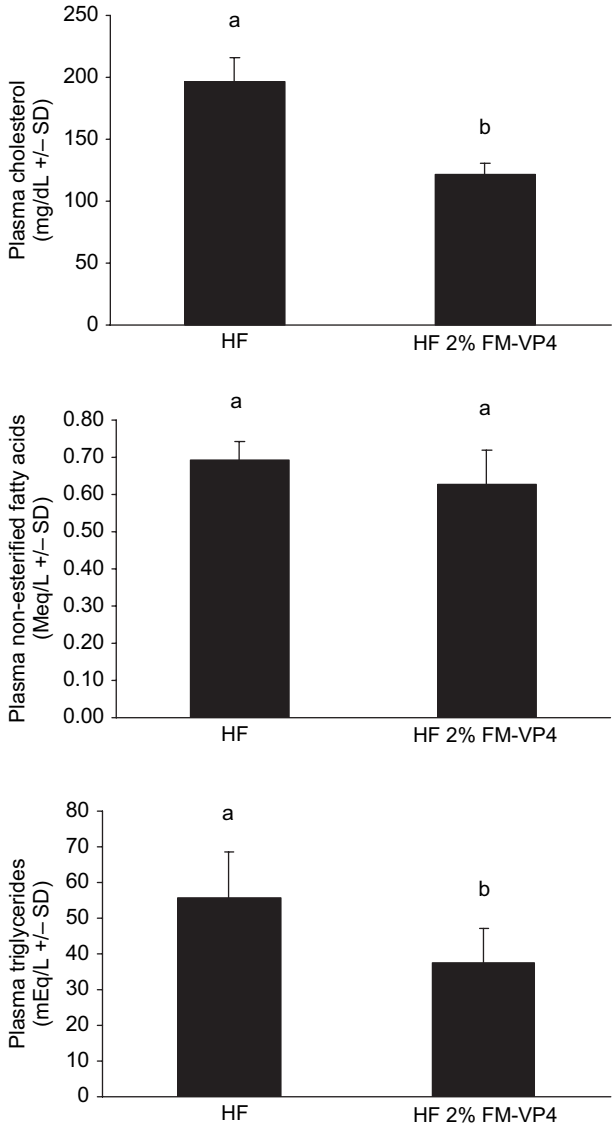

FIGURE 13. Plasma cholesterol, NEFA and triglyceride levels of previously obese animals given a daily dose of 2% FM-VP4. Cholesterol (Figure 12a; $n = 8$, $p < 0.0001$) and triglyceride (Figure 12 c; $n = 8$, $p = 0.0064$) levels are significantly lower in the HF 2% FM-VP4 group. Levels not connected by the same letter are significantly different.

difference occurring only in the 2% FM-VP4 group when compared to LF control.

Looije et al. (2005) reported that in mice fed a high fat diet, the addition of 2% FM-VP4 to the diet caused a slight reduction in food intake, suggesting that FM-VP4 might have an appetite suppressing effect. We found no evidence of a decrease in caloric intake or suppression of appetite in either the HF or LF group (Figure 1a, b). Our data also indicate that appetite suppression does not occur in previously obese animals administered 2% FM-VP4; therefore a reduction in caloric intake is not a factor in the observed decrease in mass accumulation. The diets were designed to be relatively isocaloric to reduce possible differences in caloric intake between LF and HF diets; however, mice are observed to have an incipient ability to closely regulate their energy intake, despite large changes in macronutrient content of the incoming diet (Hambly et al., 2005). Although water intake for all groups varied over the course of the study, we did not observe any

between-group differences in the control and FM-VP4 groups (Figure 2c).

To further categorize the reduction in mass accumulation and identify the target tissue(s) responsible for the loss, we evaluated the possibility of growth retardation at the organ level and found no evidence of decreased organ mass with FM-VP4 administration (spleen, liver, kidney, and heart; Figure 3). At the conclusion of the study, all animals appeared healthy with the exception of a small number of animals (6/112) that exhibited inflammation of the urinary tract; one animal was euthanized prior to the completion of the study (LF FM-VP4 2% #7). This condition was not isolated to one experimental group and did not correlate with type of diet or drug dose. On post-mortem, organs appeared healthy and free of lesions, although evidence of fatty liver was noted in the HF control group. No gross signs of growth retardation, organ atrophy or muscle wasting were observed.

To quantify skeletal development and growth, femur length was measured during post-mortem examination. Femur development is dependent on proper absorption of nutrients from the GI tract (Garcia-Sancho Tellez et al., 2001) and is used as a measure of nutritional status and growth. There was no significant difference in femur length between control groups (LF and HF), or when data from control was compared to animals receiving FM-VP4 (Figure 4), suggesting that the doses provided over the course of the study did not affect skeletal development and growth.

Body composition data obtained via NMR spectroscopic analysis showed no significant decline in the lean body mass of the experimental groups, indicating that muscle wasting or atrophy is not a contributing factor to mass loss in these animals. However, a significant decline in fat mass was observed with FM-VP4 administration, and this decline occurred in a dose-dependent fashion—most notable in HF diet animals (63% reduction in adipose with a 2% FM-VP4 dose), and the LF group (61% reduction in fat mass associated with a 2% FM-VP4 dose).

The failure to accumulate adipose tissue could be due to an increase in catabolism and utilization of dietary fuels (i.e., increased metabolic rate), a decreased absorption and/or utilization of dietary fuels, or possibly a combination of both events. To investigate these options, indirect calorimetry (metabolic studies) and fecal fat measurements were undertaken.

The lack of a significant difference in oxygen consumption at rest between the groups indicates that at the doses provided, FM-VP4 does not have a profound effect on resting metabolism and suggests that the drug is not significantly up-regulating catabolic pathways (e.g., fatty acid metabolism, glycolytic flux, etc). To further evaluate potential metabolic effects, exposure of the mice to a thermogenic atmosphere of heliox (79% helium / 21% oxygen) was undertaken to assess possible drug-induced variations in maximal metabolic rate. By challenging the animal to maintain body temperature in the face of significant and profound heat loss, both through decreased ambient temperature and increased conductive heat loss (heliox causes a six fold increase in heat conductance when compared to air), we were able to address the possibility that FM-VP4 up-regulates thermogenic pathways (Selman et al., 2001). All animals maintained a similar level of oxygen consumption when placed in a heliox environment, suggesting that alteration of metabolism at the biochemical level, if present, is not significant enough to alter metabolism at the whole animal level. Interestingly, in both the HF and LF groups, we found a significant increase in the maximal oxygen consumption in animals on 2% FM-VP4 during the swim challenge, indicating a greater aerobic capacity and suggesting an elevation of fitness at the organismal level. We can obtain an estimate of aerobic scope (an indicator of overall fitness) by evaluating the range of metabolic capacity (VO_{2swim} – RMR). Our data show that FM-VP4 treatment at 2% results in an elevation of metabolic scope when compared to control animals. The fact that animals in the FM-VP4 2% group exhibit elevated VO_{2swim} values, but show no difference in $VO_{2heliox}$ when compared to control suggest that the treatment is less likely to be affecting metabolism at the biochemical level, but instead affects factors influencing oxygen consumption at a systemic level (i.e., circulation, heart function, muscle performance, etc). A general increase in aerobic fitness is expected with decreased body fat; however, animals in the HF 2% FM-VP4 group exhibit a higher metabolic scope than animals of comparable mass in the LF group, suggesting that the increase in relative fitness is not solely attributable to a decrease in adipose stores.

Fecal fat analysis indicates that administration of FM-VP4 results in an increased excretion of cholesterol and fatty acid. At this point, we are unable to ascertain whether FM-VP4 causes an increase in fecal fat content through an inhibition of absorption, or by an elevation of efflux mechanisms; however, the net effect of FM-VP4 administration is a reduction in overall cholesterol and NEFA levels within the organism. The decreased fat retention does not appear to affect the general health and development of the animal, but instead leads to a reduction in caloric intake and results in decreased fat accumulation and provides evidence for the possible mechanism responsible for adipose tissue loss.

Previously Obese Mice

We also examined whether 2% FM-VP4 would have a mass-reduction effect on previously obese mice. Administration of the drug to obese mice for 60 days resulted in a significant decrease in overall mass, with a 41% decline in total body fat. Similar to animals in the dose-dependent FM-VP4 study, we found no adverse effects of 2% FM-VP4 administration on organ mass, skeletal development, muscle mass or general health, but observed a significant increase in aerobic fitness, as evidenced by elevation of VO_{2swim} data (Figure 10). Administration of the drug caused an immediate decrease in mass of animals fed the HF diet, which in effect turned previously obese mice into a LF diet mouse phenotype (Figure 11).

In conclusion, our findings suggest that FM-VP4 treatment decreases cholesterol and FFA uptake, likely across the epithelium, and leads to decreased adipose tissue accumulation without any apparent adverse effects on development, organ size, muscle mass or metabolic rate. These findings suggest that further investigation into the use of this compound for the treatment of obesity is warranted.

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