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Diets enriched in *trans*-11 vaccenic acid alleviate ectopic lipid accumulation in a rat model of NAFLD and metabolic syndrome☆,☆☆

M. Miriam Jacome-Sosa[a], Faye Borthwick[a], Rabban Mangat[a], Richard Uwiera[b], Martin J. Reaney[c], Jianheng Shen[c], Ariel D. Quiroga[d], René L. Jacobs[a], Richard Lehner[d], Spencer D. Proctor[a,*]

[a]*Metabolic and Cardiovascular Disease Laboratory, Group on Molecular and Cell Biology of Lipids, Alberta Diabetes and Mazankowski Heart Institutes, University of Alberta, Edmonton, AB, Canada*
[b]*Department of Agricultural Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada*
[c]*Department of Plant Science, University of Saskatchewan, Saskatoon, SK, Canada*
[d]*Department of Pediatrics, Group on Molecular and Cell Biology of Lipids, University of Alberta, AB, Canada*

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Abstract

*Trans*11-18:1 (vaccenic acid, VA) is one of the most predominant naturally occurring *trans* fats in our food chain and has recently been shown to exert hypolipidemic effects in animal models. In this study, we reveal new mechanism(s) by which VA can alter body fat distribution, energy utilization and dysfunctional lipid metabolism in an animal model of obesity displaying features of the metabolic syndrome (MetS). Obese JCR:LA-*cp* rats were assigned to a control diet that included dairy-derived fat or the control diet supplemented with 1% VA. VA reduced total body fat (−6%), stimulated adipose tissue redistribution [reduced mesenteric fat (−17%) while increasing inguinal fat mass (29%)] and decreased adipocyte size (−44%) *versus* control rats. VA supplementation also increased metabolic rate (7%) concomitantly with an increased preference for whole-body glucose utilization for oxidation and increased insulin sensitivity [lower HOMA-IR (−59%)]. Further, VA decreased nonalcoholic fatty liver disease activity scores (−34%) and reduced hepatic (−27%) and intestinal (−39%) triglyceride secretion relative to control diet, while exerting differential transcriptional regulation of SREBP1 and FAS amongst other key genes in the liver and the intestine. Adding VA to dairy fat alleviates features of MetS potentially by remodeling adipose tissue and attenuating ectopic lipid accumulation in a rat model of obesity and MetS. Increasing VA content in the diet (naturally or by fortification) may be a useful approach to maximize the health value of dairy-derived fats.

Keywords: Fat redistribution; Energy expenditure; Insulin resistance; Triglyceride secretion; Saturated fat

1. Introduction

Trans-11 vaccenic acid (VA) is one of the most predominant *trans* fatty acid (TFA) in the food chain, accounting for up to 70% of total TFA in ruminant-derived fats [1,2]. It is produced naturally by bacterial biohydrogenation of dietary unsaturated fatty acids in ruminant animals and is found in products such as beef, lamb and dairy. VA is also the precursor to endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in animals and humans [3]. Thus, VA can be endogenously converted to CLA with purported health benefits in animal models and humans [4,5]. However, in addition to being acknowledged as a potential source of CLA, there remains a void of information regarding the independent bioactivity of VA *per se*.

Studies using VA-enriched butter in different animal models of cardiovascular disease (CVD) were the first to provide insights into VA's hypolipidemic properties [6–8]. There is now accumulating evidence that suggests dietary supplementation of purified synthetic VA can improve fasting and postprandial dyslipidemia and the proinflammatory state in animal models of CVD and the metabolic syndrome (MetS) [9–13]. Most recently, it has been demonstrated that synthetic VA is a partial agonist to peroxisome proliferator activated receptors (PPAR-α and -γ) *in vitro*, acting with similar affinity to PPAR agonists commonly used for the treatment of dyslipidemia and type 2 diabetes [14]. Despite this proposed mechanism of action, putative benefits of VA to insulin resistance and energy use and storage remain unclear.

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☆☆ All authors have declared no conflict of interest.

* Corresponding author. 4-002J Li Ka Shing Centre for Health Research Innovation, University of Alberta, Edmonton, AB, T6G 2E1, Canada. Tel.: +1 780 492 4672; fax: +1 780 492 9270.

E-mail address: spencer.proctor@ualberta.ca (S.D. Proctor).

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Dairy products are the predominant (natural) source of VA in the food chain, yet conversely are also characterized for containing a high proportion of select saturated fatty acids (SFA) including myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acid, as well as a high proportion of the monounsaturated fatty acid oleic acid (OA) (C18:1). While contentious, SFA have historically been associated with negative effects on health. For instance, high SFA intake is associated with insulin resistance and postprandial lipemia in normolipidemic patients with nonalcoholic steatohepatitis [15]. The literature also documents that replacing SFA (specifically palmitic acid) with polyunsaturated fatty acids (PUFA) [16] or low-fat dairy products [17] lowers low-density lipoprotein/high-density lipoprotein (HDL) cholesterol and total/HDL cholesterol ratios. Given the recent evidence for the health effects of VA, the logical approach would be to produce VA-enriched animal-derived foods that could provide an additional health benefit. However, to date, the literature has only documented effects of purified synthetic VA on a vegetable oil background diet. Therefore, in this study, we investigated whether supplementation of dairy fat with synthetic VA (fortification) could increase the putative health value of dairy-derived SFA specifically during conditions of MetS. Additionally, we used an integrative physiological approach to determine the effects of VA-supplemented dairy fat on adipose tissue distribution, energy utilization and intestinal and hepatic lipid homeostasis in a rodent model of obesity that displays features of both nonalcoholic fatty liver disease (NAFLD) and the MetS, the JCR:LA-*cp* rat.

2. Methods and materials

2.1. Animals and diets

Rats of the JCR:LA-*cp* strain that are homozygous for the corpulent trait (*cp*/*cp*) have a complete absence of the leptin receptor in the plasma membrane and spontaneously develop symptoms associated with the MetS and the prediabetic state typically observed in humans, including obesity, insulin resistance and dyslipidemia [18]. Heterozygous rats (+/?) from the same strain are lean and metabolically normal and were used as controls. Male obese JCR:LA-*cp* rats were raised in our established breeding colony at the University of Alberta as previously described [19]. At 8 weeks (wk) of age, rats were randomized and assigned to either control diet (obese control group) or control diet supplemented with VA (obese VA group) for 8 wk and had free access to food and water. Age- and weight-matched lean littermates were fed the control diet to represent normal metabolic conditions. Food consumption and body weight (BW) were monitored weekly throughout the study. All tests were done on the same batch of animals except for the *in vivo* hepatic and intestinal triglyceride (TG) secretion experiments, which were performed on a separate group of animals.

The control diet was prepared by adding 1% w/w cholesterol and 15% w/w of fat to an 85% basal mix diet (Harlan laboratories, TD.06206) and contained 42% of energy from carbohydrate, 23.7% of energy from protein and 34.3% of energy from fat. The fat portion of the diet was adjusted by using predominantly SFA from dairy fat (Supplemental Table 1), thus reflecting the typical composition of a Western diet. The amount of cholesterol in the diet was chosen based on previous published studies [9,10], allowing for direct comparison of dietary effects while altering the source of fat in the diet. The VA diet was prepared by adjusting the fatty acid composition (replacing OA by VA) of the control diet to provide 1.0% w/w of VA. Both diets had a constant PUFA to SFA ratio of 0.4 and a constant n6 to n3 PUFA ratio of 8. Purified VA was synthesized by chemical alkali isomerization from linoleic-acid-rich vegetable oil [20]. Fatty acid composition of diets was confirmed by gas chromatograph analysis [2] of the fat blend samples (Supplemental Table 2). Animal care and experimental procedures were

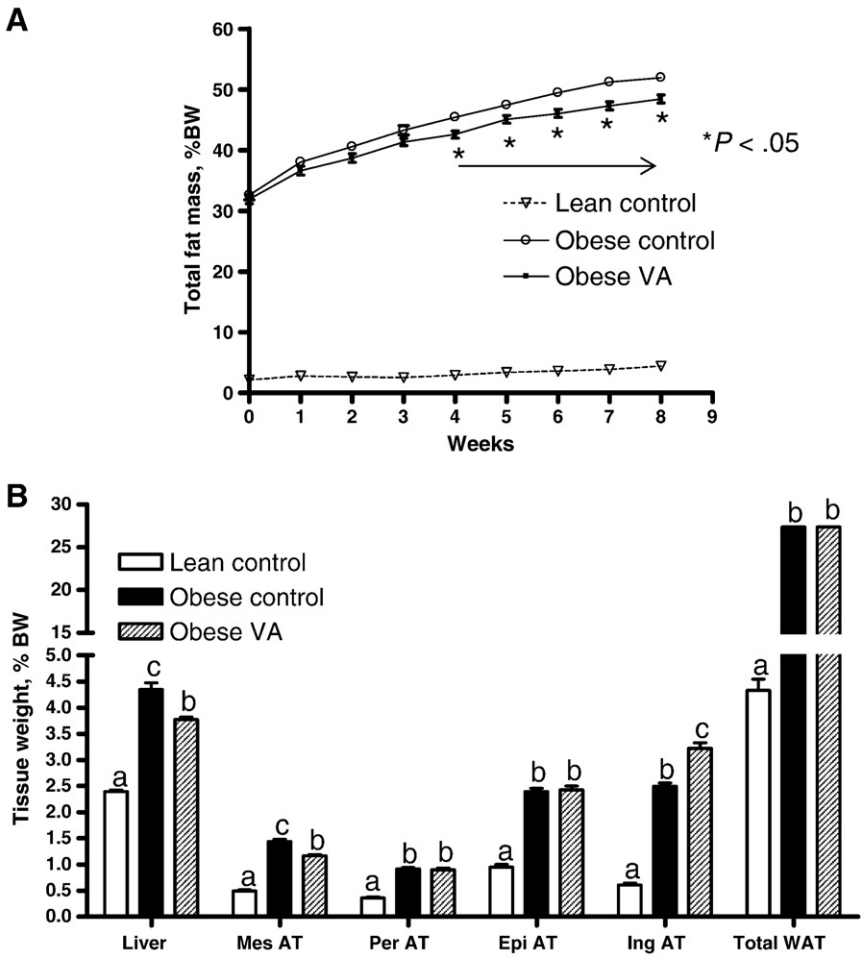

Fig. 1. Body composition of lean (open squares), obese control (open circles) and obese VA rats (black circles). (A) Total fat mass assessed by NMR (% BW) throughout the study and (B) liver and adipose tissue weight assessed during sacrifice. Values are means, with standard errors (S.E.M.) represented by vertical bars (n=8). *Total fat mass of obese VA rats was significantly reduced compared with that of obese control rats after 4 wk of feeding, as indicated by the arrow (P<.05). Means without a common letter differ (P<.05) as assessed by one-way ANOVA followed by Tukey's *post hoc* test. Mes, mesenteric; Per, perirenal; Epi, epididymal; Ing, inguinal; WAT, white adipose tissue.

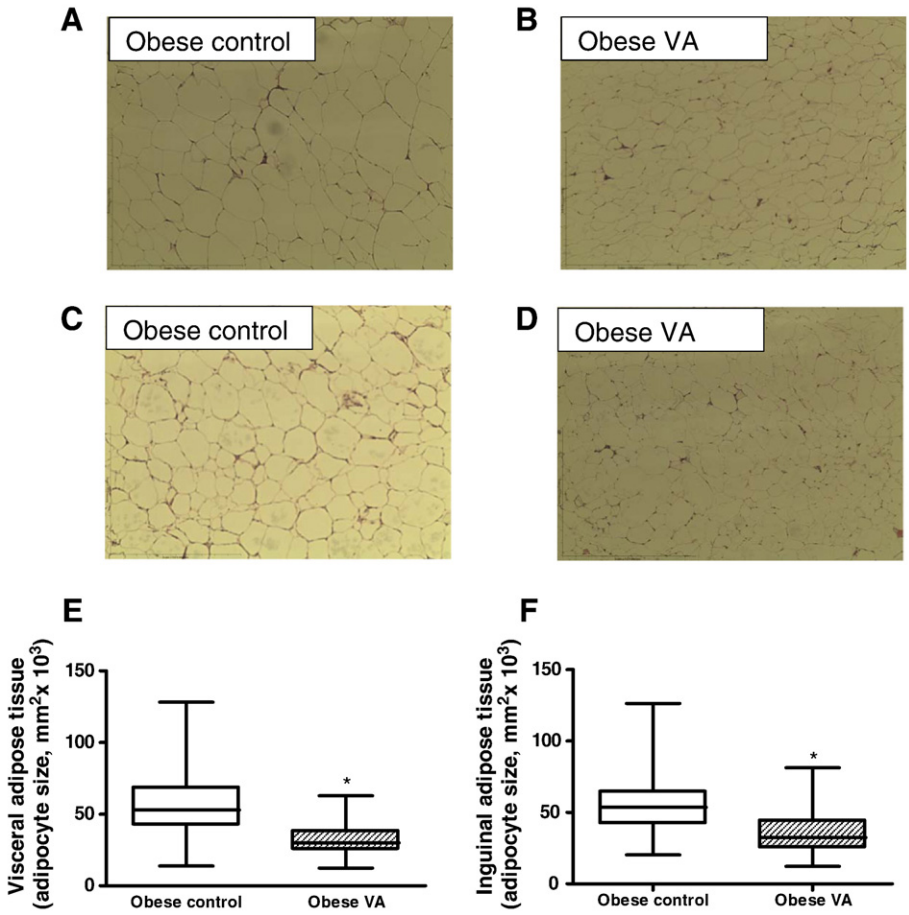

Fig. 2. Representative images of visceral (A and B) and inguinal (C and D) adipocytes (magnification 10×) and the respective adipocyte size distributions (E and F) of obese control and obese VA rats (n=5). Mean adipocyte size values (μm^2) of obese VA group differ relative to those of obese control rats (*P<.001) as assessed by two-tailed, unpaired Student's t test and a nonparametric test (Mann–Whitney test). In panels (E) and (F), error bars denote maximum and minimum of size distribution.

conducted in accordance with the Canadian Council on Animal care and approved by the University of Alberta Animal Care and Use Committee — Livestock.

2.2. Body composition analysis

Body composition was assessed noninvasively at baseline and every subsequent wk by nuclear magnetic resonance (NMR) for small animals (Minispec LF90 Body Composition Analyzer; Bruker, Germany). Body weight was assessed prior to measurements to calculate fat mass percentage. After sacrifice, visceral and subcutaneous adipose tissue depots were excised and weighed to determine body fat distribution.

2.3. Adipocyte size of visceral and inguinal depots

Sections (5 μm) were prepared from paraffin-embedded fat pads of obese control and obese VA rats (n=5) and subjected to hematoxylin and eosin (H&E) staining using standard procedures. Histology sections were viewed at 10× magnification, and images were obtained with a Westover Digital AMID-1 [D] microscope. The cell area (μm^2) of 130 adipocytes per rat was measured with the open-source image analysis software (ImageJ, National Institutes of Health, http://rsbweb.nih.gov/ij).

2.4. Adipose tissue culture and measurement of mRNA expression of PPARγ and associated target genes

We assessed mRNA expression of PPARγ and CD36 in adipose tissue collected during fasting conditions after chronic ingestion of VA, and we did not observe statistically significant differences relative to obese rats fed control diet (Supplemental Fig. 1). To further verify the potential for VA to acutely stimulate PPAR pathways in adipose tissue, we measured the expression of PPARγ and target genes in cultured adipose tissue. Adipose tissue samples from obese JCR:LA-*cp* rats were immediately placed in serum-free medium 199 (M199; Gibco, Invitrogen) [21]. Fragments (~2 mm^3) of adipose tissue were incubated in M199, and 24 h later, media were replaced with M199 media enriched with either OA or VA complexed with bovine serum albumin (250 μM) for 48 h, as previously described [22]. Total RNA was isolated from cultured adipose tissue and reversed transcribed into complementary DNA. The expression of PPARγ and downstream targets relative to the housekeeping gene *Actb* (β-actin) was assessed by quantitative real-time polymerase chain reaction (PCR) using SYBR Green (Applied Biosystems). Data were expressed as a fold change in mRNA expression relative to control (OA) $^{(2-\Delta\Delta Ct)}$.

2.5. Assessment of energy expenditure by indirect calorimetry

Oxygen consumption (VO_2) and respiratory exchange ratio (RER) were measured by indirect calorimetry in rats at the end of the study. Rats were fed *ad libitum* the control and experimental diets and were maintained under 12-h light and dark cycles beginning at 6:00 p.m. and 6:00 a.m., respectively. Rats were acclimatized for 24 h before recording, and measurements of VO_2 were recorded every 14 min over 24 h using the Oxymax System (Columbus Instruments, Columbus, OH, USA). To adjust for metabolic body size, VO_2 was then expressed in ml/h/kg of $BW^{0.75}$. The RER equals volumes of CO_2 released by volumes of O_2 consumed.

2.6. Meal tolerance test (MTT) and the postprandial response of insulin and glucose

Insulin and glucose concentrations were measured in conscious, unrestrained rats after consuming a standard test meal (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA) in order to mimic an oral glucose tolerance test in humans as described previously [23]. Briefly, 0.5 ml of blood was taken from the tip of the tail as t=0 min after an overnight fasting. Rats (n=6) were then given 5 g of the test meal. Timing was started when 50% of the test meal had been consumed, and two additional samples of blood were taken at 30 and 60 min following the initial consumption of the food pellet. The homeostasis model assessment — insulin resistance (HOMA-IR) index was used as an estimate of insulin resistance. The HOMA-IR index was calculated as fasting insulin (μU/ml)×fasting glucose concentrations (mg/dl)/405.

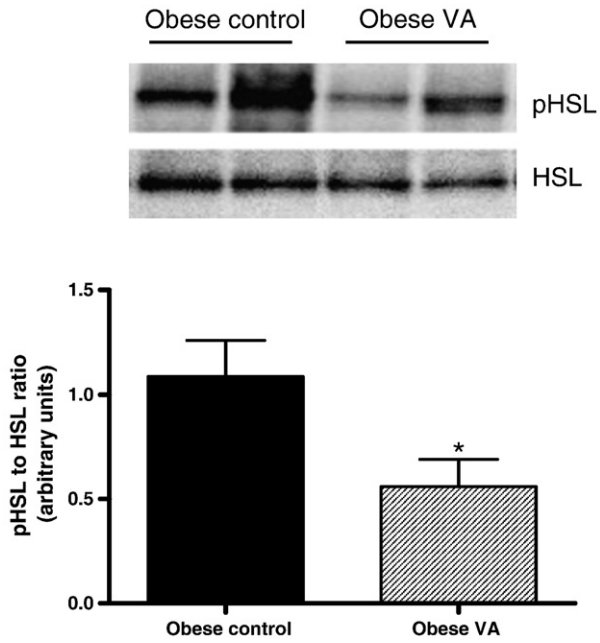

Fig. 3. Protein abundance of the active form of HSL (pHSL) was assessed via immunoblot analysis. pHSL protein was measured in the adipose tissue of obese control and obese VA groups and expressed as a ratio of pHSL to total HSL. A representative blot and graph, presented as a measure of arbitrary units, are shown. Values are means, with standard errors (S.E.M.) represented by vertical bars (n=5). Mean values of the obese VA group were significantly different (*P<.05) from those of the obese control group as assessed by two-tailed, unpaired Student's t tests.

2.7. Liver histology

Sections (5 μm) were prepared from paraffin-embedded liver portions and subjected to H&E staining using standard procedures. Slides from lean, obese control and obese VA rats (n=5–6) were blindly scored for steatosis, hepatocellular ballooning and portal and lobular inflammation by an independent veterinary pathologist as previously described [24]. The NAFLD activity score was determined by adding the scores for steatosis, ballooning and lobular inflammation and was used to assess the progression of NAFLD [25,26].

2.8. Tissue homogenization

Adipose tissue, intestine and liver samples were harvested after an overnight fast (16 h). Samples of the small intestine were washed with phosphate-buffered saline, and a 2-cm section from the middle of jejunum was cut. Samples (0.25 g) were homogenized in 500 μl of homogenization buffer [10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 1% protease inhibitor cocktail (P8340, Sigma Aldrich)]. Protein concentration of the homogenate was measured colorimetrically with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

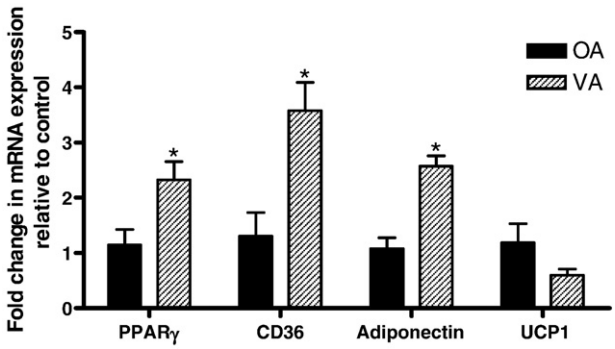

Fig. 4. Effect of VA on the mRNA expression of PPARγ and its target genes in cultured adipose tissue from JCR:LA-cp rats (n=6 replicates). Means differ (*P<.05) relative to those of control (OA) as assessed by two-tailed, unpaired Student's t test.

2.9. Quantification of phosphorylated hormone-sensitive lipase (pHSL) protein

Phosphorylation of HSL Ser^{660} was detected in adipose tissue homogenates by Western blotting. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 3%–8% Tris-acetate polyacrylamide gels (Invitrogen), transferred to a polyvinylidene fluoride membrane and incubated with an anti-pHSL (Ser 660) rabbit polyclonal (1:2000, catalogue no. 4126, Cell Signaling Technology) antibody. Detection was achieved using an anti-rabbit secondary antibody and the ECL advance detection system (Amersham Biosciences). Equal loading of total HSL was confirmed using an anti-HSL rabbit polyclonal (1:1000, catalogue no. 4107, Cell Signaling Technology) antibody. Results are expressed as a ratio of pHSL to total HSL protein.

2.10. Quantification of intestinal and hepatic lipids

The mass of intestinal and hepatic TG and cholesteryl esters (CE) was determined by gas chromatography as described previously [27] in tissues collected after an overnight fast. Briefly, lipids from tissue homogenate (0.5 mg protein) were extracted by the Folch method [28] in the presence of tridecanoylglycerol as the internal standard. Extracted lipid samples were derivatized with Sylon BFT (Supelco, Bellefonte, PA, USA) and analyzed by gas chromatography (6890 Series equipped with a flame ionization detector; Agilent Technologies, Palo Alto, CA, USA). The concentration of each lipid class was adjusted by the protein concentration of the tissue homogenate.

2.11. Hepatic and intestinal gene expression

An array of target genes (n=44) involved in lipid synthesis, oxidation and transport was measured by performing real-time PCR using a quantitative 'high-throughput' method [29] in tissues harvested following an overnight fast.

Total RNA was isolated from frozen segments of both the liver and intestine using TRIzol (Invitrogen, Canada) as described in the manufacturer's protocol and reversed transcribed into cDNA. Forty-eight gene assays (44 target genes+4 housekeeping genes) and cDNA samples were loaded into separate wells on a 48.48 format Chip gene expression assay (Fluidigm). Target gene expression was detected by quantitative PCR (40 amplification cycles) using the Fluidigm Biomark system. Relative mRNA expression for each target gene was normalized to the housekeeping genes, β-actin (Actb) and 36b4 (Arbp) and quantified using the comparative cycle threshold (Ct) method. Data were expressed as a fold change in mRNA expression relative to obese control rats $^{(2-\Delta\Delta Ct)}$. All assays were performed in triplicate.

2.12. Assessment of hepatic and intestinal TG secretion in vivo

Hepatic and intestinal TG secretion *in vivo* was conducted in a separate group of rats. Hydrolysis of plasma lipoprotein TG was inhibited by an intraperitoneal injection of poloxamer-407 (P-407) in fasted rats (14-h overnight fast). P-407 is a nonionic detergent that inhibits mainly lipoprotein lipase, reducing the enzymatic activity by 95% at 3 h following administration [30]. For the hepatic TG secretion study, blood samples were collected from the tip of the tail and plasma was obtained before (0 h) and 1, 2, 3 and 4 h after the P-407 injection. An aliquot of plasma was frozen for assessing TG by a commercially available colorimetric method (Wako Chemicals USA, Inc.).

In the case of the intestinal TG secretion study, following the P-407 injection (30 min), rats were orally gavaged with 10 μCi [^{3}H] triolein in 500 μl of olive oil. Blood samples were then collected and plasma was obtained at 0 h (before P-407 administration) and 1, 2, 3 and 4 h after the oral gavage. An aliquot of plasma was frozen for assessing radioactivity by liquid scintillation counting as described below.

2.13. Plasma lipid extraction and radioactivity counting

Total lipids were extracted from plasma samples by the Folch method and separated by thin layer chromatography using heptane/isopropyl ether/acetic acid (60:40:4, by vol) to separate neutral lipids as described previously [31]. The lipid classes were visualized by exposure to iodine vapor, the bands were scraped, and the associated radioactivity was determined by liquid scintillation counting. Radioactive counts in TG were expressed as dpm per milliliter of plasma.

2.14. Plasma biochemical assays

The concentrations of select biochemical variables in fasting plasma from lean and obese groups were assessed using commercially available enzymatic colorimetric assays. TG and free fatty acids (FFA) (Wako Chemicals USA, Inc.) were measured using direct colorimetric chemical enzymatic reactions. Plasma insulin [Insulin (Rat) Ultrasensitive EIA, AIPCO Immunoassays, 80 INSRTU-E01] was determined using commercially available enzymatic immunoassays for rodents, and plasma glucose was measured as per the glucose oxidase/peroxidase method (Genzyme Diagnostics P.E.I. Inc.). Samples were analyzed in triplicate using assay kits from a single lot and performed in one batch.

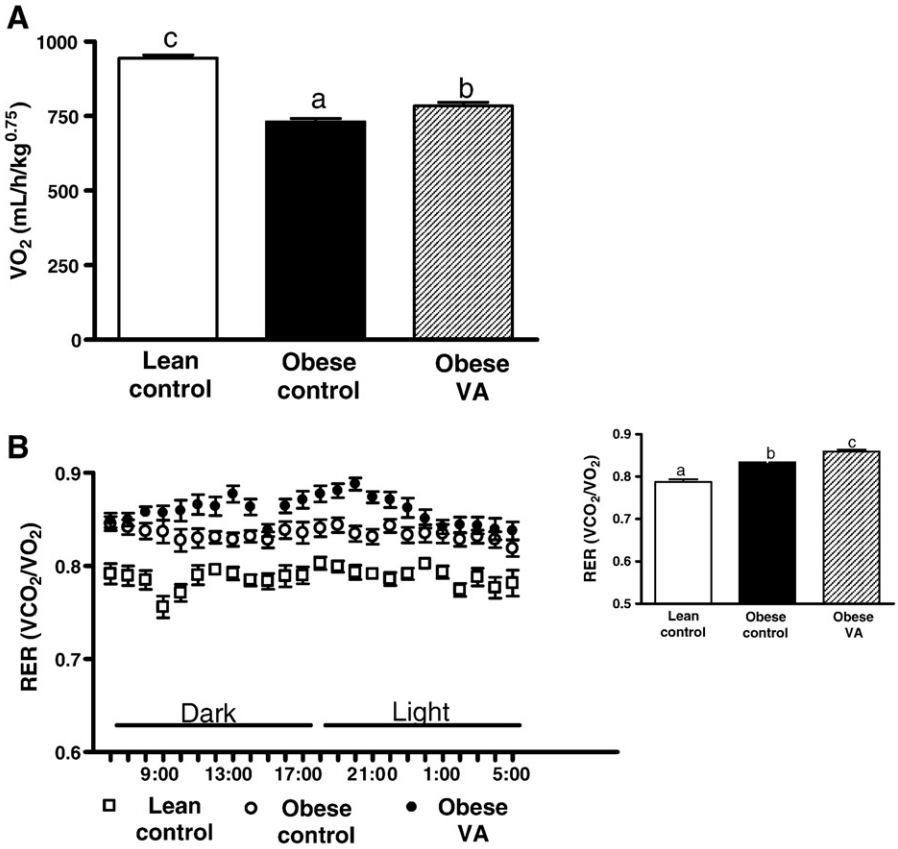

Fig. 5. Oxygen consumption (A) and RER (B) of lean (open squares), obese control (open circles) and obese VA rats (black circles) with inset showing means ±S.E.M. over a 24-h period (n=8). Means without a common letter differ (P<.05) as assessed by one-way ANOVA followed by Tukey's *post hoc* test.

2.15. Statistical analysis

All results are expressed as mean±S.E.M. Statistical comparisons between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test when experiments included more than two groups (Graph Pad Prism 5.0, USA). Changes in plasma TG concentrations over time (*in vivo* secretion studies) were analyzed via two-way ANOVA (variables: treatment and time). Two-way ANOVA and two-tailed, unpaired Student's *t* tests were used as indicated. Postprandial glucose and insulin metabolism were assessed by area under the curve (AUC) analysis. Fasting concentrations of these parameters were further subtracted from the total AUC to yield the incremental area under the curve (iAUC). The level of significance was set at P<.05.

3. Results

3.1. VA decreases total body fat mass and favorably alters fat distribution and adipocyte size

In the present study, enrichment of dairy fat with VA did not affect food intake or BW relative to control diet in obese JCR:LA-*cp* rats (Supplemental Fig. 2). However, body composition analysis by NMR revealed that obese VA rats had a modest but significant reduction in total body fat mass (6%, P<.01) relative to obese control rats after 4 wk of feeding (Fig. 1A). Furthermore, VA supplementation resulted in changes in body fat distribution as assessed by dissection of truncal fat depots during sacrifice (Fig. 1B). Obese VA rats had lower mesenteric (17%, P<.001) but higher inguinal fat mass (29%, P<.001) relative to obese control rats. In addition to this selective adipose redistribution effect, VA reduced adipocyte size in both visceral and inguinal fat pads by 44% and 37%, respectively, relative to control diet (P<.001, Fig. 2). Interestingly, total white adipose tissue (WAT) was not different (P>.05) between obese groups. VA was also observed to reduce liver weight (13.2%, P<.001) as compared to control diet.

3.2. VA decreases the active form of HSL in adipose tissue

The protein expression of pHSL, the active form of the rate-limiting enzyme in adipose tissue lipolysis, was significantly reduced (25%, P<.01) in the adipose of obese VA rats relative to obese controls (Fig. 3).

3.3. VA up-regulates mRNA expression of PPARγ and its target genes in cultured adipose tissue

To validate PPARγ activation in adipose tissue specifically, we measured the expression of cultured adipose tissue PPARγ, the fatty acid transporter CD36, adiponectin and uncoupling protein (UCP) 1 mRNA (Fig. 4). As expected, the mRNA levels of PPARγ (2.3-fold, P<.05), CD36 (3.5-fold, P<.01) and adiponectin (2.6-fold, P<.001) were significantly up-regulated in adipose tissue treated with VA compared to control adipose tissue incubated with OA for 48 h. The mRNA expression of the marker of adipose browning, UCP1, did not differ (P>.05) relative to control.

3.4. Enrichment of dairy fat with VA increases energy expenditure

Total energy expenditure was measured by indirect calorimetry at the end of the study to determine whether decreased total fat mass by VA was associated with increased metabolic rate. Results confirmed increased total energy expenditure in obese VA rats relative to obese control rats, as measured by oxygen consumption (Fig. 5A). This modest increase in oxygen consumption cannot be explained by lean mass which did not differ between obese control and obese VA rats (Supplemental Fig. 3). Interestingly, increased metabolic rate in obese VA rats was concomitant with higher RER (Fig. 5B).

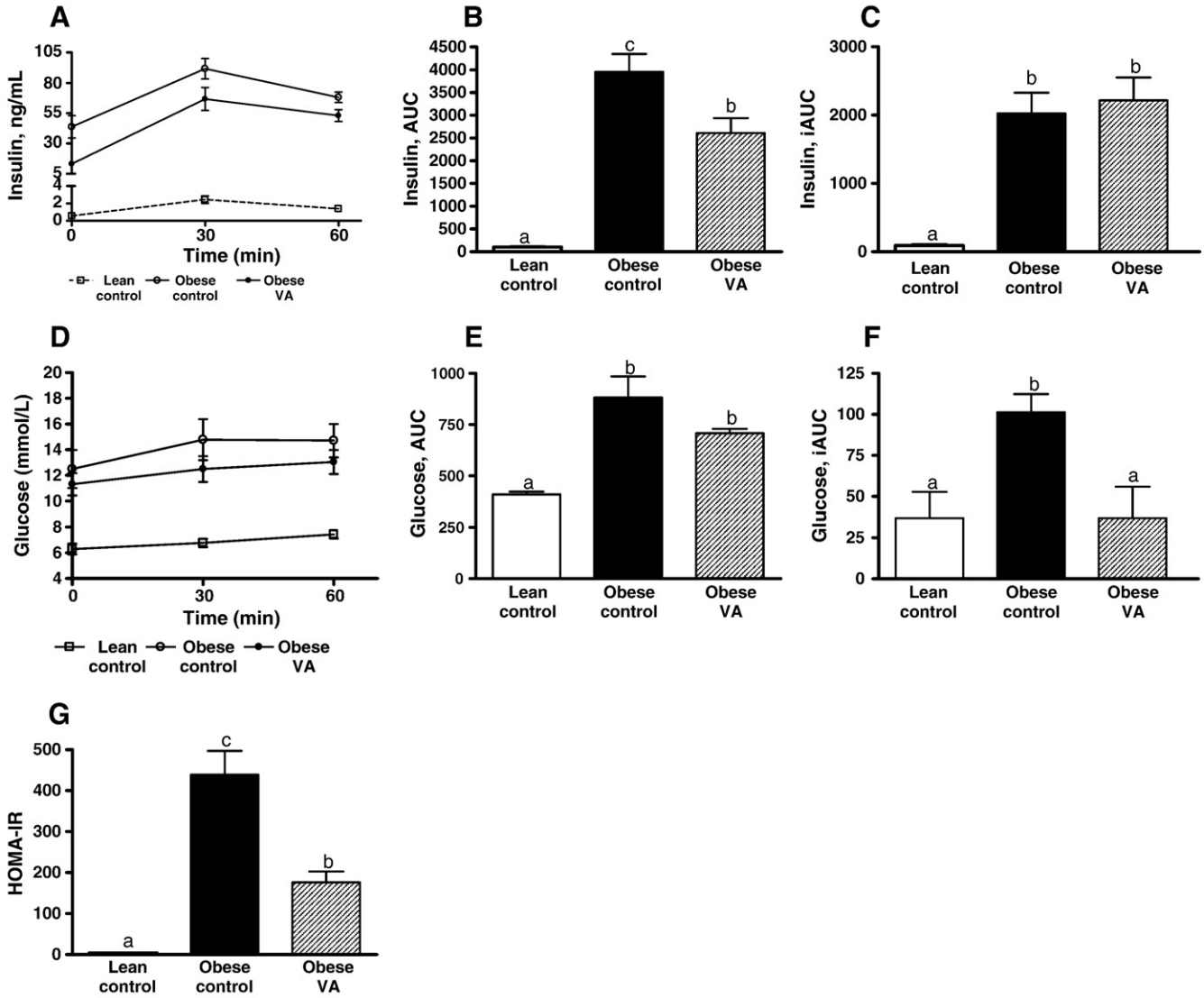

Fig. 6. Postprandial insulin (A) and glucose (D) responses after an MTT of lean (open squares), obese control (open circles) and obese VA rats (black circles) and their corresponding AUC and iAUC. The HOMA-IR index (G) was calculated as fasting insulin (μU/ml)×fasting glucose concentrations (mg/dl)/405. Obese VA rats had lower fasting insulin concentrations (A; $P<.001$). Values are means, with standard errors (S.E.M.) represented by vertical bars ($n=5$). Means without a common letter differ ($P<.05$) as assessed by one-way ANOVA followed by Tukey's *post hoc* test.

3.5. VA decreases fasting insulin and improves glucose response after a meal

Previous studies using the JCR:LA-*cp* rat have shown no effect of VA on insulin or glucose metabolism [9,10]. In the present study, enrichment of dairy fat with VA in obese rats resulted in a significant reduction in fasting insulin concentrations relative to control diet ($P<.001$) (Fig. 6A). This was consistent with lower total insulin concentrations after the MTT ($P<.05$) as determined by the analysis of total AUC (Fig. 6B).

Postprandial incremental changes in plasma insulin concentrations (iAUC, $P>.05$) (Fig. 6C) or total glucose concentrations (AUC, $P>.05$) (Fig. 6E) did not differ between obese control and obese VA rats. However, obese VA rats had lower postprandial glucose response relative to obese control rats (iAUC, $P<.05$) (Fig. 6D and F). Notably, obese VA rats showed a lower HOMA-IR index relative to obese control rats (Fig. 6G), suggesting improved whole-body insulin resistance.

3.6. VA attenuates the progression of NAFLD

Enrichment of dairy fat with VA prevented the hepatic accumulation of lipids, an effect that was clearly evident in the H&E-stained histological sections (Fig. 7A). Histopathological analysis revealed that obese VA rats had decreased steatosis (42%, $P<.01$) and NAFLD activity scores (34%, $P<.01$) as compared to obese control rats (Fig. 7B and E). There was no significant difference ($P>.05$) in inflammation or hepatocellular ballooning between obese control and VA fed rats (Fig. 7C and D).

3.7. VA affects intestinal and hepatic lipid deposition, but has a unique opposing effect on the regulation of mRNA expression for select genes

The effect of VA on reducing lipid accumulation in peripheral tissues was confirmed by quantitative analysis of intestinal and hepatic lipids. Results revealed that VA decreased intestinal TG to the level of lean rats ($P>.05$) and by 43% ($P<.05$; two-tailed, unpaired

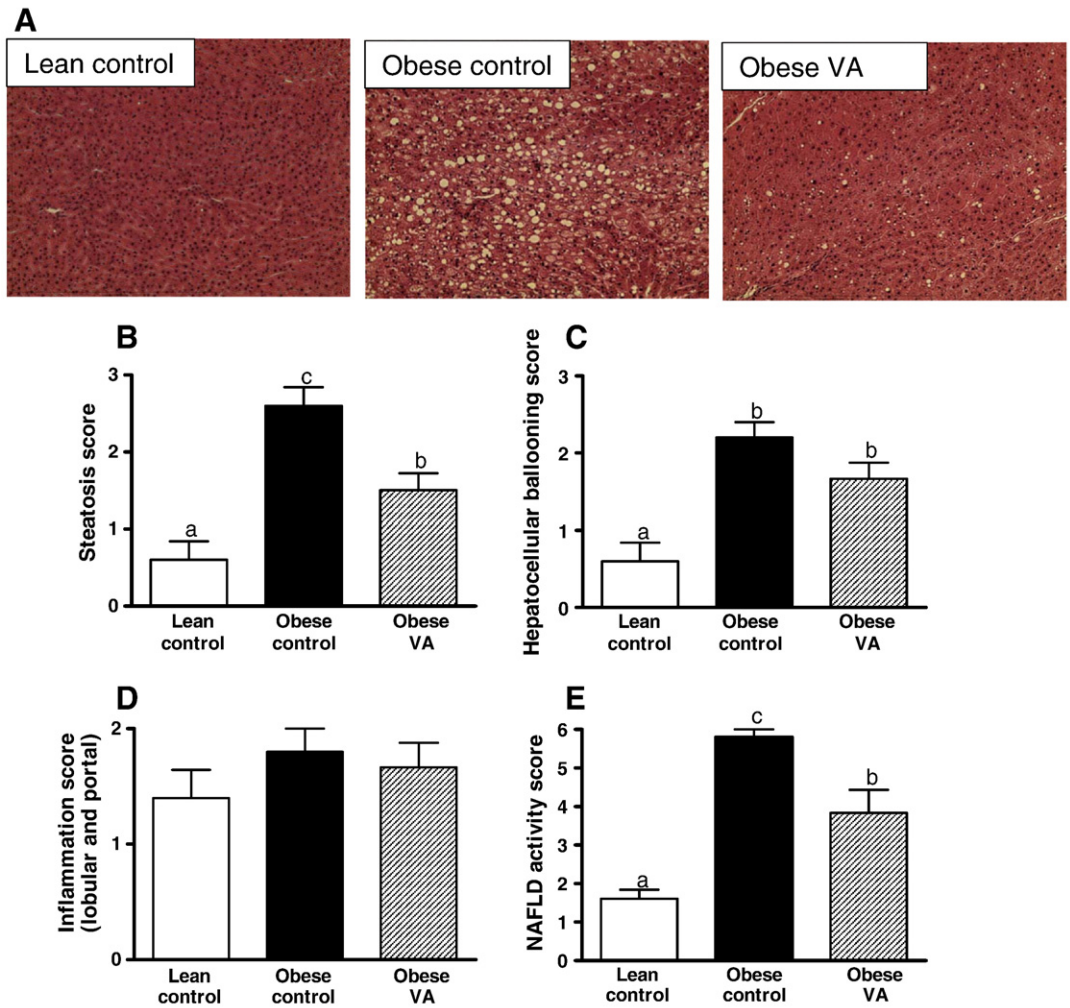

Fig. 7. Progression of NAFLD in lean and obese JCR:LA-*cp* rats fed a control or VA-supplemented diet. Liver sections were stained with H&E and histopathologically graded for (B) steatosis, (C) hepatocellular ballooning and (D) inflammation; (E) NAFLD activity scores; (A) original magnification 20×. Values are means ±S.E.M.; $n=5$. Means without a common letter differ ($P<.05$) as assessed by one-way ANOVA followed by Tukey's *post hoc* test.

Student's *t* test) relative to obese control rats. Hepatic TG and CE were also decreased in obese VA rats (68% and 76.5%, respectively; $P<.001$) relative to obese control rats (Table 1).

Gene expression analysis demonstrated opposing transcriptional regulation by VA in the intestine and the liver (Fig. 8A and B). VA was observed to down-regulate the expression of genes involved in *de novo* lipogenesis, SREBP1 and its downstream target FAS in the intestine — but not in the liver — relative to control diet ($P<.05$). The mRNA expression of genes involved in intestinal/hepatic fatty acid oxidation and transport was not affected by the dietary treatment (Supplemental Table 3 and Table 4), except that for UCP2 which was decreased in the intestine and I-FABP which was increased in the liver of obese VA relative to obese control rats. With regard to the mRNA expression of genes involved in TG synthesis and secretion, VA was observed to up-regulate DGAT2 in the liver relative to control diet ($P<.05$). Interestingly, VA down-regulated the mRNA expression of apoA1, the main apolipoprotein constituent of HDL in the intestine, while up-regulating its expression in the liver as compared to control diet ($P<.05$). Conversely, the expression of SRB1, the receptor that mediates HDL-dependent cholesterol efflux, was higher in the liver and the intestine of obese VA relative to obese control rats ($P<.05$).

Table 1
Intestinal and hepatic lipid composition

	Lean control	Obese control	Obese VA
Intestinal TG (μg/g protein)	93.2 ± 16.2[a]	193.2 ± 43.8[b]	109.7 ± 7.7[ab]
Intestinal CE (μg/g protein)	15.2 ± 0.8	12.6 ± 1.4	16.2 ± 1.1
Hepatic TG (μg/g protein)	47.5 ± 4.0[a]	385.8 ± 44.3[c]	123.3 ± 24.8[b]
Hepatic CE (μg/g protein)	29.0 ± 3.7[a]	145.3 ± 23.1[b]	34.1± 2.1[a]

Values are means ± S.E.M.; $n=6$–8. Means without a common letter differ ($P<.05$) as assessed by one-way ANOVA followed by Tukey's *post hoc* test. Tissues were collected after an overnight fasting (16 h).

3.8. Enrichment of dairy fat with VA significantly reduces hepatic and intestinal TG secretion

VA tended to lower fasting plasma FFA relative to obese control rats; however, this did not reach statistical significance ($P=.08$) (Fig. 9A). Consistent with previous findings [9,10], obese VA rats had lower fasting plasma TG (45%) relative to obese control rats ($P<.05$) (Fig. 9B). Results from the functional secretion studies revealed that obese VA rats had a decreased hepatic (27% lower) and intestinal (39% lower) TG secretion relative to obese control rats postinjection of the lipase inhibitor P-407 ($P<.05$) (Fig. 9C and D). Interestingly, obese VA rats had similar ($P>.05$) intestinal TG secretion relative to lean rats (Fig. 9D).

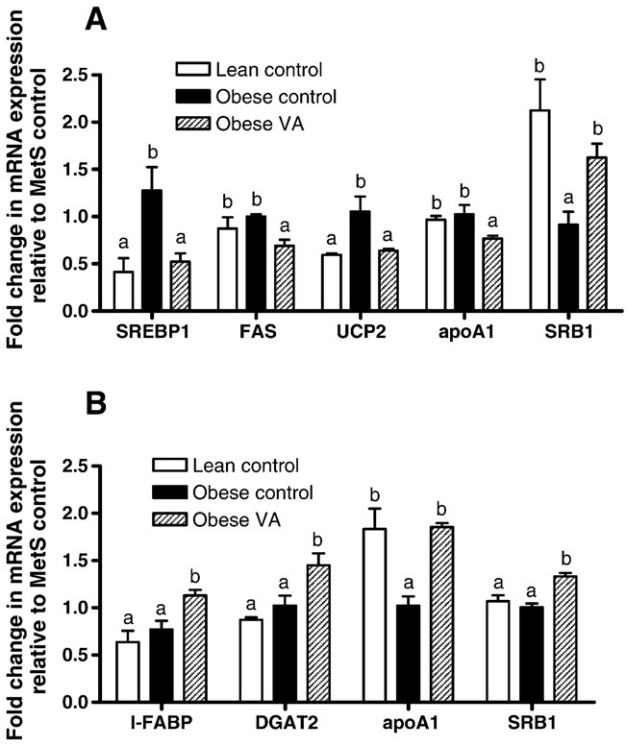

Fig. 8. Intestinal (A) and hepatic (B) mRNA expression of genes involved in lipid synthesis, oxidation and transport that were regulated by VA. Values are means ±S.E.M.; $n=5$. Means without a common letter differ ($P<.05$) as assessed by one-way ANOVA followed by Tukey's *post hoc* test.

4. Discussion

4.1. Improved insulin sensitivity by VA may be associated with body fat redistribution and decreased ectopic fat accumulation

The lipid centric hypothesis of obesity proposes that insulin resistance is causally related to peripheral ectopic fat accumulation and subsequent lipid toxicity [32]. Longstanding evidence also suggests that dietary SFA can cause insulin resistance via pleiotropic mechanisms (reviewed in Refs. [33] and [34]). However, results from the present study demonstrate that VA (supplemented in the FFA form) can favorably attenuate dairy-derived SFA-induced whole-body insulin resistance in the JCR:LA-*cp* rat (Fig. 6). These effects of VA were concomitant with changes in body fat distribution and decreased ectopic lipid accumulation, as demonstrated by measurements of total fat mass by NMR. It is important to note that we did not include a control group fed VA in the TG form in experiments presented in this study. However, additional experiments (Supplemental Fig. 4) support that VA in the TG form has similar bioactivity on improving whole-body insulin resistance, whilst it is less effective at reducing hepatic lipid storage than VA in the FFA form.

Improved whole-body insulin resistance by VA was concomitant with an increase in RER (Fig. 5B), indicating increased whole-body glucose utilization over lipid as a substrate for oxidation for energy production. It is noteworthy that the indirect calorimetric analysis was performed in rats fed *ad libitum,* suggesting increased fuel availability (mainly glucose) in the hyperphagic JCR:LA-*cp* rats. Thus, results indicate an improved (whole-body) capacity to match fuel oxidation to fuel availability following VA supplementation, a condition that is blunted in insulin-resistant and type 2 diabetic subjects [32,35]. Furthermore, enrichment of dairy fat with VA also decreased the active form of HSL in adipose tissue (Fig. 3), a known effect of insulin action [36]. Therefore, it is plausible that an increase in insulin sensitivity by VA prevents activation and subsequent lipolytic activity of HSL. We hypothesize that these insulin-sensitizing effects of VA may be associated with its potential to bind and activate PPARγ-regulated pathways in adipose tissue that we have confirmed *in vitro* in the present study. Indeed, we have previously demonstrated by competitive binding assays that VA has similar affinity to PPARγ relative to the insulin sensitizer pioglitazone [14], a thiazolidinedione class drug that exerts its effects via PPARγ-activated pathways in adipose tissue. However, the mRNA expression of PPARγ and CD36 in the adipose tissue of obese control and VA-fed rats did not differ (Supplemental Fig. 1); this could be attributed to chronic exposure of VA *versus* acute *in vitro* experiments.

Consistent with findings from this study, Mohankumar *et al.* [37] have recently shown that VA reduces adipocyte size in *fa/fa* Zucker rats, which is reminiscent of PPARγ-induced adipose differentiation and improved function [38]. Therefore, it is plausible that activation of PPARγ by VA preferentially in inguinal adipose tissue may ameliorate adipose lipid storage capacity, thereby partitioning lipids from other insulin sensitive tissues (such as the liver).

4.2. Enrichment of dairy fat with VA decreases hepatic and intestinal lipid accumulation and results in opposing transcriptional control in the liver and the intestine

VA has recently been shown to reduce fasting and postprandial triglyceridemia and to lower hepatic TG [9,10,39]. The lipid-lowering properties of VA have been recently attributed to the activation of both PPARα- and PPARγ-regulated pathways [14]; however, putative benefits to insulin resistance and mechanisms of energy use and storage have not been investigated. In this study, we report the beneficial effects of VA on the progression of NAFLD. Specifically, we have demonstrated that enrichment of dairy fat with VA alleviates the putative stimulatory effects of SFA on hepatic steatosis and NAFLD progression (Fig. 7). Considering that diminished adipose tissue storage capacity can contribute to excessive ectopic lipid accumulation [40], it is plausible that the action of VA in adipose tissue via PPARγ may alleviate NAFLD under these dietary conditions.

Hepatic steatosis can also occur as an imbalance between lipid availability (lipid synthesis) and disposal (oxidation of fatty acids or very low density lipoprotein secretion) [41]. We hypothesized that VA may further exert its effects by regulating endogenous synthesis and/or oxidative pathways. However, gene expression analysis revealed that reduced hepatic lipid accumulation by VA could not be explained at the transcriptional level (Fig. 8, Supplemental Table 4). With the exception of the up-regulation of hepatic PPARα targets, apoA1 and SRB1 [42,43], our data suggest that reduced hepatic lipid accumulation by VA is independent of PPARα-mediated lipid oxidation. Interestingly, previous findings have demonstrated lower protein expression of hepatic lipogenic enzymes and up-regulated hepatic citrate synthase activity in JCR:LA-*cp* rats fed VA- [10] or VA/CLA- [39] supplemented diets. We therefore propose that VA may regulate *de novo* lipid synthesis and fatty acid turnover at a functional level but this may result in a compensatory transcriptional response by the liver.

In contrast to our hepatic observations, decreased intestinal TG accumulation by VA can potentially be explained by a lower mRNA expression of SREBP-1 and its lipogenic target gene FAS (Fig. 8). During insulin resistance, SREBP-1 is thought to remain insulin responsive [44], and in the present study, VA improved whole-body insulin resistance (decreased fasting insulin; Fig. 6), which may result in the reduced levels of intestinal SREBP-1 mRNA (Fig. 8). Furthermore, VA also lowered intestinal mRNA expression of UCP2, a mitochondrial uncoupling protein that has also been

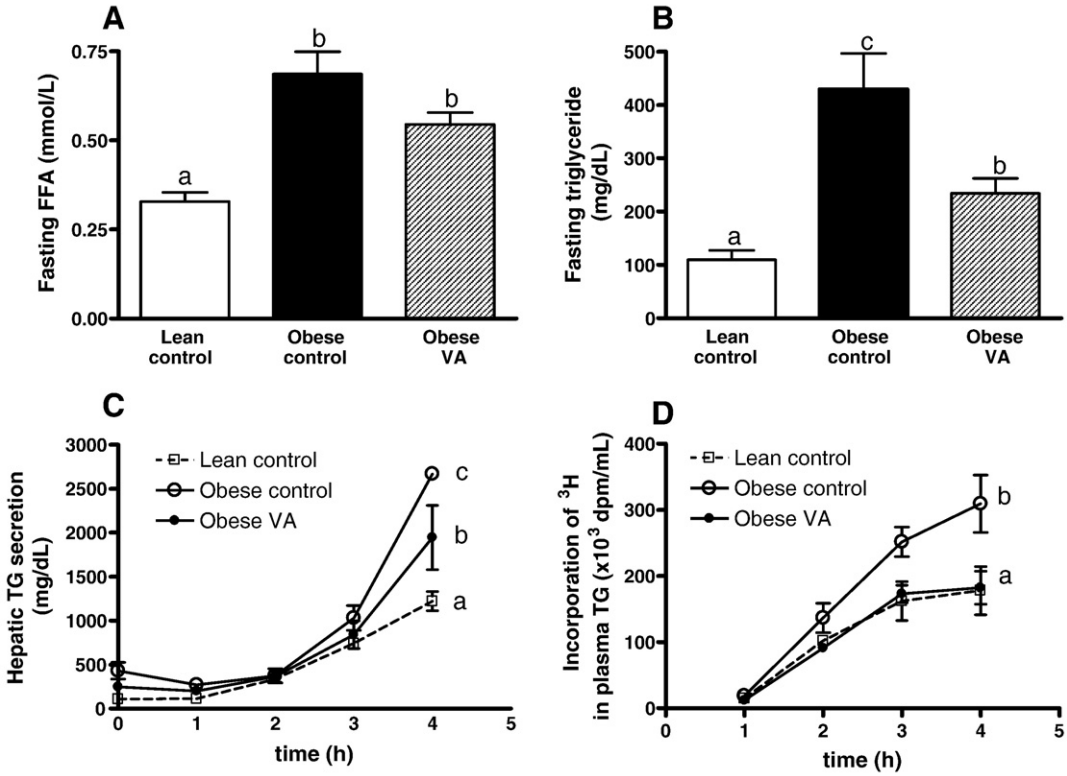

Fig. 9. Fasting lipids (A and B) and TG secretion after injection of the lipase inhibitor (P-407). Differences between groups in the TG secretion *in vivo* study were analyzed using two-way ANOVA followed by Tukey's *post hoc* test. (C) Hepatic TG secretion was assessed during fasting conditions. Means of obese control (open circles) *versus* obese VA (black circles) were significantly different at 4 h after P-407 injection ($P<.05$); (D) intestinal TG secretion was assessed after an oral gavage of [^{3}H] triolein in olive oil following lipase inhibition. Mean values of obese control and obese VA rats differ at 4 h after gavage ($P<.05$). Means of obese VA *versus* lean rats (open squares) did not differ ($P>.05$). Values in the TG secretion *in vivo* study are means ±S.E.M.; $n=3$. Means without a common letter differ ($P<.05$).

shown to be induced by high-fat feeding [45]. Interestingly, in addition to its thermoregulatory uncoupling function, UCP2 has been demonstrated to attenuate oxidative damage [46,47], and its expression is up-regulated during conditions of oxidative stress [48–50]. We speculate that normalization of intestinal UCP2 by VA to the level of healthy lean rats may be an adaptive response to lower high-fat-feeding-induced oxidative stress milieu.

4.3. Enrichment of dairy fat with VA significantly improves intestinal and hepatic TG secretion

VA has been shown to exert lipid-lowering effects without altering body fat distribution or insulin metabolism [9,10,13,39]. In this study, we provide evidence that enrichment of dairy fat with VA improves insulin resistance and reduces TG secretion from both the liver and the intestine (Fig. 9). Specifically, we have shown that VA normalizes intestinal TG secretion to the level of healthy lean control rats. These striking effects of VA on intestinal lipid metabolism could possibly be due to decreased lipid synthesis (by the intestine) and/or intestinal TG accumulation. It is noteworthy that gene expression and intestinal lipid mass were measured in tissue collected after an overnight fast, and this may not necessarily reflect the physiological status in response to a lipid load. However, cumulative evidence suggests that increased intestinal TG accumulation due to impaired intestinal *de novo* lipogenesis is associated with exacerbated lipoprotein synthesis resulting in postprandial dyslipidemia [51–53]. Therefore, the resulting TG peak in response to a meal is largely determined by the metabolic priming of the enterocyte during fasting conditions.

In conclusion, enrichment of dairy fat with VA alleviates exacerbated symptoms of the MetS and the prediabetic state in the JCR:LA-*cp* rat. Specifically, these results suggest that VA can modulate energy utilization and adipose tissue compartments while simultaneously reducing ectopic lipid accumulation. We also demonstrate that VA can re-equilibrate intestinal and hepatic lipid homeostasis while exerting differential transcriptional regulation in both organs. Indeed, we find a disconnect between transcriptional regulation and functional effects of VA in the liver that may be explained at the posttranscriptional level. Collectively, findings from this study provide further insights into the beneficial effects of VA and suggest that enriching dairy products with VA (naturally or by fortification) may be used as an approach to increase health value of dairy-derived fats, especially to those individuals with MetS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2014.02.011.

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