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Lipidomic evidence that lowering the typical dietary palmitate to oleate ratio in humans decreases the leukocyte production of pro-inflammatory cytokines and muscle expression of redox-sensitive genes ^{*}, [†], [‡]

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Abstract

We recently reported that lowering the high, habitual palmitic acid (PA) intake in ovulating women improved insulin sensitivity and both inflammatory and oxidative stress. *In vitro* studies indicate that PA can activate both cell membrane toll-like receptor-4 and the intracellular Nucleotide Oligomerization Domain (Nod)-Like Receptor protein, NLRP3. To gain further insight into the relevance to human metabolic disease of dietary PA, we studied healthy, lean and obese adults, enrolled in a randomized, cross-over trial comparing 3-week, high PA (HPA) and low PA/high oleic acid (HOA) diets. After each diet, both hepatic and peripheral insulin sensitivity were measured, and we assessed cytokine concentrations in plasma and in supernatants derived from

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Authorship

CLK researched the data, contributed to discussion, and wrote the manuscript. JYB, NKF, VA, DEM, REP, and MEP researched the data, contributed to discussion, and wrote the manuscript. KIC, DBE, AND ET researched the data. CLK is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis. No author has a conflict of interest.

Data on the effects of these diets on PBMC secretion of cytokines were presented at EB 2013, April 20, 2013, Boston, MA and published in abstract form The FASEB J 27, 46.2. 2013.

LPS-stimulated peripheral blood mononuclear cells (PBMCs) as well as pro-inflammatory gene expression in skeletal muscle. Insulin sensitivity was unaffected by diet. Plasma concentration of tumor necrosis factor- α was higher during the HPA diet. Lowering the habitually high PA intake by feeding the HOA diet resulted in lower secretion of interleukin (IL)-1 β , IL-18, IL-10, and tumor necrosis factor- α by PBMCs, as well as lower relative mRNA expression of *cJun* and *NLRP3* in muscle. Principal components analysis of 156 total variables coupled to analysis of covariance indicated that the mechanistic pathway for the differential dietary effects on PBMCs involved changes in the PA/OA ratio of tissue lipids. Our results indicate that lowering the dietary and tissue lipid PA/OA ratio resulted in lower leukocyte production of pro-inflammatory cytokines and muscle expression of redox-sensitive genes, but the relevance to diabetes risk is uncertain.

Keywords

fatty acids; palmitic acid; oleic acid; inflammation; innate immunity; cytokines

1. Introduction

The saturated fatty acid (FA), palmitic acid (PA; C16:0), and the monounsaturated FA, oleic acid (OA; C18:1), are the most prevalent FAs in the American diet. Understanding their effects, when fed in amounts comparable to conventional dietary patterns, may contribute to better understanding of human physiology and health and may be important to understanding the etiology of human metabolic disease, including type 2 diabetes, vascular disease, and Alzheimer's Disease [1-6]. Human diets are complex, and reported health benefits of diets rich in OA and low in PA (e.g. Mediterranean Diet) are not solely due to FA intake *per se* [1,7]. However, accumulation in tissues of specific FAs and perhaps lipids such as ceramides derived from them [8] could be an important proximal event to inflammation, which has been linked to the aforementioned diseases [5,9,10]. While gaining insight into optimal diets is important, our work has largely focused on manipulating dietary FA consumption to produce changes in tissue FA composition, in order to ascertain the differential metabolic effects of PA and OA, which are so ubiquitous in the diet and cellular milieu of humans[8].

Studies in cultured cells suggest that exposure to a high PA concentration causes pro-inflammatory signaling events, in part by activating the cell surface pattern recognition receptor, toll-like receptor-4 (TLR4) [9,11], which results in increased transcription of genes including tumor necrosis factor- α (TNF α), pro-interleukin (IL)-1 β , pro-IL-18, IL-6, and Nucleotide Oligomerization Domain (NOD)-Like Receptor Protein (NLRP3). NLRP3 is an intracellular receptor, activated by a variety of stimuli, including PA, ceramide, reactive oxygen species, and amyloid proteins [9]. Activation of NLRP3 causes sequential recruitment of apoptosis-associated speck-like protein and caspase-1 to form the NLRP3 inflammasome complex; multimerization of caspase-1 leads to its autocatalytic cleavage into an active form, which then is liberated from the inflammasome and subsequently cleaves pro-IL-1 β and pro-IL-18 into the secretable, mature IL-1 β and IL-18 capable of activating their respective receptors [9,12,13]. Among the effects of IL-1 β is the activation

of c-Jun N-terminal kinase (JNK) [14]. We [8] have reported that lowering the habitually high intake of PA, associated with the Western Diet, was associated with lower levels of phosphorylated JNK in skeletal muscle and lower serum concentrations of IL-6 and ferritin, results indicative of decreased inflammatory and oxidant stress.

Despite *in vitro* and animal model data predicting that a high PA diet would enhance inflammation, exposure of cells to high concentrations of PA may not reflect normal physiology [15], raising doubts about the clinical relevance of such experiments and mandating translation of these findings to the human under physiological conditions. Therefore, in this study we advanced the hypothesis that greatly reducing the habitual dietary intake of PA in our volunteers by substituting OA [8] would decrease biomarkers indicative of TLR4 and NLRP3 inflammasome activation, as assessed in both LPS-stimulated PBMCs and biopsied skeletal muscle tissue. Activation of JNK by cytokines and/or oxidative stress may lead to phosphorylation of c-Jun and c-Fos, which heterodimerize to form the Activator Protein-1 complex [16]. The Activator Protein-1 complex binds to the promoters of several genes related to cell injury, repair, and inflammation, including *cJun* and *cFos* as well as inflammatory cytokines *IL-8* and *IL-6*. Thus, we assessed muscle expression of *cJun* and *cFos* as biomarkers for enhanced JNK activity in skeletal muscle [8] that may then lead to inflammatory gene expression.

Accelerated FA oxidation during the HPA diet and associated mitochondrial dysfunction (incomplete FA oxidation)[8,17] could enhance reactive oxygen species production and activation of JNK, independent of IL-1 β [18]. Endoplasmic reticulum (ER) stress (unfolded-protein response) also can induce JNK activation [19]. PA may induce ER stress by altering fusion/fission events of membranes, but inhibiting ceramide synthesis from PA partially blocks induction of ER stress [20]. In liver cells, PA induces ER stress resulting in decreased insulin action, but in obese mice, ER stress was not detected in skeletal muscle [21,22]. Other studies suggest that PA but not OA induces ER stress in mouse and human muscle cells [23-25]. However, it also has been reported that PA only weakly induces ER stress in human myotubes [20]. Particularly in view of our previous data demonstrating that feeding a high PA diet increased muscle concentration of ceramide and phosphorylation of JNK [8], we assessed ER stress in skeletal muscle biopsies, using both mRNA and protein expression techniques.

Finally, we assessed peripheral and hepatic insulin sensitivity to determine if effects of dietary FA on innate immunity might alter risks for type 2 diabetes.

2. Materials and Methods

2.1 Subjects, screening, and overall design

This study was approved by institutional committees associated with the University of Vermont Clinical Research Center (CRC). This study has been registered at <http://www.clinicaltrials.gov/> as University of Vermont Protocol Record R01DK082803.

This study was conducted as part of a trial designed to examine the effects of dietary PA on whole body and skeletal muscle PA oxidation, as well as on inflammation, as reported here.

The actual data on PA oxidation are extensive and will be reported elsewhere, but a general outline of these tracer studies are given below, both to convey the timing of the relevant experimental procedures and because the experimental results related to PA oxidation were included in the principal components analysis (PCA) (see below).

This was a double-masked, cross-over study evaluating two diets, one based on the FA composition of the Western Diet and high in PA (HPA) and one reflecting more the Mediterranean Diet FA composition, low in PA and high in OA (HOA)[8].

Figure 1 depicts the consort diagram for this trial. Healthy, lean or obese, but non-diabetic subjects aged 18 - 40 yrs. were recruited. Exclusion criteria included use of prescription medication except for contraception, regular use of non-prescription medication, regular aerobic exercise training, dyslipidemia [26], and evidence of type 2 diabetes [27]. We also excluded subjects who were insulin resistant as defined by manifesting a value for the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) > 4.65 [28]. Screening for study eligibility included: history and physical, fasting laboratory tests for evidence of metabolic dysfunction, dyslipidemia, and hepatitis or HIV infection. The results in this paper relate to healthy males (n = 8) and females (n = 8), aged 22 – 39 yrs. (average 29.9 yrs.), with a body mass index > 18 < 25 (n = 6 males, n = 5 females) or > 30 (n = 2 males, n = 3 females). In this cohort, there was one South Asian (male), 3 blacks (2 males, one of whom is a native African), and 12 whites. There were 3 Hispanics and 13 non-Hispanics.

After screening to rule out relevant health problems, all subjects ingested a low fat/low-PA, baseline/control diet for 7 d. (Protein, 19.7 % kcal; Carbohydrate, 51.6% kcal; Fat, 28.4% kcal; PA, 5.3% kcal; OA, 15.9% kcal) [26]. On the morning of day 8 of the baseline/control diet, in the fasted state, blood and muscle tissue (vastus lateralis) were collected at 0700; a blood sample also was obtained in the fed state, 3 hr. after the meal for measurement of the fatty acid composition of serum phospholipids [29]. Then, the subject participated in a cross-over study of two, 3-wk. experimental diets; energy intake during the study was adjusted, as necessary, to prevent change in body weight [8]. These experimental diets were separated by a one week period on the baseline/control diet. Our dietary model was described in detail previously [8,17]. One experimental diet was designed to resemble the habitual diet and was high in PA (HPA)(Fat, 40.4% kcal; PA, 16.0% kcal; OA,16.2% kcal); the other experimental diet was low in PA and high in OA (HOA)(Fat, 40.1% kcal; PA, 2.4% kcal; OA, 28.8% kcal) [8]. Both of these diets are characterized by a low-glycemic load [8]. We stimulated and monitored compliance with the diets in three ways: written attestation; daily verbal guidance and diet history; monitoring any food returns (usually absent or minimal) [8,17,30].

On day 18 of each experimental diet, after an evening meal at 1800 h served in the CRC and an overnight stay in the CRC, we obtained a 20-min measurement of measurements of oxygen consumption (VO_2), and CO_2 production (VCO_2) (Vmax SPECTRA 29 , Sensor Medics Corp, Yorba Linda, CA). Then, we conducted a euglycemic/hyperinsulinemic clamp as previously described [31,32]. At approximately 0530, catheters were placed in an antecubital vein for infusion and retrograde in a dorsal hand vein (heated) for blood draws [33,34]. At 0555, a baseline blood sample was obtained for measurement of glucose

enrichment, and at 0600, a primed (3.46 mg/kg), continuous (0.048 mg/kg/min) infusion of [6,6-d₂]-glucose was started and maintained for 240 min. Blood was collected for glucose enrichment at 90, 100, 110, and 120 min after beginning the glucose tracer infusion and at 100, 110, and 120 min for glucose concentration. At 120 min, we initiated an insulin infusion (40 mU/ m²/ min)(278 pmol/ m²/ min) as well as a variable rate infusion of 20% dextrose with the goal of obtaining a steady-state plasma glucose level of 80 - 90 mg/ dL[35]. [6,6-²H₂] was added to the 20% dextrose infusate prior to the study to preserve steady-state plasma enrichments. Plasma glucose levels were monitored every 5 minutes (or more often) during the clamp, and the dextrose infusion rate was adjusted to achieve the euglycemic target. Serum insulin was monitored at “0” minutes prior to the insulin infusion and at 30 min intervals during the clamp. Glucose enrichment was again monitored at 150, 180, 210, 220, 230, and 240 min after beginning the tracer infusion.

The glucose disposal rate (“M value”) was equated to the sum of the average “plateau” glucose infusion rate (estimated individually, but usually representing the values each 5 min for the last 50 – 60 min) (I_{glucose}).

The hepatic glucose production rate (hepatic glucose output, HGO) in the fasted state (HGO Fasted) was assessed using the following equation:

$$HGO \text{ Fasted (mg/min)} = \left(E_{\text{inf}} \bullet I_{\text{inf}} \bullet E_{\text{p } 90-120\text{min}}^{-1} \right)$$

Where E_{inf} is moles fraction excess of the deuterated glucose tracer, I_{inf} is the rate of infusion (mg/min) of the deuterated glucose tracer, $E_{\text{p } 90-120}$ is the plateau plasma glucose enrichment (moles fraction excess) during the 30 minutes prior to initiating the insulin infusion.

The post-insulin, hepatic glucose production rate (hepatic glucose output, HGO) was assessed using the following equation:

$$HGO \text{ (mg/min)} = \left(E_{\text{inf}} \bullet I_{\text{inf}} \bullet E_{\text{p } 210-240}^{-1} \right) - I_{\text{inf}} - I_{\text{glucose}}$$

Where E_{inf} is moles fraction excess of the deuterated glucose tracer, I_{inf} is the rate of infusion (mg/min) of the deuterated glucose tracer, $E_{\text{p } 210-240}$ is the plateau plasma glucose enrichment (moles fraction excess) during the last 30 min of the clamp, and I_{glucose} is the plateau, rate of infusion of unlabeled glucose (glucose infusion rate).

The insulin sensitivity index was defined as the M value (mg/min) divided by the product of the steady state glucose concentration and the average insulin concentration (μU/ml) at 30, 60, 90, and 120 min. post-start of the insulin infusion [36,37].

Indirect calorimetry was repeated for 20 min. from approximately 80 – 100 min after the beginning of the insulin infusion. All infusions were stopped at 240 min except for the

dextrose infusion, which was continued and tapered until no longer required to maintain normal glycemia (approximately one hour).

On days 19 – 21 of each experimental diet, the subjects participated in studies of PA oxidation [38,39]; these data will be reported separately. During all three days of tracer studies, daily food intake was given as a liquid formula over a period of 9 hrs., while they rested in the CRC. The composition of this formula was identical to the solid food diet for that experimental diet period, and the energy intake (total formula intake) was equal to the solid food intake for at least several days prior to this study period.

On day 21, measurements of O₂ consumption, and CO₂ production (Vmax SPECTRA 29, Sensor Medics Corp, Yorba Linda, CA) were obtained in the fed state after starting the liquid formula feeding) and once again the next morning in the fasted state (50-min. measurement at 0500).

Repeat blood collection in the fasted and fed state and biopsy of the vastus lateralis in the fasted state were carried out on the 22nd day of each experimental diet (HPA and HOA) as previously described [8].

On the first day of the Baseline Diet and on day 16 of the HPA and HOA diets, body composition was assessed by dual-energy x-ray absorptiometry, including upper body (android) and lower body (gynoid) regions (GE Lunar Prodigy Densitometer, Version 5.6) [40]. In extremely obese individuals (1 male; 2 females) who were too wide to fit adequately on the scanning table, we used the technique of Tataranni [41] to perform a half-body scan to estimate body fat and fat-free mass.

2.2 Metabolic assays

Serum concentrations of total cholesterol, HDL cholesterol, and triacylglycerol were measured at the Clinical Chemistry Laboratory at The University of Vermont Medical Center using a colorimetric method, and LDL cholesterol was calculated. Glucose concentration was measured using a YSI 2300 Stat Plus glucose analyzer (YSI Inc., Yellow Springs, OH), and serum insulin concentration was measured in the CRC Core Laboratory, using a radioimmunoassay (Linco Research Inc., St. Charles, Missouri). HbA1C was measured at The University of Vermont Medical Center by high performance liquid chromatography. Serum concentrations of estrogen and progesterone in females and testosterone in males were measured by The University of Vermont Medical Center using competitive, chemiluminescent immunoassays (Centaur Assay Manual Protocols for estradiol, progesterone, and testosterone, Siemens Diagnostics Corp., Malvern, PA).

Plasma [6,6-2H₂]glucose enrichment was measured by gas chromatography-mass spectrometry in the electron impact mode (Agilent 5973; Agilent Technologies, Palo Alto, CA), as described previously [42]. Prior to measurement, glucose was derivatized to the butylboronate acetate derivative. Injections of butylboronate acetyl glucose were made isothermally while monitoring the [*M*-57] ions at a mass-to-charge ratio (*m/z*) of 297 and 299 for unlabeled and [6,6-2H₂]glucose, respectively. Analyses and equations pertaining to

the studies of PA oxidation, which were incorporated into our lipidomic analyses (see below) will be described elsewhere.

We assessed the FA composition of serum phospholipids as well as that of muscle phospholipids, diacylglycerol, and triacylglycerol in order to ascertain that, in this study, we observed the expected changes in the PA and OA content of these lipids with the respective dietary interventions (HPA and HOA diets)[8]. These assays also were incorporated into our principal components analysis as noted below. The concentration of non-esterified serum FA, the FA composition of skeletal muscle diacylglycerol and triacylglycerol, as well as serum and muscle phospholipids (phosphatidylcholine, phosphatidylethanolamine, and cardiolipin), were analyzed by thin layer chromatography followed by gas chromatography [8,29].

2.3 Measurement of cytokines in plasma and secreted by PBMC

On day 8 of the Control/baseline diet and on the 22nd day of each experimental diet (HPA and HOA), blood was collected in the fasted state for measurement of cytokines in plasma and from LPS-stimulated, PBMCs. We enriched lymphocytes and monocytes from blood (PBMCs) using LSM Lymphocyte Separation Medium (MP Biomedicals, Solon, OH), and the mononuclear cell layer was collected, washed, and counted. Freshly-collected cells were plated at 10⁶ cells/ml and treated in duplicate under control conditions or were stimulated by the addition of 1ng/ml ultra-pure lipopolysaccharide (LPS; from *E. coli* 0111:B4, InvivoGen, San Diego, CA). After 24-hr., cell-free supernatants were collected and stored at -80° C until analysis. After pilot studies were conducted demonstrating which cytokines were responsive to LPS stimulation in our assays, the following cytokines were measured in these supernatants using a Luminex-based assay: TNF α , IL-1 β , IL-6, and IL-10 (Bio-Rad, Hercules, CA) [8]. IL-18 was measured by ELISA (R&D Systems, Minneapolis, MN).

The following cytokines were measured in plasma using a Luminex-based assay: IL-1 β , IL-6, IL-8, IL-10, and TNF α (R&D Systems, Minneapolis, MN).

2.4 Assessment of gene expression in skeletal muscle

Total RNA was isolated from muscle biopsies of 16 subjects, after both the baseline diet and each experimental diet using Trizol extraction followed by clean-up using RNeasy Columns, Qiagen Corp.). The RNA was then DNase treated and quantified using a NanoDrop ND-1000 spectrophotometer. RNA was reverse transcribed into cDNA using iSCRIPT reverse transcriptase (Bio-Rad). Real-time quantitative RT-PCR was performed with 25 ng of cDNA using iQ SYBR Green Supermix (Bio-Rad) on a Bio-Rad Chromo4 Sequence Detection System or an Applied Biosystems CFX1000 (Foster, City, CA). Primer sets (Table 1) were purchased from IDT (Coralville, IA). Levels of gene expression are presented as average expression relative to β -Actin from the same subject as calculated using the C_T method [43-46]. Briefly, the threshold cycle (C_T) was determined for the gene of interest and β -Actin in each sample and the C_T was calculated for each sample by subtracting the C_T of β -Actin from the C_T of the gene of interest. The C_T values were calculated by subtracting the C_T of the experimental samples from the values on the

baseline diet. The C_T values were then transformed into relative quantity (rq) values using the equation: $rq = 2^{-C_T}$.

2.5 Western Blot Analysis

In 10 subjects, the muscle biopsy samples were lysed in buffer containing 137mM Tris·HCl (pH 8.0), 130mM NaCl, and 1% NP-40. Proteins were prepared in the same buffer. Insoluble proteins were pelleted via centrifugation, and following protein quantitation of the supernatant, samples were re-suspended in loading buffer with dithiothreitol and resolved by SDS-PAGE. Proteins were transferred to PVDF and membranes were probed using a standard immunoblotting protocol using the following primary antibodies: ATF6, CHOP (both from Abcam, Cambridge, MA), ERp57, GRP94 (both from Stressgen Biotechnologies Corporation, San Diego, CA), and β -Actin (Sigma-Aldrich, St. Louis, MO). Densitometric analyses were performed utilizing BioRad® VersaDoc™ Imager Software (Minneapolis, MN). Briefly, Western Blot x-ray films were imaged and rectangles of identical size were traced around each band and assessed for density (intensity/mm²). Values were normalized to corresponding β -Actin bands following background subtraction.

2.6 Statistics

All data are expressed as mean $\pm$ SEM, and a P value < 0.05 was selected as statistically significant. Cytokine concentrations derived from PBMC secretion were first log transformed before statistical analysis. Analyses were performed with SAS, version 9.2. This study employed a two-treatment, two-period, two-sequence cross-over design. Diet effects were analyzed using a maximum likelihood approach to repeated measures analysis of variance, including sequence, period, and treatment effects, with the baseline value as a covariate, when available. Preliminary analysis of cytokine and gene expression data revealed no statistically significant interaction term for diet and sex or for diet and obesity; thus, data are presented for the entire data set. For some analyses, we employed the same model using ranks. All correlations reported are Spearman rank correlations.

PCA was used to reduce the dimensionality of the data and to aid in explaining the highest variance within the overall dataset [8]. Orthogonal rotation was employed in the interpretation of the components. The following classes of variables were included in the PCA: serum and muscle phospholipids and muscle diacylglycerol and triacylglycerol in the fasted state (including total amounts of each as well as the fractional content of various FA); whole body fat mass, fat-free mass, and per cent body fat; in the fed state, whole body PA oxidation rate and relative rate of PA accretion (equated to PA intake minus PA oxidation); in the fasted state: serum, serum LDL, HDL, triacylglycerol, total cholesterol concentration, total serum concentrations of non-esterified FA, serum concentrations of glucose and insulin, and calculated HOMA-IR; in both the fasted and fed states: respiratory exchange ratio, fat oxidation rate, and resting energy expenditure, all derived from indirect calorimetry. Data pertaining to palmitate metabolism, indirect calorimetry, serum FA, and serum concentrations of HbA1C, glucose, and insulin will be specifically reported elsewhere.

An additional purpose of the PCA was to examine how components derived from the PCA might impact the diet effect on the PBMC cytokine secretion data. Therefore, diet differences in component scores were examined using the repeated measures analysis of variance methods described above. In addition, select components were included as time-varying covariates in the analysis to examine the relationship between dependent variables of interest and the component scores.

3. Results

3.1 Body composition, serum lipids, and muscle lipids

When males and females were combined, diet did not affect body weight and body mass index (body weight in kilograms divided by the height in meters squared) (BMI). There also were no significant effects of the diets on fat mass or per cent body fat, but fat-free mass was 1% higher during the HOA diet ($P = 0.05$). In the three individuals in whom half-body dual-energy x-ray absorptiometry scans were performed, regional fat mass was not estimated. In the remaining 13 subjects, we observed no diet effects on body fat in the android, gynoid, or truncal regions.

Table 2 provides data on body weight, BMI, and body composition after the baseline diet and after the experimental diets, separately for the male and female subjects. In males, during the HOA diet, both body weight and BMI were significantly higher compared to the HPA diet, and fat-free mass trended upward by 1% ($P = 0.08$), perhaps explaining the modest effects of diet on body weight and BMI (Table 2).

Serum concentrations of the following lipids were significantly lower during HOA compared to the HPA diet: total cholesterol (11%; $P = 0.0016$); LDL cholesterol (16%, $P = 0.0006$); HDL cholesterol (6%, $P = 0.039$). The LDL/HDL ratio also was 12% lower during HOA ($P = 0.0014$). Figure 2 shows that the HPA diet was associated with an increased PA/OA ratio in serum and muscle phospholipids.

3.2 Secretion of cytokines by LPS-stimulated PBMC in the fasted state

We used an *in vitro* assay of PBMC stimulated by ultrapure LPS to interrogate the consequences of TLR4 stimulation, as may occur in response to PA itself, gram-negative bacterial infections, or endogenous, damage-associated patterns that signal through TLR4 [9]. Unstimulated PBMCs secreted very low levels of all cytokines measured, whereas LPS stimulation substantially increased levels of all of the measured cytokines. Figure 3 shows the cytokine concentrations in the supernatant after LPS stimulation of PBMC. The values for IL-1 β , IL-10, IL-18, and TNF α were all significantly higher during HPA.

We also examined the difference in secretion of cytokines (concentration, pg/ml, in the supernatant after LPS stimulation) in each diet compared to the baseline value. The concentration of IL-1 β was significantly lower during HOA versus baseline (738 ± 170 versus 1069 ± 279 , $P = 0.05$).

The dimension reduction strategy of PCA was used to reduce all metabolites measured into a smaller number of orthogonal variables. PCA identified a total of 21 factors among 156

total variables. Principal Components Factor-1 (PCF-1) was significantly different by diet ($P < 0.0001$) and correlated with the PBMC secretion of IL-1 β , IL-18, IL-10, and TNF α ($P < 0.05$). PCF-1 variables with loading scores $> |0.40|$ mainly reflected the PA or OA content of muscle or serum phospholipids and muscle diacylglycerol and triacylglycerol as well as the rate of whole body PA oxidation and retention (Table 3). Greater PA content of lipids or a higher rate of PA oxidation or retention generated a positive loading score, whereas a higher OA content generated a negative loading score (Table 3). When the effect of diet on the secretion of IL-1 β , IL-18, IL-10, and TNF α by PBMCs was adjusted for PCF-1, in addition to the baseline value, the P values for a diet effect were > 0.05 for these four cytokines, implying that alteration in the PA/OA content of cellular lipids was a plausible mechanistic pathway for how the diets differentially altered cytokine secretion.

3.3 Plasma cytokine concentrations

Plasma TNF α concentration was significantly lower during the HOA diet (12.5 ± 1.2 pg/ml) compared with the HPA diet (14.8 ± 1.7) ($P = 0.044$). Otherwise, there were no effects of diet on plasma cytokine concentrations.

3.4 Inflammatory and redox-sensitive gene expression in skeletal muscle in the fasted state

Figure 4 shows that during the HPA diet, the relative mRNA expression of *cJun* and *NLRP3* were significantly increased in skeletal muscle. There also was a trend, during the HPA diet, for an increased relative expression of *cFos* ($P = 0.097$). However, there were no significant effects of diet on the expression of the following genes: *IL-1 β* , *IL-18*, *IL-10*, *IL-6*, and *TNF α* (data not shown).

3.5 ER stress in skeletal muscle

In myoblasts, PA in physiological (blood) concentrations induces ER stress (e.g. increased mRNA expression of XBP-1 [23,25] and increased protein expression of CHOP [20,24]). However, we observed no diet effects on the mRNA expression in skeletal muscle of the following genes (fasted state): *Xbp-1*, *ATF6 α* , *GRP78C*, *GRP94C*, and *ER-p57C* (data not shown). Likewise, there was no effect of diet on the expression of the following proteins: ATF6, CHOP, ERp57, and GRP94 (data not shown).

3.6 Hepatic and peripheral insulin sensitivity

There was no significant difference between diets in the blood HbA1C concentration. There also were no significant effects of the diets on peripheral insulin sensitivity (M value or insulin sensitivity index) or hepatic insulin sensitivity (fasting or post-insulin HGO or per cent suppressibility of HGO by the insulin infusion) with the ratios of the HOA/HPA values close to unity except for the insulin sensitivity index (1.12, $P = 0.19$). Because our previous study of ovulating women suggested that insulin sensitivity was higher during HOA in women only [8], we also examined males and females separately, and in females, also used serum estrogen or progesterone concentration at the time of study as a covariate, but again, there were no significant diet effects. Finally, we observed no significant correlations between diet change in cytokines secreted by PBMCs and diet change in clamp variables.

4. Discussion

In this cross-over study of healthy young adults, we compared a Western-type dietary FA pattern (HPA) to one with a much lower PA/OA ratio (HOA) in order to investigate how the PA/OA ratio in tissue lipids affects inflammatory and oxidant stress pathways. We show here that lowering the PA/OA ratio of the diet resulted in lower secretion of IL-1 β , IL-18, IL-10, and TNF α by LPS-stimulated PBMCs and also decreased the circulating concentration of TNF α . To our knowledge these effects on IL-1 β and IL-18 are the first data showing in healthy humans that dietary FA composition can influence cytokines modulated by the NLRP3 inflammasome, but a recent paper did suggest that inhibition of lipolysis led to a lower circulating level of IL-18 [47]. The present study was not designed to address the mechanism of this effect but rather to determine if the pro-inflammatory response of monocytes could be altered by diet-induced changes in tissue FA composition. However, it is relevant that in freshly isolated mouse macrophages, treated with Kdo2-lipid A (a TLR4 agonist), mRNA expression of IL-1 β and IL-6 and the secretion of cytokines were enhanced in those mice injected biweekly (for 4 or 8 weeks) with an antisense oligonucleotide in order to knockdown stearoyl-CoA desaturase (which desaturates stearic acid, synthesized via elongation of PA, to produce OA) [48]. Thus, in that study, raising the PA/OA ratio by effectively decreasing the endogenous conversion of PA to OA enhanced apparent TLR4 activity in monocytes.

It seemed important in studies such as this one to monitor metabolic effects of the diets in order to determine if the diets were having the expected effects, suggesting that the subjects were responding in a predictable fashion to changes in the level of PA in the diet. Thus, it is of interest that the effects of the HOA versus the HPA diets on serum lipid concentrations were quite similar to results from our previous trials [17,29,49]. Our lipidomic (PCA) analysis also suggested that the diets were having the expected, pervasive, differential effects on the FA composition of serum and muscle lipids, as previously observed [8]. This PCA indicated that the PA/OA ratio of tissue lipids appeared to mediate the changes we observed in cytokine secretion by PBMCs. Indeed, based on the PCA, a diet-induced change in the PA/OA ratio of blood and muscle lipids and the rate of body accretion of PA comprised a crucial mechanistic step for the differential effects of our experimental diets on cytokine secretion by LPS-stimulated PBMCs. *In vitro* studies indicate that PA activates TLR4 and leads to increased transcription of the gene for IL-1 β (and production of pro-IL-1 β) [9]. We observed increased mRNA expression of NLRP3 during the HPA diet, which also is suggestive of either different cellular composition of muscle tissue or TLR4 activation upstream [9], a finding that may be relevant to the mechanism for how obesity coupled to excessive ingestion of saturated fat leads to increased TLR4 expression and nuclear factor- κ B signaling in skeletal muscle [11]. We believe that our results are consistent with these previous data from isolated cells [9,12], suggesting that a higher tissue lipid ratio of PA to OA facilitates augmented ability to activate the NLRP3 inflammasome as well as TLR4.

Secretion of IL-1 β and TNF α have been linked to both impaired insulin signaling and peripheral insulin resistance, in part via activation of JNK [9,12]. Previously, in a different cohort of healthy women, we reported that JNK activation in muscle was lower during the

HOA diet [8]. A major biological effect of IL-1 β is to activate JNK, which leads to enhanced transcription of *c-Fos* and *c-Jun* [9,16]. Our results suggest that *c-Jun* and perhaps *c-Fos* were both relatively down-regulated during the HOA diet.

OA appears to be neutral with respect to the pro-inflammatory effects of PA, but there are insufficient data to determine if this FA has antagonistic effects on activation of either TLR4 or the NLRP3 inflammasome, as has been reported for docosahexaenoic acid (C22:6n3) and eicosapentaenoic acid (C20:5n3) [50-52]. Docosahexaenoic acid and eicosapentaenoic acid seem to diminish activation of the NLRP3 inflammasome via the G protein-coupled receptor 120 (Gpr120) [51,52]. It has been reported that OA does not have a similar effect, but in that study, the dose of OA used (20 μ M in cell medium) seemed disproportionately low compared to docosahexaenoic acid or eicosapentaenoic acid, considering usual dietary intakes and circulating levels [52]. In another study involving intraventricular injection of OA, Gpr120 was activated [6]. Moreover, in a breast cancer cell line, OA at a high concentration (400 μ M) promoted proliferation via Gpr120 [53]. Thus, it is plausible that, *in vivo*, OA is anti-inflammatory via activation of Gpr120, but further studies would be required to isolate a particular effect of OA. In our study, we selectively manipulated the intakes of PA and OA, but the intakes of other FA as well as total protein, fat, and carbohydrate remained the same. In order to maintain a constant intake of protein and specific amounts of other FA, including PA, linoleic acid, and n-3 polyunsaturated FA, the only way one could explore if OA has effects *per se* on any biological property is to compare a lower fat/higher carbohydrate diet with a higher fat/lower carbohydrate, high OA diet. Relevant to this issue is our observation that the HOA diet was associated with less IL-1 β secretion compared to the low fat, high complex carbohydrate, control diet.

Besides showing proof of concept that dietary FA composition can influence the ability to activate TLR4 and NLRP3 affecting inflammatory pathways and oxidant stress-sensitive signaling mediators (JNK), these data are important because of possible insights into how dietary FA composition, via changes in IL-1 β and JNK activation, could alter insulin sensitivity and, thus, the pathophysiology of metabolic diseases, such as type 2 diabetes [1,9,18], atherosclerosis [2,3], obesity (via changes in food intake, sleep, and physical activity [6,30,54,55]) and Alzheimer's disease [4-6]. Since we did not observe effects of the diets on circulating cytokines, other than TNF α , it is unclear if the observed effects in PBMC have relevance to insulin signaling in liver or skeletal muscle; moreover, in this cohort of healthy young adults, including females who generally were receiving hormonal contraception, we did not observe significant diet effects on peripheral or hepatic insulin sensitivity. However, monocytes do migrate to the intima of the vascular endothelium, where they are transformed into macrophages; cholesterol crystals within the macrophage can activate the NLRP3 inflammasome leading to the chronic inflammation characteristic of atherosclerosis [10,56]. Whether the dietary effects on PBMC inflammatory pathways, which we report here, have relevance to the controversy about the role of PA, OA, and polyunsaturated fat in the pathogenesis of atherosclerosis will require additional studies [2,3]. Impaired insulin signaling in brain along with activation of the NLRP3 inflammasome and JNK also has been linked to Alzheimer's disease and cognitive decline in both humans and mice [4-6]. However, the relative role of peripheral blood-derived macrophages versus

resident microglia in non-infectious inflammation in the brain is controversial [57-59]. Finally, IL-1 β and IL-18 are also biomarkers for activation of the NLRP3 inflammasome and caspase-1 *per se*; genetic knock-out of caspase-1 causes an increased resting energy expenditure, which could be relevant to our observation that lowering the PA content of the diet resulted in higher resting energy expenditure, which we also attributed to lower ceramide production [8,30,60,61].

In conclusion, our data suggest that enhancing the PA/OA ratio in tissue lipids leads to enhanced secretion by PMBCs of IL-1 β , IL-18, TNF α , and IL-10; moreover, a greater dietary and tissue PA/OA ratio also reversibly increased mRNA expression in skeletal muscle of *NLRP3* and *cJun*. These data provide evidence that a low saturated/high monounsaturated diet (HOA), compared to the Western dietary FA pattern (HPA diet), may decrease activation of TLR4 and the NLRP3 inflammasome, leading to decreased secretion of IL-1 β , IL-18, and TNF α by PBMCs and decreased plasma concentration of TNF α , along with less activation of JNK in skeletal muscle. Elevated IL-1 β and TNF α secretion and/or JNK activation can lead to serine phosphorylation of insulin receptor substrate-1 [9,14], which is apt to impair insulin signaling (Akt serine phosphorylation) in tissues such as skeletal muscle, liver, and brain [4,5,9,12,54,55], but we observed no effects on hepatic or peripheral insulin sensitivity in this cohort. However, while *in vivo*, cellular insulin signaling is apt to be negatively impacted by the secretion of IL-1 β and TNF α , IL-18 and IL-10 may enhance insulin sensitivity [9,62-64]. Thus, varying distribution of functionally distinct tissue macrophages as well as other factors altering JNK activation, such as sex hormones and physical exercise level [8,63], may determine whether Western Diet-induced activation of inflammation reduces insulin signaling in tissues such as skeletal muscle or brain. Our observations that normal variations in dietary intake in PA and OA can affect innate immunity in human subjects could have relevance to myriad diseases involving the immune system, and show that accounting for dietary FA composition in humans subjects is critical to understanding disease pathogenesis and responsiveness to potential therapeutic interventions.

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Abbreviations

ER	endoplasmic reticulum
FA	fatty acid
Gpr120	G protein-coupled receptor 120
HGO	hepatic glucose output

LA	linoleic acid (18: 2 n 6)
HPA	high palmitic acid diet
HOA	high oleic acid and low palmitic acid diet
IL	interleukin
JNK	c-Jun N-terminal kinase
M value	glucose disposal rate
OA	oleic acid
NEFA	non-esterified FA
NEOA	non-esterified oleic acid
NEPOA	non-esterified palmitoleic acid
NEPA	non-esterified palmitic acid
NLRP3	Nucleotide Oligomerization Domain (Nod)-Like Receptor protein
OA	oleic acid
PA	palmitic acid
PA oxidation	whole body rate of PA oxidation, measured using a [1- ¹³ C]PA tracer
PBMCs	peripheral blood mononuclear cells
PC	phosphatidylcholine
PCA	principal components analysis
PCF-1, PCF-5	Principal Components Factor -1 and -5
PE	phosphatidylserine
TLR4	toll-like receptor-4
TNFα	tumor necrosis factor- α

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Highlights

- Human cross-over study of the effects of dietary palmitic acid (PA).
- Decreased PA intake caused lower secretion of cytokines by blood monocytes.
- PA intake can influence cytokines modulated by the NLRP3 inflammasome.

Figure 1.
Consort flow diagram for the trial.

Figure 2.

Lowering the palmitic acid (PA) content of the diet by replacing it with oleic acid (OA) reduces the PA/OA ratio in both serum and skeletal muscle phospholipids. On day 22 of the high PA diet (HPA) and again on day 22 of the high OA diet (HOA), blood was withdrawn and a muscle biopsy was obtained in the fasted state. The fatty acid content of the serum and muscle phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) was measured using thin layer chromatography followed by gas chromatography. Results are mean $\pm$ SEM (n = 16). * $P < 0.05$, ** $P = 0.01$ for diet effects (repeated measures analysis of variance).

Figure 3.

Lowering the ratio of palmitic acid/oleic acid in the diet affects LPS-stimulated cytokine production by PBMCs. PBMCs were collected from overnight-fasted subjects on day 22 of the high PA diet (HPA) and again on day 22 of the high OA diet (HOA) and stimulated *in vitro* for 24 hr. with 1 ng/ml LPS. Secreted cytokines were measured by BioPlex or ELISA. In order to display all the cytokines in the same graph, actual cytokine concentrations were multiplied by the respective correction factors, shown on the abscissa. Results are mean $\pm$ SEM (n = 16). * P < 0.05 for diet effects (repeated measures analysis of variance).

Figure 4.

Lowering the ratio of palmitic acid/oleic acid in the diet affects skeletal muscle gene expression. Skeletal muscle biopsies were collected from overnight-fasted subjects, on day 22 of the high PA diet (HPA) and again on day 22 of the high OA diet (HOA). Quantitative RT-PCR was used to measure relative mRNA expression. Results are mean $\pm$ SEM (n = 16).

* P < 0.05 for diet effects (repeated measures analysis of variance).

Table 1

Primers used for measuring mRNA expression in skeletal muscle biopsies.

Gene	Forward	Reverse
IL-1 β	5' ACGCTCCGGGACTCAGCA 3'	5' TGAGGCCCAAGGCCACAGGT 3'
IL-6	5' CAGACAGCCACTCACCTCTTC 3'	5' GGGTCAGGGGTGGTTATTGC 3'
IL-10	5' ACATCAAGGGCGCATGTGAAC 3'	5' GCCACCCTGATGTCTCAGTT 3'
IL-18	5' GCTGAAGATGATGAAAAACCTGGA 3'	5' GCCATACCTCTAGGCTGGCT 3'
TNF α	5' TTCTCGAAACCCCGAGTGACAAG 3'	5' CCCTTCTCCAGCTGGAAAGAGC 3'
NLRP3	5' CACTTCCAGTTTTTGCCGGG 3'	5' CAAGGCAITCTCCCCCACAT 3'
cJun	5' TCCTGCCAGTGTGTTGT 3'	5' GACTTCTCAGTGGGCTGTCC 3'
cFos	5' CAGACTACGAGGCGTCATCC 3'	5' AGTTGGTCTGTCTCCGCTTG 3'
β -actin	5' CGATCCGCCGCCGTCACACA 3'	5' CGTCACCGGAGTCCATCACG 3'
sXbp-1, mature	5' GGAGTTAAGACAGCGCTTGG 3'	5' ACTGGGTCCAAGTTGTCCAG 3'
GRP78	5' CGGTCTACTATGAAGCCCGT 3'	5' CATCTGGGTTTATGCCACGG 3'
GRP94	5' ATGTGCGCGTGTATTCATC 3'	5' GAGTCTCGCGGGAACATTC 3'
P4HB	5' CAAAATTGTTCGGGCTTCA 3'	5' CATCGGCTTCAAGGACG 3'
ERp57	5' CACTTAATTACGGGCCACCT 3'	5' AAGCTCAGCAAGACCCAAA 3'
ERp57	5' CACTTAATTACGGGCCACCT 3'	5' AAGCTCAGCAAGACCCAAA 3'
ATF6	5' GCCTTTATTGCTTCCAGCAG 3'	5' TGAGACAGCAAAACCGTCTG 3'

Table 2

Demographic and metabolic characteristics

	MEN		MEN		WOMEN		WOMEN		WOMEN	
	BASELINE	HPA	HPA	HOA	BASELINE	HPA	HPA	HOA	BASELINE	HOA
Body weight	78.51 ± 6.62	77.96 ± 6.54 [†]	24.73 ± 1.57 [†]	78.47 ± 6.49	81.16 ± 11.78	80.46 ± 11.37	28.63 ± 3.81	80.39 ± 11.38	28.60 ± 3.81	80.39 ± 11.38
Body mass index	24.89 ± 1.57	24.73 ± 1.57 [†]	24.73 ± 1.57 [†]	24.89 ± 1.56	28.88 ± 3.95	28.63 ± 3.81	28.63 ± 3.81	28.60 ± 3.81	28.63 ± 3.81	28.60 ± 3.81
Body Fat (%)	24.46 ± 2.71	24.66 ± 2.80	24.66 ± 2.80	24.39 ± 2.81	37.65 ± 3.68	37.65 ± 3.88	37.65 ± 3.88	36.90 ± 3.95	37.65 ± 3.88	36.90 ± 3.95
Fat-free mass (kg)	60.24 ± 4.97	59.23 ± 4.74	59.23 ± 4.74	59.83 ± 4.63	49.03 ± 4.48	48.42 ± 4.03	48.42 ± 4.03	48.86 ± 3.97	48.42 ± 4.03	48.86 ± 3.97
Fat Mass (kg)	19.12 ± 2.95	19.07 ± 3.00	19.07 ± 3.00	18.96 ± 2.94	32.36 ± 7.70	32.22 ± 7.75	32.22 ± 7.75	31.68 ± 7.78	32.22 ± 7.75	31.68 ± 7.78

* Mean ± SEM (n = 16 ; n = 8 males, n = 8, females).

[†] P < 0.05 for diet differences (HPA versus HOA)(Fat-free mass, males: P = 0.08).

Table 3

Variables with loading scores for PCF-1 > |0.40|

Variable	Loading Score
Serum PC OA*/Total FA Fed	<u>-0.91305</u>
Serum PE OA/Total FA Fed	<u>-0.87324</u>
Serum CL SA/PA Fed	<u>-0.82751</u>
Serum CL OA/Total FA Fed	<u>-0.82355</u>
Skeletal muscle TAG OA/ Total FA Fst	<u>-0.78001</u>
Serum NEOA/Total NEFA Fst	<u>-0.77188</u>
Serum PE OA/SA Fed	<u>-0.73338</u>
Skeletal muscle DAG OA/Total FA Fst	<u>-0.71149</u>
Skeletal muscle PC OA/Total FA Fst	<u>-0.69995</u>
Serum PE SA/PA Fed	<u>-0.67853</u>
Serum PC SA/PA Fed	<u>-0.67229</u>
Skeletal muscle TAG SA/PA Fst	<u>-0.66998</u>
Serum PC OA/SA Fed	<u>-0.57537</u>
Skeletal muscle DAG OA/SA Fst	<u>-0.56648</u>
Skeletal muscle PC SA/PA Fst	<u>-0.54095</u>
Skeletal muscle DAG SA/PA Fst	<u>-0.50395</u>
Skeletal muscle PE OA/Total FA Fst	<u>-0.48105</u>
Skeletal muscle PC OA/SA Fst	<u>-0.47756</u>
Skeletal muscle DAG LA/Total FA Fst	<u>-0.42094</u>
Serum CL OA/SA Fed	<u>-0.41047</u>
Serum NEPOA/Total NEFA Fst	<u>0.45081</u>
Serum NEPA conc. μ M Fst	<u>0.51793</u>
Serum PE PA/Total FA Fed	<u>0.63511</u>
Skeletal muscle DAG PA/OA Fst	<u>0.71076</u>
Skeletal muscle DAG PA/ Total FA Fst	<u>0.75332</u>
Skeletal muscle PC PA/OA_Fst	<u>0.78623</u>
PA oxidation	<u>0.80661</u>
PA retention	<u>0.86067</u>
Skeletal muscle TAG PA/Total FA Fst	<u>0.81129</u>
Skeletal muscle TAG PA/OA Fst	<u>0.82725</u>
Serum NEPA/NEOA Fst	<u>0.85539</u>
Serum PE PA/OA Fed	<u>0.8602</u>
Serum NEPA/Total NEFA Fst	<u>0.88836</u>
Serum PC PA/Total FA Fed	<u>0.89677</u>
Serum CL PA/OA Fed	<u>0.91973</u>
Serum CL PA/Total FA Fed	<u>0.92832</u>

Variable	Loading Score
Serum PC PA/OA Fed	0.94588

* Abbreviations used: CL, cardiolipin; conc., concentration; DAG, diacylglycerol, FA, fatty acids; Fed, fed state; Fst, fasted state; LA, linoleic acid (18: 2 n 6); OA, oleic acid; NEFA, non-esterified FA; NEOA, non-esterified oleic acid; NEPOA, non-esterified palmitoleic acid; NEPA, non-esterified palmitic acid; PA, palmitic acid; PABALC1, palmitic acid balance (i.e. retention), measured using a [1- ¹³]PA tracer (retention; see text); PAOXWBC1, whole body rate of PA oxidation, measured using a [1- ¹³]PA tracer; PC, phosphatidylcholine; PE, phosphatidylserine; POA, palmitoleic acid (16:1 n9); SA, stearic acid (18:0); SM, skeletal muscle; TAG, triacylglycerol.