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Permalink

https://escholarship.org/uc/item/5rt1r4qr

Journal

Journal of Nutrition, 146(7)

ISSN

0022-3166

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Publication Date

2016-07-01

DOI

10.3945/jn.115.223909

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Postprandial Inflammatory Responses and Free Fatty Acids in Plasma of Adults Who Consumed a Moderately High-Fat Breakfast with and without Blueberry Powder in a Randomized Placebo-Controlled Trial[1–4]

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Abstract

Background: Saturated fatty acids (FAs) released from triglyceride-rich lipoproteins (TGRLs) activate Toll-like receptor 2 (TLR-2) and induce the expression of proinflammatory cytokines in monocytes. Certain plant polyphenols inhibit TLR-mediated signaling pathways.

Objective: We determined whether plasma free FAs (FFAs) after a moderately high-fat (MHF, 40% kcal from fat) breakfast modulate the inflammatory status of postprandial blood, and whether blueberry intake suppresses FFA-induced inflammatory responses in healthy humans.

Methods: Twenty-three volunteers with a mean ± SEM age and body mass index (in kg/m^2) of 30 ± 3 y and 21.9 ± 0.4, respectively, consumed an MHF breakfast with either a placebo powder or 2 or 4 servings of blueberry powder in a randomized crossover design. The placebo powder was provided on the first test day and the blueberry powder doses were randomized with a 2-wk washout period. Plasma concentrations of lipids, glucose, and cytokines were determined. To determine whether FFAs derived from TGRL stimulate monocyte activation, and whether this is inhibited by blueberry intake, whole blood was treated with lipoprotein lipase (LPL).

Results: The median concentrations of FFAs and cytokines [tumor necrosis factor-α, interleukin (IL)-6 and IL-8] in postprandial plasma (3.5 h) decreased compared with fasting plasma regardless of the blueberry intake ($P < 0.001$ for FFAs and $P < 0.05$ for cytokines). However, concentrations of FFAs and cytokines including IL-1β increased in LPL-treated whole blood compared with untreated blood samples from participants who consumed the placebo powder. Blueberry intake suppressed IL-1β and IL-6 production in LPL-treated postprandial blood compared with the placebo control when fasting changes were used as a covariate.

Conclusions: The plasma FFA concentration may be an important determinant affecting inflammatory cytokine production in blood. Supplementation with blueberry powder did not affect plasma FFA and cytokine concentrations; however, it attenuated the cytokine production induced by ex vivo treatment of whole blood with LPL. This trial was registered at clinicaltrials.gov as NCT01594008. *J Nutr* 2016;146:1411–9.

Keywords: diet and dietary lipids, antioxidants, postprandial lipemia, plasma free fatty acids, postprandial inflammation, lipoprotein lipase, cytokines, monocyte activation, blueberries

Introduction

Chronic metabolic diseases are associated with elevated FFAs and prolonged postprandial hyperlipidemia particularly with TG-rich lipoproteins (TGRLs)[10] (1–3). High-fat meal-induced postprandial lipidemia is often associated with enhanced concentrations of proinflammatory marker proteins in the plasma (4–8). However, the mechanism by which postprandial hyperlipidemia affects inflammatory responses is unclear.

Our previous studies revealed that saturated FAs can activate Toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain receptor 2-mediated signaling pathways, whereas the n-3 FA DHA and certain plant polyphenols that are abundant in fruits and vegetables can inhibit TLR4 and nucleotide-binding oligomerization domain receptor 2-mediated inflammatory signaling pathways (9–19). Animal studies demonstrated that TLR4- or TLR2-deficient mice were protected from high-saturated fat

© 2016 American Society for Nutrition.
Manuscript received September 10, 2015. Initial review completed October 20, 2015. Revision accepted May 6, 2016.
First published online June 15, 2016; doi:10.3945/jn.115.223909.

diet-induced inflammation and insulin resistance, suggesting that inflammation and insulin resistance induced by dietary saturated fat are at least in part mediated through the activation of TLR4 and TLR2 (20–25). In addition, activation of TLR2 by FFAs released from TGRLs derived from human subjects who consumed a high fat meal leads to inflammasome-mediated secretion of IL-1β in isolated primary blood monocytes or whole blood (26).

FAs derived from dietary saturated fat from people who consume a high-saturated fat meal are transported in TGRLs as TGs in the postprandial state. The predominant FAs released from this TGRL after LPL treatment are saturated FAs (27). Our previous mechanistic study (26) revealed that both exogenous palmitic acid and endogenous FFAs released from TGRLs derived from the human subjects who consumed a high-fat meal directly activate TLR2 and induce inflammasome-mediated secretion of IL-1β in isolated primary blood monocytes or whole blood. These results suggest that the plasma concentration of FFAs is an important determinant in modulating TLR-mediated cytokine production in blood.

In a microenvironment where secreted LPL can hydrolyze TGs in plasma TGRLs to release FFAs, elevated plasma FFAs can enhance the propensity of monocyte activation. Monocytes possess constitutively active caspase-1 and thus can release IL-1β if the expression of pro-IL-1β is induced by the activation of TLRs (28). Thus, saturated FAs in plasma FFAs may be sufficient to induce inflammasome-mediated IL-1β release, a hallmark of monocyte activation. Activated monocytes transmigrate into peripheral tissues and are differentiated into macrophages that generate inflammatory signals. Therefore, alleviation of monocyte activation by dietary constituents may be an effective strategy to suppress the triggering of inflammation that leads to enhanced inflammation in peripheral tissues.

In this study, we determined whether changes in plasma FFA concentrations induced by a single moderately high-fat (MHF) breakfast modulate plasma cytokine concentrations and the propensity of monocyte activation in blood and whether supplementation with blueberry (BB) powder that contains anti-inflammatory polyphenols inhibits these processes.

Methods

Ethics statement and recruitment

This investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the Institutional Review Board for Human Subjects at the University of California Davis. Written informed consent was obtained from all subjects who were recruited from the greater Sacramento, California area. The health screening and study visits were conducted at the Western Human Nutrition Research Center (WHNRC) in Davis, California. This trial was registered at clinicaltrials.gov as NCT01594008.

Inclusion criteria included: *1*) age between 18 and 60 y and *2*) a normal BMI (in kg/m^2, 18–24.9). Exclusion criteria included: *1*) total blood cholesterol >240 mg/dL, *2*) TGs >300 mg/dL, *3*) hemoglobin <11.5 mg/dL, *4*) abnormal results in clinical chemistry and hematology panels, *5*) inflammatory or metabolic diseases, *6*) use of nonsteroidal anti-inflammatory drugs including asthma and allergy medications, *7*) unwillingness to discontinue use of dietary supplements before and during the study period, and *8*) vegetarianism. In total, 27 subjects participated in the study (women n = 18, men n = 5); 4 subjects did not complete the study (**Figure 1**).

Study design

This study was designed as a placebo-controlled crossover study. A schematic of the study design is depicted in **Figure 2**. Subjects were blinded to the dose of BB powder they were given. Researchers handling samples and data did not know what dose of BB powder subjects received on test day 2 or 3. The frozen BB yogurt supplemented with either placebo control or BB powder was coded by letter, and samples were coded by test day. The provision of the placebo control powder on the first day to all subjects was done to mitigate potential carryover effects of the BB powder. The registered dietitian coded the different treatments (placebo control, 2 and 4 servings of BB powder). The randomization scheme was generated by using the website Randomization.com's "second generator." The registered dietitian created a randomized list of the 2 and 4 servings codes (designed for 42 subjects divided into 7 blocks) and assigned subjects sequentially as subject numbers were assigned. If a subject dropped out after being randomized, the next subject was assigned to the treatment schedule of the dropped subject.

Subjects were instructed by a registered dietitian to follow a low-polyphenol and low-omega-3 FA diet and limit consumption of fruits, vegetables, soy, fatty fish (e.g., salmon), whole grains, nuts, coffee, tea, and chocolate starting 3 d before each test day. The night before each test day, subjects consumed a standardized dinner provided by the WHNRC that included a burrito (consisting of tortilla, chicken, cheese, oil, and pinto beans) as well as yogurt and lemonade (**Table 1** and **Supplemental Table 1**) to minimize masking effects of previous diet and variations in fasting levels of endpoints caused by the different dinners consumed by the subjects (29). On each test day, subjects arrived at the WHNRC after a 12-h overnight fast. Subjects had their body temperature, blood pressure, and weight measured and then had blood drawn. Subjects consumed the breakfast meal (Table 1) with either placebo control or BB powder prepared in yogurt as described below under the test meals section. Subjects were given 20 min to consume the entire breakfast, after which they were instructed not to eat or drink anything other than water and were allowed to return to their normal daily activities. Subjects returned to the WHNRC 3.5 h after consumption of the test meal for a postprandial blood draw. Postprandial peaks of the plasma concentration of TGs occur on average 3.5 h after the consumption of a high-fat breakfast (30). Following the postprandial blood draw, subjects were allowed to return to their normal dietary habits until 3 d before their next test day. Subjects consumed the placebo control powder on their first test day. On the test days 2 and 3, subjects consumed varying amounts of the BB powder that were equivalent to 2 or 4 servings of fresh BBs (24.1 and 48.2 g of BB powder, respectively) in a random order.

Test meals

The MHF breakfast (not including the BB powder) contained 650 kcal and met the following nutrient specifications: 40% kcal derived from fat, primarily from food sources containing long chain FAs (20% derived from saturated fats); 15% kcal derived from protein; and 45% kcal derived from carbohydrates (Table 1 and Supplemental Table 1).

The MHF breakfast consisted of a bagel sandwich (containing butter, cheese, beef sausage, and egg) and sucrose-sweetened yogurt, which served as the vehicle for administering the BB or placebo control powder. Table 1 presents the nutrition information separately for the base MHF meal with and without the addition of the placebo control/BB powder,

[1]Supported in part by a grant from the US Highbush Blueberry Council and USDA, Agricultural Research Service, Western Human Nutrition Research Center project funds (5306-51530-017-00D to DHH; 5306-51530- 018-00D to SJZ), USDA/NIFA competitive grant (2013-03477 for DHH), and NIH (R01AG045541 to JCR).

[2]Author disclosures: KD Ono-Moore, RG Snodgrass, S Huang, S Singh, TL Freytag, DJ Burnett, EL Bonnel, LR Woodhouse, SJ Zunino, JM Peerson, JY Lee, JC Rutledge, and DH Hwang, no conflicts of interest.

[3]Supplemental Tables 1 and 2 and Supplemental Figures 1–3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

[4] The USDA is an equal opportunity provider and employer.

[9] These authors contributed equally to this study.

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[10] Abbreviations used: BB, blueberry; MHF, moderately high fat; PBMC, peripheral blood mononuclear cell; RANTES, regulated upon activation, normal T-cell expressed and secreted; TGRL, TG-rich lipoprotein; TLR, Toll-like receptor; WHNRC, Western Human Nutrition Research Center.

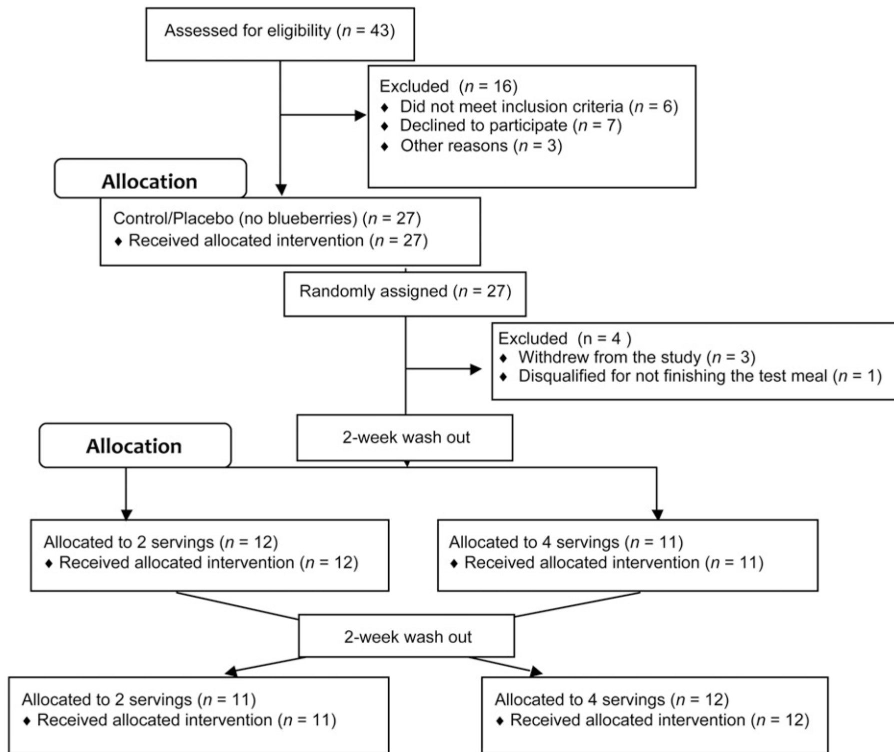

FIGURE 1 CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials.

which provided an additional 191 kcal. The addition of the control/BB powder decreased the percentage of fat content of the overall meal from 40% to 31.5%. The amount of total fat in grams remained the same including a small amount of fat from the BBs and the matched control powder (29.6 g) for each test meal. The placebo control and BB powder mixed in yogurt were prepared in advance and frozen, and each of them was served in a separate cup, so that each subject consumed all of the BB or placebo control powder. The rationale for doing so was to realistically simulate the consumption of BBs with an MHF meal while maintaining the same nutrient composition of each test meal. A registered dietitian designed the pretest dinner and the MHF breakfast by using the Nutrient Data System for Research software version 2011, developed by the Nutrition Coordinating Center of the University of Minnesota in Minneapolis, Minnesota and ProNutra (Viocare Technologies, Inc.) software.

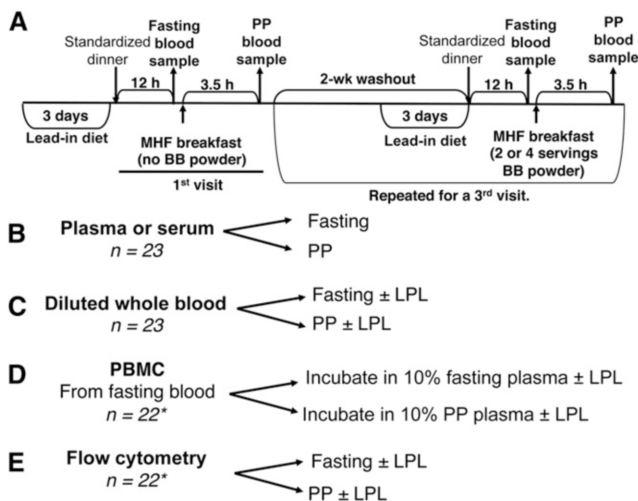

FIGURE 2 Study design and sample allocation. (A) Schematic of the study design. (B) Fasting and postprandial plasma or serum samples were isolated for lipid, insulin, FFAs, and cytokine analyses. (C) Diluted whole blood treated with or without LPL was used to assess monocyte activation induced by endogenously released FFAs from blood TGs. (D) PBMCs isolated from fasting blood were cultured in both 10% heat-inactivated autologous fasting and PP plasma and treated with or without LPL to assess monocyte activation induced by endogenously released FFAs from plasma TGs. (E) Diluted whole blood treated with or without LPL for 120 min to assess expression of adhesion markers by flow cytometry. *, PBMCs and/or data were not collected from one subject because of technician error. BB, blueberry; MHF, moderately high fat; PBMC, peripheral blood mononuclear cell; PP, postprandial.

The BB powder was provided by the US Highbush Blueberry Council and was composed of a 50/50 mixture of 2 varieties of highbush BBs, Tifblue (*Vaccinium ashei*) and Rubel (*Vaccinium corymbosum*). Whole BBs were freeze-dried, milled, and stored in sealed aluminum cans with a desiccant at −20°C. The placebo control powder and 2 different servings [2 (24. 1 g) and 4 (48.2 g) servings] of BB powder preparations were made to match: color (artificial blue and red; Lorann Oils), flavor (artificial BB Weber Flavors), fat (corn oil), carbohydrates (sucrose and maltodextrin, M100), protein (Beneprotein; Nestlé Health Science), and fiber (Benefiber; Novartis Consumer Health, Inc.), and vitamin C (Fruit Fresh; Ball) of the preparation containing 4 servings of BB powder (Table 1). Nutrient content was confirmed by chemical analysis at Medallion Labs.

Cytokines and lipid analyses in fasting or postprandial serum or plasma

EDTA-treated plasma was collected at fasting and postprandial blood draws and concentrations of plasma cytokines (IL-8, TNF-α, IL-6, and IL-1β) were determined by using the Human ProInflammatory II 4-plex ultra-sensitive kit from Meso Scale Discovery. Plasma glucose and serum TGs, total cholesterol, and HDL cholesterol were measured by using standard clinical chemistry techniques on a COBAS Integra 400 plus analyzer according to the manufacturer's instructions (Roche Diagnostics). Serum FFAs were measured by using NEFA-HR2 reagents (Wako Diagnostics) on the COBAS Integra 400 plus. LDL cholesterol was calculated by using the Friedewald equation (31). Serum insulin was measured by using an Immulite analyzer (Diagnostic Products Corp.).

TABLE 1 Nutrient contents of the MHF test breakfast, the placebo control, and BB powder supplementation, the MHF breakfast with BB powder, and the standardized dinner[1]

Nutrients	MHF breakfast[2]	Placebo control or BB powder			MHF meal + BB powder[6]	Standardized dinner
		Placebo control[3]	2 servings[4]	4 servings[5]		
Total energy, kcal	650	191	191	191	841	799
Carbohydrate, g	73.6	45.3	45.2	45.1	119	115
Energy, %	44.6	94.4	94.6	94.9	56.5	57.7
Fat,[7] g	29	0.6	0.6	0.6	29.6	24
Energy, %	40.0	2.7	2.7	2.6	32	26
SFAs, g	14	0.1	0.1	0.0	14	6.5
MUFAs, g	10	0.2	0.1	0.1	10	8
PUFAs, g	2	0.3	0.3	0.4	2	8
trans Fats, g	1.2	0	0	0	1.2	1.4
Cholesterol, mg	175	—	—	—	175	38
Protein, g	24	1.3	1.2	1.2	25	31
Total dietary fiber, g	1.7	9.8	10	10	~11.8	8.0
Vitamin C, mg	0.79	4.6	4.8	5.0	~5.8	10

[1] BB, blueberry; MHF, moderately high-fat.
[2] Nutrition information from the Nutrient Data System for Research without added BB/placebo powder.
[3] Placebo control total 48.2 g = 0.0 g of BB powder + 48.2 g of placebo control powder.
[4] 2 servings total 48.2 g = 24.1 g of BB powder + 24.1 g of placebo control powder.
[5] 4 servings total 48.2 g = 48.2 g of BB powder + 0.0 g of placebo control powder.
[6] MHF meal + BB powder 412 g = 363.8 g of MHF + 48.2 g of powder.
[7] See Supplemental Table 1 for the composition of FAs.

Monocyte activation by FFAs released from TGRLs

Whole blood assay. Fasting and postprandial heparinized venous blood was diluted 1:1 with RPMI 1640 (American Type Culture Collection), and 2 mL of diluted whole blood was treated with 4 U/mL of LPL (Sigma) or left untreated for 24 h at 37°C and 5% CO_2. Supernatants were clarified by centrifugation at 1300 × g for 10 min at 4°C and stored at −80°C.

Peripheral blood mononuclear cells isolation from whole blood and treatment with LPL. Peripheral blood mononuclear cells (PBMCs) were incubated with heat-inactivated autologous plasma with or without LPL. PBMCs were prepared from fasting blood as described elsewhere (26). The concentration of monocytes in the PBMC fraction was determined by using a Cell-Dyn 2200 (Abbott Laboratories), and 1 million monocytes/well were plated in a 12-well plate in RPMI 1640 with 10% fasting or postprandial heat-inactivated (55°C for 30 min) plasma. Next, PBMC cultures were treated with 4 U/mL of LPL for 24 h at 37°C and 5% CO_2. Supernatants were clarified and stored at −80°C.

Supernatant cytokine concentrations (TNF-α, IL-6, IFN-γ, and IL-1β) were determined by using the human ProInflammatory I 4-plex ultra-sensitive kit from Meso Scale Discovery. FFAs were measured by using the microtiter protocol provided by Wako Diagnostics with the use of NEFA-HR2 reagents (Wako Diagnostics).

Flow Cytometric analyses of CD11b, CD11c, and CD62L in blood monocytes. Fasting and postprandial heparinized venous blood were diluted 1:1 with RPMI 1640 and treated with or without 4 U/mL of LPL for 120 min. Diluted blood samples were stained with CD14-FITC, CD11b-phycoerythrin, CD11c-yallophycocyanin, and CD62L-peridinin chlorophyll protein-Cy5.5 (BioLegend) for 30 min on ice. Red blood cells were lysed by using a red blood cells lysis buffer (0.8% NH_4Cl, 0.1 mM EDTA, buffered with $KHCO_3$ to achieve a final pH of 7.2–7.6), and then samples were fixed in 1% paraformaldehyde in PBS. The samples were stored covered at 4°C until analysis on a FACSCalibur flow cytometer (Becton Dickinson). Five thousand monocytic events were collected. Data were analyzed by using Flow Jo v7.6.5 (Tree Star, Inc.).

Statistical analysis

For sample size determination, we used the results showing the concentrations of a proinflammatory cytokine (RANTES) were suppressed after anthocyanin consumption in humans. Karlsen et al. (32) found that an intake of 300 mg/d of Medox (anthocyanins) for 3 wk decreased RANTES by 12% compared with baseline. We assumed that there would be a 30% decrease in cytokine concentrations following the consumption of an MHF meal supplemented with BBs as compared with the MHF meal alone. We determined that a sample size of 25 subjects would be needed to detect this difference with 85% power at the 5% level of significance (SAS 9.2 for Windows).

Statistical analyses were performed with SAS software version 9.2 for Windows. Variables that were not normally distributed were ranked to fit a normal distribution for statistical analyses. For plasma and serum variables, mixed models analysis (SAS MIXED procedure) was run to assess whether there was a main effect of BB, time (fasting and postprandial), and a time-BB interaction (time × BB). To further assess the effect of BBs, the difference between postprandial and fasting was compared between BB treatments, with fasting concentrations as a covariate. For ex vivo studies of LPL treatments, mixed model analysis was used to assess whether there was a main effect of BB, time, treatment (± LPL), time by BB (time × BB) (2-way), treatment by BB (LPL × BB) (2-way), time by treatment (time × LPL) (2-way), and time by treatment by BB (time × LPL × BB) (3-way) interactions. The differences between + LPL and − LPL samples at each time point were compared between BB treatments with mixed model analysis, by using the fasting difference as a covariate. The random effects of subject and subject by BB serving were included in all mixed models. When significance was detected, Tukey-Kramer's test was used for post hoc analysis. Pdmix 800 was used to assign letters to different test medians. Data are represented as median and IQR unless otherwise specified. Graphs were designed in GraphPad Prism (version 6). Data are graphed on box-and-whisker plots by using the Tukey's method. Tukey outliers are not shown. Differences were considered significant at $P < 0.05$.

Results

Postprandial changes in serum lipids, insulin, and plasma glucose concentrations. General characteristics of the 23 subjects are presented in **Table 2**. Postprandial TG and insulin

TABLE 2 General characteristics of 23 healthy participants in the fasting state at the time of screening[1]

	Values
Age, y	30 ± 3
BMI, kg/m^2	21.9 ± 0.4
Systolic blood pressure, mm Hg	109 ± 2.4
Diastolic blood pressure, mm Hg	70 ± 2.0
Total cholesterol,[2] mg/dL	180 ± 6.3
Total cholesterol:HDL cholesterol ratio	3.2 ± 0.2
HDL cholesterol,[2] mg/dL	60 ± 3
LDL cholesterol,[2] mg/dL	106 ± 5.3
TGs,[2] mg/dL	72.7 ± 6.7
Glucose,[2] mg/dL	80.5 ± 1.4

[1] Values are means ± SEMs.
[2] Serum.

concentrations after the MHF breakfast were significantly elevated compared with fasting concentrations (median increase of 34% and 125%, respectively, $P < 0.0001$; **Figure** 3A, B), whereas postprandial FFA and blood glucose concentrations were decreased compared with fasting concentrations (median decrease of 59% and 4%, respectively, $P < 0.05$; Figure 3C and **Supplemental Table 2**, respectively). The addition of BB powder to the MHF breakfast did not affect postprandial blood glucose, or serum TGs, insulin, FFA, total cholesterol, LDL cholesterol, or HDL cholesterol concentrations (Figure 3 and Supplemental Table 2).

Cytokines in postprandial plasma were decreased after the MHF breakfast. The concentrations of proinflammatory cytokines (TNF-α, IL-6, and IL-8) in postprandial plasma were significantly lower than fasting plasma in parallel with decreased concentrations of FFAs in the postprandial plasma compared with fasting plasma (median decreases of 11%, 25%, 12%, and 59%, respectively, $P < 0.05$; Figure 3D–F). IL-1β was below the level of detection in the majority of the plasma samples (data not shown). There was no substantial effect of BB powder on the postprandial plasma cytokine concentrations (Figure 3).

Monocyte activation was enhanced by endogenous FFAs derived from postprandial blood TGs after the treatment with LPL. To assess the propensity of blood monocyte activation in response to endogenous FFAs derived from TGRLs, fasting or postprandial whole blood samples were treated ex vivo with LPL or vehicle control. Treatment of both fasting and postprandial blood with LPL significantly increased FFA concentrations compared with untreated blood (median increase of 368%, untreated compared with treated; $P < 0.001$): the magnitude of the increase was greater in postprandial blood compared with fasting blood (**Supplemental Figure 1**). Whole blood treated with LPL also increased secretion of IL-1β (median increase of 653%), IL-6, and TNF-α compared with untreated blood, regardless of the blood draw (fasting or postprandial) (untreated compared with LPL-treated, $P < 0.001$) (Supplemental Figure 1). In addition, secretion of IL-1β in the postprandial blood samples was significantly elevated compared with fasting blood samples, regardless of treatment with LPL ($P < 0.05$).

The concentration of FFAs released after LPL treatment of PBMC cultured in 10% autologous plasma was also greater in postprandial plasma compared with that of fasting plasma (**Supplemental Figure 2**). Thus, the amounts of FFAs released after LPL treatment reflected the concentration of TG in plasma. Furthermore, secretion of IL-1β, TNF-α, and IL-6 by PBMCs incubated with LPL in the presence of postprandial autologous plasma was greater than that secreted from PBMCs incubated in fasting plasma and LPL (Supplemental Figure 2). These results demonstrate that cytokine concentrations in blood may be dependent on the concentration of plasma FFAs.

Supplementation with BB powder decreased the propensity of monocyte activation induced by endogenous FFAs derived from postprandial TGRLs. To further assess the effect of BB intake on the propensity of monocyte activation, whole blood or PBMCs incubated in autologous plasma were treated with LPL to elevate FFA concentrations. If the treatment of blood samples with LPL causes increased FFA and cytokine concentrations compared with untreated samples, such results would further support the notion that cytokine production in

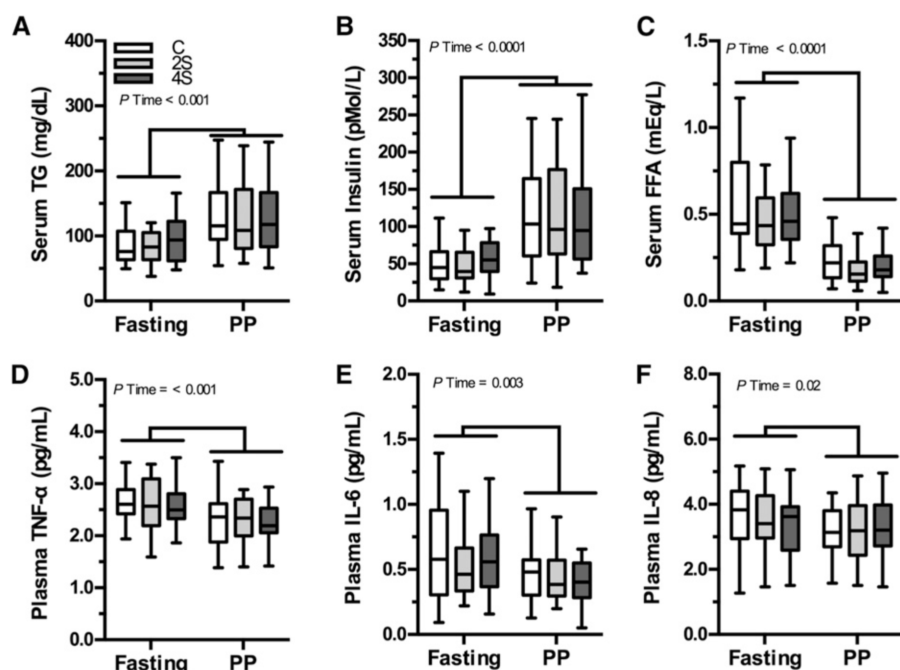

FIGURE 3 Effect of a moderately high-fat breakfast including a placebo control powder or 1 of 2 levels of BB powder on postprandial TGs (A), insulin (B), free FAs (C), and cytokine (D–F) concentrations in healthy adults. Values are medians, n = 23. Data are graphed on box-and-whisker plots by using the Tukey's method. Tukey outliers are not shown. The variables are displayed on the original scale. The statistical analysis was completed on normalized variables. C, placebo control; PP, postprandial; 2S, 2 servings of BB powder; 4S, 4 servings of BB powder.

blood at least in part depends on FFA concentrations. In diluted whole blood treated with LPL, 4 servings (48.2 g) of BB powder intake decreased concentrations of IL-6 (median decrease of 65%) when fasting concentrations were used as a covariate compared with the placebo control powder ($P < 0.05$), and 2 servings (24.1 g) of BB powder intake decreased LPL-induced secretion of IL-1β (median decrease of 55%) when fasting concentrations were used as a covariate compared with the placebo control powder ($P < 0.05$) (**Figure 4**). The addition of BB powder to an MHF meal did not change cytokine production in PBMCs cultured with autologous postprandial plasma (data not shown).

LPL treatment of whole blood significantly increased surface expression of adhesion molecules. As an additional readout for monocyte activation, surface expression of CD14, CD11b/c, and CD62L was examined. Monocyte surface expression of CD14 and CD11b/c was increased with LPL treatment of whole blood compared with time-matched untreated samples. The expression of CD62L was also increased by LPL treatment of whole blood compared with time-matched untreated samples (**Table 3**). Consumption of BB powder did not have a significant effect on adhesion marker expression. Again, these results demonstrate that increased concentrations of FFAs may stimulate monocyte activation as assessed by the expression of surface adhesion molecules.

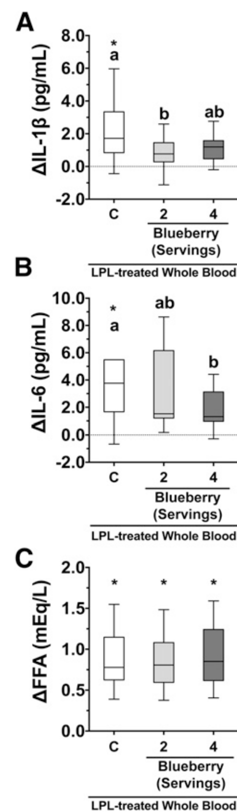

FIGURE 4 Changes in cytokine (A, B) and FFA (C) concentrations caused by LPL treatment of postprandial whole blood from healthy adults who consumed an MHF breakfast including a placebo control powder or 1 of 2 levels of BB powder. The median differences (Δ) between postprandial +LPL and postprandial −LPL samples are graphed on box-and whisker plots by using the Tukey's method (n = 23). Tukey outliers are not shown. *Different from 0 (representing no change), $P < 0.05$. Labeled medians without a common letter differ, $P < 0.05$. The variables are displayed on the original scale. The statistical analysis was completed on normalized variables. C, placebo control.

Discussion

Humans spend the majority of the day (18 h) in the postprandial state. Increased plasma TGs, insulin surge, and accompanying decrease in plasma FFAs are general features of immediate (3–4 h) postprandial changes in blood (33). Decreased postprandial plasma FFA concentrations are generally preceded by a meal-induced postprandial insulin surge, which suppresses lipolysis and stimulates re-esterification of FFAs into TGs. Whether and how plasma concentrations of FFAs modulate postprandial inflammation are not clear.

Saturated FAs can activate TLR2 and TLR4 leading to the expression of markers of inflammation in cell culture systems (9–19, 26, 34, 35). TLR2- or TLR4-deficient mice were protected from high saturated fat diet-induced inflammation and insulin resistance (20–25), suggesting that saturated FAs derived from dietary fat can induce TLR-mediated inflammation. The concentrations of plasma FFAs and TGs are temporally regulated in concert with endocrine changes (e.g., insulin level) in fasting and postprandial states. Our previous mechanistic study showed that both exogenous palmitic acid and endogenous FFAs, hydrolyzed by added LPL from TGRLs isolated from human subjects who consumed a high fat meal, induce the activation of TLR2 in primary blood monocytes and whole blood (26). TLR2 activation was assessed by the receptor dimerization (a direct readout for receptor activation) and the expression of the monocyte-specific TLR2/inflammasome-mediated IL-1β as a biochemical readout. Treatment of primary monocytes or whole blood with LPL also caused increased expression of IL-1β, providing a mechanistic insight for the causal role of plasma FFAs on cytokine production (26). Together, these results suggest that the concentration of plasma FFAs is an important determinant for the expression of TLR target gene products in blood monocytes and possibly other blood leukocytes that express TLR2 or TLR4.

The results of our current study show that the concentrations of cytokines (TLR target gene products) decreased in 3.5-h postprandial plasma after the MHF breakfast as the concentrations of FFAs declined compared with those of fasting plasma (Figure 3), suggesting that eating breakfast may attenuate acute postprandial inflammation. However, when postprandial blood samples were treated with LPL, the pattern of FFA and cytokine concentrations was reversed. The FFA concentrations of LPL-treated postprandial blood samples were greater than those of LPL-treated fasting blood samples (likely because of much higher concentrations of TGs in postprandial blood) (Supplemental Figure 1). Similarly, the cytokine concentrations from PBMCs incubated in postprandial autologous plasma treated with LPL increased compared with those from PBMC incubated with fasting plasma (Supplemental Figure 2). The concentrations of IL-1β in postprandial whole blood treated with LPL were also increased compared with those from fasting whole blood treated with LPL (Supplemental Figure 1). Taken together, the results from the current study and our previous mechanistic study (26) reveal that plasma FFA concentration may be an important modulator of cytokine production in human blood.

The decreased FFA concentrations in postprandial plasma compared with fasting plasma may result from the postprandial surge of insulin, which suppresses lipolysis and stimulates esterification

TABLE 3 Effect of LPL treatment of fasting or postprandial whole blood on the expression of monocyte adhesion markers from healthy adults who consumed an MHF breakfast including a placebo control powder or 1 of 2 levels of BB[1]

Markers	Fasting		Postprandial		ANOVA *P* values[2]		
	− LPL 120 min	+ LPL 120 min	− LPL 120 min	+ LPL 120 min	Time	LPL	Time × LPL
CD14, MFI					0.95	<0.001	<0.05
BB C	57.4 (52.0–66.7)[b]	63.6 (53.6–66.9)[a]	57.8 (54.5–64.3)[b]	64.8 (56.2–68.7)[a]			
BB 2S	56.8 (53.2–62.1)[b]	61.2 (58.3–65.6)[a]	56.6 (53.0–61.9)[b]	61.7 (57.7–68.6)[a]			
BB 4S	58.2 (52.9–63.2)[b]	62.4 (54.7– 70.0)[a]	56.1 (48.4–62.5)[b]	63.0 (51.9–68.4)[a]			
CD11b, MFI					0.02	<0.0001	0.67
BB C	278 (214–352)	411 (326–486)	252 (210–271)	385 (348–434)			
BB 2S	239 (211–261)	369 (326–400)	231 (192–256)	316 (301–409)			
BB 4S	259 (201–286)	400 (336–465)	248 (187–260)	369 (325–405)			
CD11c, MFI					0.96	<0.001	0.12
BB C	36.5 (32.8–49.7)	47.5 (42.5–62.5)	32.8 (30.7–44.1)	50.0 (45.5–66.5)			
BB 2S	31.9 (27.0–35.8)	44.0 (38.3–53.6)	31.8 (27.8–37.1)	42.5 (39.9–56.3)			
BB 4S	36.5 (31.1–42.1)	47.2 (43.6–56.4)	34.7 (28.8–38.9)	49.1 (41.6–56.6)			
CD62L, MFI					<0.001	<0.001	0.39
BB C	247 (227–277)	270 (248–302)	229 (184–251)	243 (217–292)			
BB 2S	254 (242–277)	285 (250–299)	216 (193–234)	241 (207–264)			
BB 4S	247 (221–288)	276 (241–294)	204 (184–246)	230 (207–266)			

[1] Values are medians (IQRs), $n = 22$ for CD14, CD11c, and CD62L, $n = 21$ for CD11b. BB, blueberry powder (C, 2S, 4S); C, placebo control; MFI, median fluorescence intensity; T, time (fasting compared with postprandial); 2S, 2 servings; 4S, 4 servings.

[2] Three-factor ANOVA was used to assess the effect of time (fasting compared with postprandial), LPL (−/+), and BB (C, 2S, or 4S) on the expression of cell surface adhesion markers. Labeled medians for a variable without a common letter differ, $P < 0.05$. The variables are displayed on the original scale. The statistical analysis was completed on normalized variables.

of FFAs to TGs (33), leading to decreased production of cytokines. Other studies showing varying results on postprandial cytokine concentrations (6–8, 36–39) have not focused on the relation of the postprandial cytokine concentrations with those of plasma FFAs. Such varying results may reflect in part differences in sampling time of postprandial blood, dietary composition, and heterogeneous health status of the subject populations. The subjects enrolled in our study were mostly young (mean age of 30 y) and generally healthy, with a BMI of 18–25, reflecting a relatively homogeneous group.

Saturated FAs are the predominant FAs esterified in TGs of TGRLs isolated from subjects who consumed a meal high in saturated fat (27). Thus, the result that increased plasma concentrations of endogenous FFAs released by the treatment of LPL correlated with elevated production of cytokines in the blood samples suggests that saturated FAs in the FFA fraction predominate in modulating proinflammatory signals (e.g., TLR2/4) in blood. The breakfast meal used in this study contained 14.3 g of saturated fat, which represents 19.8% and 15.3% of the total calories from the breakfast alone and breakfast plus BB powder, respectively. Thus, the saturated fat content is more than twice the level recommended by the American Heart Association. The reason for using the elevated level of saturated fat was that, because saturated FAs can induce TLR-mediated monocyte activation, we intended to use this breakfast as a challenge meal. In spite of such a high saturated fat content of the breakfast, the postprandial plasma FFAs and cytokine concentrations declined compared with those of the fasting plasma. This result suggests that a meal-induced insulin surge may predominate in regulating plasma FFAs and, in turn, FA-induced cytokine production in healthy subjects given the type of breakfast described in our study. The surface expression of adhesion markers CD11b/c and CD14 were increased after treatment of whole blood with LPL (Table 3). This result further supports the possibility that plasma FFAs derived from dietary fats can activate monocytes and may lead to increased adherence of monocytes to vascular endothelial cells.

The concentrations of TGs in postprandial (3.5 h) plasma were greatly increased compared with those of fasting plasma (Figure 3). FFAs, not TGs, can induce the expression of the TLR target gene product, COX-2 in macrophages (**Supplemental Figure 3**). This finding is consistent with the result that the concentrations of postprandial (3.5 h) cytokines follow FFA levels but not TG levels.

Plasma FFAs are derived from TGs in adipose tissue and in part from dietary fat. However, in a microenvironment where TGRLs are exposed to LPL secreted from endothelium, local concentrations of FFAs can be increased before FFAs are taken up by endothelial cells for re-esterification to TGs or incorporation into lipid droplets (40). Thus, cell surface TLRs expressed in blood monocytes will be exposed to the increased FFAs and can subsequently be activated, leading to production of proinflammatory gene products including cytokines. Therefore, elevated plasma TGs could enhance the propensity of monocyte activation.

Polyphenols are extensively metabolized by gut microbiota, enterocytes, and liver enzymes (41). Postprandial whole blood should contain any nonmetabolized polyphenols and their metabolites derived from the ingested BB powder. Therefore, to assess the effects of these absorbed polyphenols and their metabolites on TLR-mediated monocyte activation, fasting and postprandial whole blood were stimulated with endogenous FFAs derived from plasma TGs by LPL treatment in this study. Certain polyphenol compounds found in fruits and vegetables can inhibit the activation of pattern recognition receptors (10, 15, 16, 18). BBs contain high concentrations of these polyphenolic compounds including anthocyanins (42, 43). Cyanidin-3-glucoside (one of the major anthocyanins present

in BBs) and their metabolites suppress LPS-induced cytokine production in THP-1 monocytes (44).

BB supplementation did not affect postprandial FFA and cytokine concentrations (Figure 3); however, it attenuated the propensity of monocyte activation induced by elevated concentrations of FFAs in LPL-treated blood ex vivo (Supplemental Figure 1). Supplementation with 2 and 4 servings of BBs with the breakfast meal resulted in decreased LPL-induced secretion of IL-1β and IL-6, respectively, in postprandial whole blood without affecting concentrations of FFAs released (Figure 4). These results suggest that the consumption of BB powder with the breakfast meal could decrease the propensity of postprandial monocyte activation in the microenvironment where blood monocytes directly interact with endothelial cells that secrete LPL. The inhibitory effect of the BB powder on LPL-induced cytokine production is likely due to the inhibitory effects of polyphenols on FFA-induced activation of pattern recognition receptors including TLRs rather than potential off-target effects on LPL because BB intake did not alter the concentrations of FFAs released. It was reported that intake of strawberries (45, 46), orange juice (47), tomatoes (48), or blackcurrants (49) with a high-fat meal was cardioprotective by decreasing selective inflammatory markers (e.g., postprandial IL-6, oxLDL cholesterol, mRNA of TLR4/2, and NFκB activation). Because postprandial FFA concentrations may change in time, the impact of polyphenol-rich BBs on FFA-induced postprandial inflammatory status will need to be studied in the future with blood samples taken at multiple time points, including the period when FFA concentrations rebound close to or exceed fasting concentrations.

The study has several limitations. The study was not powered to look at interactions between sex and consumption of BB powder. In addition, the subjects were allowed to return to their normal daily activities after consumption of the test meal until their return for the postprandial blood draw. Because sex differences and physical activity could have affected postprandial responses, recruitment strategies to create a balanced sex distribution and control of physical activity may need to be implemented in future studies.

In summary, consumption of an MHF breakfast decreased postprandial plasma FFA and cytokine concentrations compared with those of fasting plasma, suggesting that eating breakfast acutely attenuates the inflammatory status in postprandial blood. Plasma FFA concentrations may be an important determinant modulating monocyte activation as assessed by TLR-mediated IL-1β secretion and the expression of adhesion molecules (CD11b/c). These results corroborate the results from our previous mechanistic studies that both palmitic acid and endogenous FFAs can directly activate TLR2 and induce the expression of proinflammatory cytokines in primary monocytes and whole blood. A corollary of these results is that the concentration of plasma FFAs may be one of the important determinants affecting the inflammatory status in blood. Plasma FFA concentrations are generally elevated in obesity and diabetic patients. Thus, our results lead to the next translational question as to whether plasma FFA concentrations can be a target of dietary or pharmacological intervention to alleviate the increased inflammation in metabolic diseases. Supplementation with BB powder did not affect the postprandial FFA and cytokine concentrations; however, it suppressed FFA-induced cytokine production in LPL-treated blood.

Acknowledgments

KDO-M, RGS, SH, DJB, ELB, LRW, SJZ, JCR, and DHH designed the research; KDO-M, RGS, SH, SS, TLF, DJB, and JYL conducted the research; KDO-M and JMP analyzed the data; KDO-M and DHH wrote the paper; and DHH had primary responsibility for the final content. All authors read and approved the final manuscript.

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