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Markers of systemic exposures to products of intestinal bacteria in a dietary intervention study

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Abstract

Purpose—Systemic exposures to intestinal bacteria may play a role in the etiology of the chronic, low-grade inflammation that is associated with western diets. Production of lipopolysaccharide-binding protein (LBP) is one biomarker of increased exposures to intestinal bacteria. This study evaluated whether changes in diet quality could affect serum LBP.

Methods—This was a randomized, controlled trial of Mediterranean and Healthy Eating diets over 6 months in 120 healthy subjects at increased risk of colon cancer. Blood samples obtained before and after intervention were analyzed for LBP, branched-chain fatty acids characteristic of intestinal bacteria, micronutrients and cytokines. Data were analyzed for changes in LBP over time and for predictors of LBP.

Results—Serum concentrations of branched-chain bacterial fatty acids declined significantly in both diet groups. However, there was no significant change in mean serum LBP concentrations with either diet intervention. In serum, LBP was positively associated with CRP and negatively associated with carotenoids both before and after intervention. After intervention, LBP was predicted positively by both CRP and bacterial fatty acid concentrations in serum, and negatively by serum carotenoids and the ω3/ω6 fatty acid ratio. This model accounted for 30 % of the inter-individual variation in serum LBP after intervention.

Conclusions—These results indicate that dietary intervention over 6 months was insufficient to alter serum LBP. The relationships with inflammation-related markers, however, indicate that anti-inflammatory strategies other than changes in diet quality, such as weight loss or improved

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fitness, may have more potential for reducing systemic markers of LPS exposures in well-nourished populations.

Keywords

Intestinal bacteria; Inflammation; Obesity; Mediterranean diet; High fruit and vegetable diets

Introduction

Lipopolysaccharide (LPS) is an endotoxin from Gram-negative bacteria that associates with chylomicrons in the intestine. In the colon, LPS-binding protein (LBP) together with sCD14 functions to concentrate LPS at the membrane Toll-like receptor 4, resulting in an inflammatory response [1]. Induction of LBP by LPS exposure also may have a role in detoxifying LPS via a HDL-mediated pathway [1]. LBP and products of LPS circulate systemically and are increased by ingestion of a high-fat diet [2]. LBP is synthesized in the liver, adipose and intestinal epithelial cells, and its production in the intestine is induced by cytokines [3, 4]. Thus, LBP represents both LPS load and obesity-related changes in the intestinal barrier and pro-inflammatory pathways.

LPS can be measured in human serum using a limulus amebocyte lysate (LAL) reactivity assay, but this has been fraught with technical problems when applied to analyses of serum due to interfering compounds [5, 6]. LPS-binding protein (LBP) therefore has been used as a more reliable systemic marker [7, 8]. LBP binds constituents of Gram-positive and Gram-negative bacteria, making it a more general marker of bacterial exposures than the LPS that stems only from Gram-negative bacteria [7]. Other markers of bacterial exposures, such as quantitation of unique bacterial fatty acids, have not been investigated as widely in dietary studies [9, 10].

Obesity, weight gain and high-fat diets have been shown to increase serum LBP [2, 3, 11]. Other studies have suggested that the ratio of LBP to soluble CD14 (sCD14) might be more sensitive to energy balance since sCD14 was decreased by overfeeding and can function to blunt the activity of LPS [12]. In endotoxemia induced by inflammatory bowel disease, glucose intolerance or alcoholism, however, both LBP and sCD14 were elevated making use of the LBP/sCD14 ratio as a superior biomarker of endotoxemia unclear [13–15]. The evidence that specific dietary components might affect LBP concentrations is more limited than for obesity. In rodents, a diet high in ω3 fatty acids reduced LBP, and in another study, a diet high in unsaturated fatty acids reduced both LBP and the LBP/sCD14 ratio relative to a diet high in saturated fat [16, 17]. Effects of increased fruit and vegetable intakes on LBP have not been published, but fruit and vegetables extracts did decrease LPS-stimulated inflammatory responses in vitro [18–20].

We conducted a randomized clinical trial of a Healthy Eating or a Mediterranean diet, and we evaluated changes in serum LBP and in fatty acids that are characteristic of bacterial exposures using a quantitative assay. Many aspects of the Greek Mediterranean diet, namely higher intakes of whole grains, legumes, olive oil, fish, fruits and vegetables but less red meat, ω6 fats and processed foods, could work in concert to improve the intestinal barrier. A reduction in systemic exposures to products of intestinal bacteria therefore could be one

mechanism behind the anti-inflammatory effects of a Mediterranean diet. This is relevant to persons at increased risk of colon cancer due to the strong associations of pro-inflammatory states with colon cancer risk [21]. We also evaluated predictors of serum LBP independent of intervention arm to better understand the relative impact of diet versus other factors such as cytokines on LBP concentrations.

Methods

Subjects and dietary intervention

This study utilized samples from a dietary intervention trial, the Healthy Eating Study. Briefly, 120 persons at increased risk of colon cancer were enrolled, randomized to follow a standard Healthy Eating diet or a modified Mediterranean diet, and 93 finished 6 months of study. Compliance to the dietary goals was good, as reported previously [22]. The study was approved by the University of Michigan Institutional Review Board (HUM00007622) and was listed on the Clinical Trials Web site maintained by the National Institutes of Health (NCT00475722).

Blood samples

Fasting blood samples were obtained at baseline and after 6 months. Plasma and serum were prepared promptly, and aliquots were stored at −80 °C until analysis. One serum sample from the study was not available for LBP measures at baseline. Serum LBP was analyzed by enzyme-linked immunosorbent assay (ELISA) from Cell Sciences (Canton, MA). Carotenoids were analyzed by high-pressure liquid chromatography as previously described [23]. Fatty acids characteristic in bacteria was measured by GC-MS after acid hydrolysis of serum to release fatty acids from LPS (8 N HCl, 90 °C for 4 h), followed by extraction with dichloromethane and derivatization with BSTFA:TMCS (99:1) as modified from published assays [9, 10].

Plasma cytokines (IL1β, IL6, IL8, interferon γ, TNFα, IL4, IL10 and IL13) were measured using ELISA DuoSets from R&D Systems (Minneapolis, MN). Measures of total cholesterol, HDL and triglycerides were performed using a Cobas Mira Chemistry analyzer (Roche Diagnostics Corporation, Indianapolis). High-sensitivity C-reactive protein (CRP) was measured using a latex immunoturbidimeteric assay. Four subjects had CRP values below the limit of detection and were assigned a value that was half the detection limit (0.05 mg/dl). Glucose was measured using a hexokinase colorimetric assay, and C-peptide was measured using an Immulite chemiluminescent assay (Diagnostics Products Corporation, Los Angeles, CA). The homeostasis model of assessment for insulin resistance (HOMA) was calculated from C-peptide and glucose using an online calculator (the HOMA Calculator version 2.2) [24].

Statistical analyses

All data were analyzed using IBM SPSS Statistics version 22 (IBM Corporation, Armonk, New York). Linear mixed regression models were used to evaluate average changes in iBFA and LBP with time, with diet group and the group-by-time interaction as the primary predictors (Table 1). Age was included as a covariate since mean age at baseline was higher

in the Mediterranean versus the Healthy Eating group (55 vs. 50 years, $p = 0.03$). Post hoc pairwise comparisons were done using Bonferroni correction for multiple comparisons.

To examine dietary and demographic factors associated with serum LBP and iBFA concentrations at both baseline and 6 months, Spearman correlation coefficients were calculated (Table 2). Those showing a correlation with LBP with $p < 0.10$ were further evaluated in multiple linear regression models using backward variable selection with a $p \leq 0.10$ cutoff. Only total carotenoids were used in the linear regression models. Serum and dietary measurements were log-transformed, if appropriate, to reduce deviation from the normal distribution. Models were developed using backward covariate selection using $p < 0.10$ as the criterion to retain variables in the models (Table 3).

Results

There were no significant changes in serum LBP concentrations with either a Healthy Eating or Mediterranean intervention (Table 1). These diets both roughly doubled dietary carotenoid intakes and significantly decreased saturated fat intakes, but changes in serum carotenoids and serum fatty acids were modest [22, 23]. Hydroxylated bacterial fatty acids characteristic of LPS was not detected in the serum samples, but branched-chain (iso-methyl) fatty acids (iBFA), namely 13-methylmyristic acid (i15:0),14-methylpentadecanoic acid (i16:0) and 15-methylpalmitic acid (i17:0), were quantified. These fatty acids are common in both Gram-negative and Gram-positive bacteria [25]. Serum concentrations of total iBFA declined significantly in both diet groups (Table 1).

LBP was positively correlated with iBFA, and this was significant post-intervention (Table 2). Except for CRP, there were no significant correlations of baseline LBP with any cytokine measured or the summary variable created by summing cytokine z-scores. Serum total iBFA was not correlated with CRP at baseline ($p > 0.2$). Correlations of LBP with serum and dietary parameters at baseline were generally similar to the correlations obtained post-intervention, with positive associations for BMI and CRP, and negative associations for several micronutrients and the ω3/ω6 fatty acid ratio (Table 2). These results should be interpreted with caution since they were not controlled for multiple comparisons and were primarily performed to identify variables for the subsequent multivariate analyses shown in Table 3.

Linear regression analysis indicated that before intervention, serum CRP and total serum carotenoids accounted for 13 % of the inter-individual variability in LBP (Table 3). Serum CRP, the ω3/ω6 fatty acid ratio, total carotenoids and iBFA accounted for 30 % of the inter-individual variability in LBP concentrations after dietary intervention (Table 3).

Discussion

LBP initiates the recognition of bacterial LPS exposures and amplifies the host immune response that, if continued over the long-term, results in adverse health consequences to the host [26]. LBP has been suggested to serve as a marker of inflammation in obesity-related insulin resistance [27]. In addition, LBP appears to play a role in risk of cardiovascular disease by promoting atherogenesis [28]. Although obesity and high-fat diets have been

shown to increase serum LBP [2, 3, 11], little is known about the effects of dietary components that are thought to be beneficial, such as fruits, vegetables and omega 3 fatty acids, on LBP.

In this study, serum concentrations of carotenoids and LBP were inversely correlated, but neither dietary intervention affected serum LBP (Tables 1, 2). However, a more direct measure of LPS exposure, iBFA, was reduced by either type of diet intervention. The decreases in iBFA post-intervention were small but significant. This could be due to a number of factors. Both interventions increased fiber intakes by about 50 % [22], and fiber is known to improve intestinal permeability [29]. There are, however, other sources of iBFA in addition to commensal bacteria. Endogenous synthesis of branched-chain fatty acids can occur in the skin, but they do not appear to be made in other organs [30]. Bacterial fatty acids also are known to be present in the meat and milk of ruminants and in marine animals [31]. In this study, the dietary counseling encouraged intakes of lean meat and dairy to decrease saturated fat intakes in the Healthy arm and to allow for the substitution of usual fat intakes with olive oil in the Mediterranean arm [22]. Presumably, bacterial fatty acids would be lower in reduced fat animal products versus the higher-fat products due to partitioning of LPS into fat.

Unlike iBFA, which decreased after dietary intervention, the protective effects of these types of diets on LBP may only be manifest when instituted in populations with initially poor quality diets to result in larger changes, when consumed for longer periods of time, or when accompanied by weight loss in overweight or obese individuals. Also metabolic processes related to oxidative stress and inflammation determined by genetics or environmental exposures may limit increases in carotenoids with diet, and changes in serum carotenoids post-intervention in this study were modest [23].

Our study found associations of increased BMI and CRP with increased LBP, similar to previous reports [2, 3, 11], but the effect of BMI was no longer significant in multiple linear regression models (Table 3). This indicates that obesity per se might not result in increased exposure to intestinal bacteria. A limitation of the work is that other markers of exposures to bacterial LPS, such as sCD14, were not measured. After dietary intervention, when inter-individual variability in diets was likely reduced, a positive association of serum LBP and bacterial fatty acids emerged. In addition, a negative association of LBP with the ω3 to ω6 fatty acid ratio was found post-intervention perhaps because half the subjects had been counseled to increase intakes of ω3 fatty acids.

Conclusions

Two different dietary interventions that resulted in large increases in fruits and vegetables along with changes in fat intakes reduced systemic concentrations of branched-chain bacterial fatty acids without an effect on plasma LBP. CRP, but not BMI, had strong effects on LBP. Future efforts should be made to identify other anti-inflammatory strategies, such as weight loss or improved fitness, to decrease markers of systemic exposures to products of intestinal bacteria. In addition, it may be important to determine what biological effects of

these branched-chain fatty acids are in cells, especially with regard to stimulating immune responses.

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Abbreviations

BFA	Bacterial fatty acids
CRP	C-reactive protein
LBP	Lipopolysaccharide-binding protein

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Table 1

Serum concentrations of LPS-binding protein (LBP) and branched-chain bacterial fatty acids (iBFA) in each diet group before and after dietary intervention

Variable	Healthy Eating		Mediterranean	
	Baseline	6 Months	Baseline	6 Months
LBP (μg/ml)	19.0 (0.9)	19.7 (1.1)	17.3 (0.9)	18.0 (1.1)
iBFA (μg/ml)	5.09 (0.11)	4.79 (0.11)*	5.04 (0.11)	4.69 (0.11)*

Data are mean and SE from mixed models ANOVA for 120 subjects randomized to a Healthy Eating or Mediterranean diet
Models were controlled for age. Time had a significant fixed effect for BFA $p < 0.001$
Values at 6 months that are significantly lower than baseline ($p \leq 0.001$) are starred, as determined by post hoc pairwise comparisons using Bonferroni correction

Table 2

Spearman correlations of LBP with select parameters before and after dietary intervention

Parameter	Baseline *N* = 119	6 Months *N* = 93
BMI	0.322***	0.204*
Diet		
Percent energy fat	0.072	−0.072
Percent energy MUFA	0.157	−0.097
ω3/ω6 fatty acid ratio	−0.144	−0.214*
Fiber/1000 kcal	−0.012	−0.027
Carotenoids/1000 kcal	−0.157	0.136
Serum concentrations		
TotChol	0.072	−0.167
HDL	−0.174	−0.211*
TriGlyc	0.193*	0.162
LDL	−0.082	−0.173
CRP	0.342***	0.391***
Insulin resistance	0.217*	0.036
Cytokine z-score 1[a]	0.093	0.038
Cytokine z-score 2[b]	0.051	0.081
ω3/ω6 Fatty acid ratio	−0.136	−0.237*
Leptin	0.181*	0.181
iBFA	0.133	0.280**
Serum micronutrients		
y-Tocopherol	0.239**	0.267*
α-Tocopherol	0.022	−0.021
Lutein	−0.054	−0.010
Zeaxanthin	−0.147	0.028
β-Cryptoxanthin	−0.136	−0.133
α-Carotene	−0.062	0.004
β-Carotene	−0.323***	−0.250*
Lycopene	−0.272**	−0.309**
Total carotenoids	−0.300**	−0.222*

Starred correlations were significant with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$

[a] Cytokine z-score 1 was the sum of z-scores for IL-1β, IL6, IL8, IFNγ and TNFα

[b] Cytokine z-score 2 was the sum of z-scores for IL4, IL10 and IL13

Table 3

Multiple regression analysis of LBP serum concentrations at before and after intervention using backward covariate selection

Predictive variables	R square final model	Standardized regression coefficient β	*p* value[a]
Pre-intervention			
CRP (Ln)[b]		0.252	0.006
Total carotenoids (Ln)		−0.205	0.025
Final model with both factors	0.131		<0.001
Post-intervention			
CRP (Ln)		0.421	<0.001
n3:n6 fatty acid ratio (Ln)		−0.180	0.056
Total carotenoids (Ln)		−0.161	0.083
iBFA		0.196	0.037
Final model with all four factors	0.301		<0.001

[a]The *p* value shown is for the significance of the F change in the linear regression model. Demographic factors (age, gender, smoking, NSAID use) were not significant in the models. Models were developed using backward covariate selection, and co-variates with $p \leq 0.1$ were retained. BMI, leptin, triglycerides, insulin resistance (HOMA) and γ-tocopherol did not remain in the final model for baseline ($n = 119$). BMI, γ-tocopherol and HDL did not remain in the final model for post-intervention ($n = 93$)

[b]The variables shown are serum concentrations of C-reactive protein (CRP), the ratio of serum n3:n6 fatty acids, serum carotenoids and total serum branched-chain bacterial fatty acids (iBFA, which was the sum of i15:0, i16:0 and i17:0)