Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2006, Article ID 64980, Pages 1–6
DOI 10.1155/MI/2006/64980

Research Communication

The Evaluation of the Role of Beta-Hydroxy Fatty Acids on Chronic Inflammation and Insulin Resistance

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Received 9 May 2006; Revised 19 July 2006; Accepted 7 August 2006

β-hydroxy fatty acids are a major component of lipid A moiety of lipopolysaccaride. We aimed to investigate the role of free β-hydroxy fatty acids on inflammation, as well as to evaluate their effects on cytokine release from human blood cells, and whether they exist in plasma of patients with chronic inflammatory diseases with/without insulin resistance. Peripheral venous blood was incubated with β-hydroxy lauric and β-hydroxy myristic acids (each 100 ng, 1 μg, 10 μg/mL) up to 24 hours. Cytokines were measured from culture media and plasma. Free fatty acids and biochemical parameters were also measured from patients' plasma. Only β-hydroxy lauric acid significantly stimulated interleukin-6 production at 10 μg/mL compared to control (533.9 $\pm$ 218.1 versus 438.3 $\pm$ 219.6 pg/mL, $P < .05$). However, free β-hydroxy lauric and myristic acids were not found in patients' plasma. Therefore, free β-hydroxy lauric and myristic acids do not seem to have a role on sterile inflammation in chronic inflammatory diseases associated with insulin resistance.

INTRODUCTION

Insulin resistance (IR) occurs in a number of diseases associated with chronic inflammation such as metabolic syndrome, type 2 diabetes, atherosclerosis, cancer, rheumatoid arthritis (RA), and infections, polycystic ovary syndrome (PCOS) [1–5]. There has been a growing body of laboratory and epidemiological evidence that IR and type 2 diabetes mellitus are conditions of low-grade inflammation and this inflammation is now believed to play a causative role in the pathogenesis of these disorders [6]. What causes chronic inflammation in such diseases is not clearly understood. Cytokines are important mediators of inflammation. It has been shown that there is a relationship between cytokines, in particular, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1α, IL-1β, and IR but the mechanism is not fully understood. TNF-α may cause IR by suppressing insulin-induced tyrosine phosphorylation of insulin receptor and its substrate [7, 8]. There is a correlation between IR and serum concentrations of TNF-α and IL-6 in cancer patients [9, 10]. In addition, plasma concentrations of TNF-α, TNF-receptor, plasminogen activator inhibitor-1, and IL-6 were found to be increased in obese and in type 2 diabetic patients [11–14].

Lipopolysaccaride (LPS) is a part of the outer membrane of the cell wall of Gram-negative bacteria. The lipid A moiety of LPS is responsible for its toxic effects like inflammation, fever, tissue necrosis, endotoxic shock, and activation of the complement system [15]. Inflammatory and other toxic effects of LPS are mainly via cytokines such as TNF-α, IL-1, IL-6. β-hydroxy fatty acids, especially β-hydroxy myristic acid and β-hydroxy lauric acid, constitute an essential part of lipid A [15].

We hypothesized that nonbacterial β-hydroxy fatty acids, either taken by foods or produced in the body during fatty acid metabolism, by mimicking a chronic Gram-negative bacterial infection, may cause chronic inflammation by increasing cytokine release, which may result in finally IR. This study consisted of two parts. In the first part, we investigated the effects of β-hydroxymyristic acid and β-hydroxylauric acid on cytokine release from human peripheral blood cells. In the second part of this study, we measured anthropometric and biochemical variables and searched whether free β-hydroxymyristic acid and free β-hydroxylauric acid exist in plasma obtained from patients with metabolic syndrome, cancer, RA, and PCOS.

PATIENTS AND METHODS

Healthy subjects and patients

The Ethics Committee of the University of Cumhuriyet approved the present study, and all participants gave written informed consent. Peripheral venous blood samples from healthy volunteer male subjects ($n = 10$) were used for incubation with fatty acids. Plasma samples for fatty acid measurements were obtained from patients with metabolic syndrome ($n = 26$) cancer ($n = 13$), RA ($n = 15$), and PCOS ($n = 24$) according to 2003 Rotterdam criteria [16]. Control patients ($n = 23$) were fibromyalgia since it is a noninflammatory rheumatic disorder. Exclusion criteria included having disorders or using medication known to affect insulin sensitivity and smoking. PCOS patients having hormon therapy in the last 6 months were excluded. Individuals reporting a proinflammatory condition (infections, trauma, etc) as well as those with excessive alcohol intake and unusual dietary habits were also excluded from the study. According to ATP III criteria, the metabolic syndrome was diagnosed in the presence of any three of the following: waist circumference > 102 cm in men and > 88 cm in women, triglyceride ≥ 150 mg/dL (1.7 mmol/L), HDL cholesterol < 40 mg/dL (1.0 mmol/L) in men and < 50 mg/dL (1.3 mmol/L) in women, blood pressure ≥ 130/85 mmHg, or fasting glucose ≥ 110 mg/dL (6.1 mmol/L) [17].

Blood biochemistry and HOMA test of subjects

Blood samples were obtained in the morning at 0800 after a 12-hour overnight fast. Blood specimens were centrifuged and the plasma was immediately frozen and stored at −20°C for analysis. Plasma values of free fatty acids, glucose, insulin, LDL, HDL, hsCRP (high sensitive C-reactive protein), triglyceride, total cholesterol, cytokines (TNF-α, IL-1α, IL-1β, IL-6) were measured from all subjects. Height, weight, and waist circumference (cm) were measured and BMI as well as HOMA index was calculated. Free fatty acids were measured at the University of Atatürk (Erzurum, Turkey) and the University of Yeditepe (Istanbul, Turkey) by collaborating with Dr Fikrettin Şahin by gas chromatography using Sherlock microbial identification system (MIS) software (Microbial ID, Newark, Del, USA) with a database of FAME profiles for eukaryocyte as described [18].

Plasma glucose levels were measured by glucose oxidase method using a commercial kit (IL Test TM Glucose 182508-40 (Instrumentation Laboratory, Italy)), triglyceride levels were measured by enzymatic method using a commercial kit (IL Test TM Triglyceride 182556-40 (Instrumentation Laboratory, Italy)), total cholesterol was measured by bichromatic analysis using a commercial kit (IL Test TM Cholesterol 182505-40 (Instrumentation Laboratory, Italy)), HDL cholesterol was measured using a commercial kit (IL Test TM HDL Cholesterol 182551-40 (Instrumentation Laboratory, Italy)) at an autoanalyzer (IIab 900/1800 Test (USA)) in the Department of Biochemistry of the Cumhuriyet University Hospital. hsCRP was measured by ELISA (BioCheck Inc, USA). IR was estimated by calculating homeostasis model assessment (HOMA-IR) index (fasting serum insulin (μU/mL) multiplied by fasting plasma glucose (mmol/L), then divided to 22.5) [19]. Serum insulin concentrations were measured at the Department of Nuclear Medicine, University of Cumhuriyet, by immunoassay method using a commercial kit (Diagnostic Products Corporation, USA). Sensitivity was 2.0 uIU/mL. Cytokines were measured by using ELISA (BioSource, USA).

Whole blood cytokine responses to beta-hydroxy fatty acids

β-hydroxy myristic acid and β-hydroxy lauric acid were purchased (Sigma-Aldrich, Germany), dissolved in 95% ethanol (10 mg/mL as stock solution), and incubated with whole blood as described [20]. Briefly, heparinized blood freshly taken from healthy volunteers ($n = 10$) was diluted fivefold with RPMI 1640 (Sigma-Aldrich, Germany) containing 2.5 IU heparin (Vem, Turkey), 100 IU penicillin (IE Ulagay, Turkey), and 100 μg streptomycin (IE Ulagay, Turkey) per mL. One milliliter of blood samples was used. After addition of β-hydroxy myristic acid or β-hydroxy lauric acid as 100 ng/mL (0.5 μM), 1 μg/mL (5 μM), and 10 μg/mL (50 μM), the samples were incubated in polypropylene vials in the presence of 5% CO_2 at 37°C for 0, 1, 2, 4, 8, and 24 hours. Ethanol (95%) was added to control vials as 0.0095 μL/mL, 0.095 μL/mL, and 0.95 μL/mL, respectively. Then, after shaking, the cells were pelleted by centrifugation (400 xg, 2 min) and the cell-free supernatants were stored at −80°C for cytokine measurements. Cytokines (TNF-α, IL-1α, IL-1β, IL-6) were measured by ELISA as described by manufacturer (BioSource, Germany).

Statistics

Data were analyzed by unpaired t test. Results are expressed as the mean ±SEM; $P < .05$ was considered statistically significant.

RESULTS

The results of pilot experiments have shown that the amounts of TNF-α, IL-1α, and IL-1β were not at detectable level at all concentrations and incubation times for both β-hydroxy myristic acid and β-hydroxy lauric acid. On the other hand, IL-6 reached detectable levels at 24-hour incubation with β-hydroxy lauric acid but not with β-hydroxy myristic acid and the experiments were carried out by incubation of 1 mL whole blood with 100 ng and 10 μg of β-hydroxy lauric acid for 24 hours ($n = 10$). Amount of IL-6 released displayed different profile (Table 1). At 100 ng, 5 out of 10 subjects had higher, 4 out of 10 had lower, and 1 out of 10 had equal amount of IL-6 released compared to vehicle control (537.5 ± 249.5 versus 494.5 ± 229.2 pg/mL, $P = .46$). At 10 μg, the difference was significant and 7 out of 10 had higher and the rest 3 had almost equal amount of IL-6 released compared to vehicle control (533.9 ± 218.1 versus 438, 3 ± 219.6 pg/mL, $P = .019$). 9 out of 10 subjects have

Table 1: Amounts of IL-6 released from whole blood obtained from healthy men following incubation with 100 ng or 10 μg of β-hydroxy lauric acid for 24 hours ($n = 10$, V; vehicle). Results were expressed as pg/mL (compared to vehicle, NS; not significant).

	Subjects										means ± SEM	P
	1	2	3	4	5	6	7	8	9	10		
100 ng	500	131	106	478	2115	31	56	19	67	1872	537.5 ± 249.5	NS
V1	119	81	52	385	1837	52	274	130	141	1874	494.5 ± 229.2	
10 μg	22	104	54	652	1811	92	370	256	200	1778	533.9 ± 218.1	< .05
V2	7	104	69	452	1663	23	107	37	119	1802	438.3 ± 219.6	

Table 2: Anthropometric and biochemical variables of patients with metabolic syndrome, RA, and cancer. Data were presented as means ± SEM (compared to control, $^{*}P < .001$, $^{**}P < .01$, $^{\S}P < .05$).

	Control	Metabolic syndrome	Cancer	RA
N	23	26	13	15
Age (y)	43.5 ± 2.3	45.11 ± 2.0	57.6 ± 4.3**	53.9 ± 2.4**
Sex (m/f)	6/17	7/19	7/6	4/11
BMI (kg/m^2)	29.5 ± 1.1	35.5 ± 1.3*	22.2 ± 1.2*	28.9 ± 1.3
Waist circumference (cm)	91.8 ± 3.6	105.5 ± 2.7**	90.4 ± 2.9	95.9 ± 3.2
Fasting glucose (mg/dL)	88.2 ± 2.3	96.0 ± 3.2	114.3 ± 16.1§	89.9 ± 5.0
HOMA-IR	2.3 ± 0.3	5.7 ± 1.2**	2.8 ± 0.8	3.0 ± 0.7
hsCRP (mg/L)	6.0 ± 1.1	9.0 ± 0.9§	20.7 ± 2.3*	14.3 ± 2.1**
HDL (mg/dL)	37.0 ± 1.7	44.6 ± 2.5§	31.2 ± 3.3	53.9 ± 4.8**
LDL (mg/dL)	103.1 ± 10.3	116.6 ± 7.8	84.4 ± 8.6	107.2 ± 8.6
Triglyceride (mg/dL)	135.2 ± 12.4	195.3 ± 19.3§	161.2 ± 35.8	124.4 ± 11.3
Total cholesterol (mg/dL)	170.8 ± 9.2	200.5 ± 9.5	147.7 ± 13.6	183.7 ± 10.5
TNF-α(pg/mL)	1477.3 ± 240.1	1431.8 ± 114.4	1467.7 ± 136.3	1552.1 ± 215.1
IL-6 (pg/mL)	27.7 ± 4.4	21.5 ± 4.2	63.5 ± 8.6*	41.1 ± 11.7
Weight (kg)	77.2 ± 2.4	90.4 ± 2.9**	63.2 ± 3.6**	73.7 ± 3.1

higher amount of IL-6 released at either 100 ng or 10 μg compared to vehicle control. Only 1 subject had at almost equal amount of IL-6 released at both dose.

Anthropometric and biochemical variables in patients were presented in Tables 2 and 3. BMI, waist circumference, HOMA index, the amounts of hsCRP, HDL, triglycerides, and weight were significantly higher in patients with metabolic syndrome compared to control. Age, fasting glucose, hsCRP, and IL-6 concentrations were significantly higher in cancer patients compared to control. On the other hand, BMI and weight were significantly lower in cancer patients. Age, hsCRP, and HDL concentration were significantly higher in RA patients compared to control. PCOS patients were compared with only female controls (Table 3). Age, BMI, waist circumference, the concentration of IL-6, and weight were significantly lower in patients with PCOS compared to control. The amounts of major free fatty acids, as a % of total amount, were shown in Tables 4 and 5. We were not able to detect either β-hydroxy myristic acid or β-hydroxy lauric acid in our subjects. PCOS patients were compared with only female controls (Table 5).

DISCUSSION

The relationship between dietary fatty acids and inflammation has been shown by in vivo and in vitro studies [21–24]. Saturated, particularly lauric, fatty acids seem to induce cytokine production as well as expression of cyclooxygenase-2 in vitro [25]. In addition, Weatherill et al [26] have found that lauric acid upregulated the expression of cytokines (IL-12p70 and IL-6) in bone marrow-derived dendritic cells. On the other hand, eicosapentaenoic acid ($20 : 5$, $n - 3$) and docosahexaenoic acid ($22 : 6$, $n - 3$) inhibited in vitro production of IL-6 by human endothelial cells [27]. Supplementation of a diet with EPA and DHA also reduced cytokine production in human in vivo [22, 23]. Prospective studies indicate that increased IL-6 and CRP concentrations as well as fatty acid composition are associated with IR and cardiovascular events [28–30].

This study presents a novel approach to understand the relationships between fatty acids, chronic inflammation, and IR. As far as we know, this is the first study to investigate the role of β-hydroxy fatty acids on inflammation and IR both in vivo and in vitro. We found that β-hydroxy lauric acid increased the amount of IL-6 released, proinflammatory cytokine, by human blood cells in vitro. At high concentration (10 μg/mL), the effect of β-hydroxy lauric acid reached statistical significance ($P < .05$). β-hydroxy myristic acid did not increased the amount of IL-6 released by peripheral human blood cells in vitro. Since there is a strong association between mediators of inflammation (such as IL-6) and IR, it was important to measure

Table 3: Anthropometric and biochemical variables of patients with PCOS. Data were presented as means ± SEM (compared to control, $^{*}P < .001$, $^{**}P < .01$, $^{\S}P < .05$).

	Control	PCOS
N	17	24
Age (y)	44.5 ± 2.9	24.7 ± 1.2*
BMI (kg/m^2)	31.3 ± 4.7	25.9 ± 1.3**
Waist circumference (cm)	92.8 ± 4.4	82.0 ± 3.0§
Fasting glucose (mg/dL)	90.6 ± 2.3	85.8 ± 1.9
HOMA-IR	2.4 ± 0.4	2.0 ± 0.3
hsCRP (mg/L)	6.1 ± 1.2	6.0 ± 1.0
HDL (mg/dL)	38.6 ± 2.2	42.6 ± 2.7
LDL (mg/dL)	96.9 ± 10.1	111.5 ± 9.1
Triglyceride (mg/dL)	140.8 ± 15.9	140.9 ± 13.1
Total cholesterol (mg/dL)	169.3 ± 10.2	181.0 ± 9.7
TNF-α(pg/mL)	1566.2 ± 305.3	1334.0 ± 125.1
IL-6 (pg/mL)	24.2 ± 5.4	5.2 ± 1.8**
Weight (kg)	77.5 ± 3.0	66.8 ± 3.4§

Table 4: The amounts of major free fatty acids (FFAs) as % of total amount in plasma of patients with metabolic syndrome, RA, and cancer. Results were expressed as means ± SEM (compared to control, $^{*}P < .001$, $^{**}P < .05$).

FFAs	Control ($n = 23$)	Metabolic syndrome ($n = 26$)	Cancer ($n = 13$)	RA ($n = 15$)
14 : 0	1.99 ± 0.27	2.02 ± 0.14	1.41 ± 0.29	1.82 ± 0.20
16 : 0	26.96 ± 0.64	26.95 ± 0.56	25.73 ± 0.75	29.14 ± 0.83**
16 : 1 ($n - 7$)	4.20 ± 0.51	5.21 ± 0.28	4.48 ± 0.56	4.06 ± 0.43
18 : 0	7.09 ± 0.35	5.76 ± 0.42**	4.62 ± 0.18*	8.04 ± 0.50
18 : 1 ($n - 9$)	19.43 ± 0.48	19.56 ± 0.60	19.25 ± 0.81	21.57 ± 1.00**
18 : 2 ($n - 6$)	35.06 ± 1.44	32.72 ± 0.83	36.54 ± 1.93	30.73 ± 1.64
20 : 4 ($n - 6$)	4.72 ± 0.42	5.97 ± 0.41**	7.58 ± 0.62*	4.40 ± 0.57
20 : 3 ($n - 6$)	2.19 ± 0.21	1.95 ± 0.01	1.76 ± 0.28	2.53 ± 0.42

Table 5: The amounts of major free fatty acids (FFAs) as % of total amount in plasma of patients with PCOS. Results were expressed as means ± SEM (compared to control, $^{*}P < .001$, $^{**}P < .01$, $^{\S}P < .05$).

FFAs	Control ($n = 17$)	PCOS ($n = 24$)
14 : 0	1.64 ± 0.09	2.40 ± 0.31§
16 : 0	27.19 ± 0.90	23.90 ± 0.50**
16 : 1 ($n - 7$)	4.02 ± 0.43	3.69 ± 0.29
18 : 0	7.30 ± 0.42	4.86 ± 0.19*
18 : 1 ($n - 9$)	19.47 ± 0.64	16.50 ± 0.52*
18 : 2 ($n - 6$)	35.88 ± 1.88	39.45 ± 1.21
20 : 4 ($n - 6$)	4.98 ± 0.50	7.63 ± 0.27*
20 : 3 ($n - 6$)	2.05 ± 0.24	2.00 ± 0.01

β-hydroxy lauric acid in plasma of patients with chronic inflammatory diseases associated with IR. HOMA-IR index was higher in cancer, RA, and metabolic syndrome patients, but not in PCOS patients. However, we did not detect free β-hydroxy lauric acid in plasma. This could be explained in two ways. The first one is that there was no free β-hydroxy lauric acid in samples. The second one is that the amount was not at detectable range. The method we used allowed us to measure free fatty acids as only a percent in total.

A major component of LDL is phosphatidylcholine (PC) and 40% of LDL-PC can be converted to lyso-PC by phospholipase A_2 activity during oxidative modification [31]. Lyso-PC stimulated human monocytes to produce IL-1β, on both protein and mRNA levels, in a dose and time-dependent manner [32]. The acyl chain length and saturation of lyso-PC were important for this stimulating effect. There were no effects when the acyl chain of lyso-PC was less than C16 and lyso-PC 18 : 1 had much less effect than lyso-PC 18 : 0. Although we did not determine free β-hydroxy lauric acid in plasma of patients with chronic inflammation associated with IR, it stimulated the release of IL-6 in culture conditions. Therefore, further studies may be required to investigate the existence of β-hydroxy lauric acid in the structure of complex lipids such as phospholipids, lipoproteins, and so forth. The results of those studies might be helpful to understand the involvement of fatty acids as an endogenous molecule in sterile chronic inflammation and IR.

ACKNOWLEDGMENTS

This study was supported by Cumhuriyet University Scientific Research Council (CUBAP, Project no T:219). We are grateful to Dr Fikrettin Şahin for his technical support during measurement of free fatty acids from plasma. This work was performed partly at Cumhuriyet University Medical School Research Center (CUTFAM).

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