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Comparison of postprandial endotoxemia in male adolescents and male subjects above 50 years after a fat overload

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A B S T R A C T

The objective of this study was designed to test the hypothesis that male adolescents and male subjects after the age of 50 years may be independently associated with postprandial endotoxemia and indirectly associated with atherosclerosis. 90 male adolescents below 18 years and 90 male subjects above 50 years were selected for the study were evaluated for weight, eating habits, physical activity, body circumferences, fasting blood glucose level, postprandial blood glucose level and insulin level. The lipopolysaccharide (LPS) levels and circulating lipopolysaccharide-binding protein (LBP) were determined in serum at fasting, 1 hr, 2 hrs, 3 hrs, and 4 hrs after a fat overload and their levels related in the above groups. Serum LPS concentrations were measured by endotoxin assay, based on a Limulus amoebocyte extract with a chromogenic LAL assay. The male group aged 50 years plus showed a significant increase ($P < 0.05$) in LPS levels and circulating LBP in serum after the fat overload, but the increase was less so in the male adolescent group. Elevated LPS and circulating LBP were associated significantly with male subjects age 50 years and above, especially after a fat overload. These findings suggested a role of LPS and LBP in atherosclerosis and other diseases. Prospective studies are needed to confirm these results.

Introduction

Endotoxin (lipopolysaccharide [LPS]) is one of the potent virulence factors of Gram-negative bacterial species and has a major role in both acute and chronic infections [1]. Circulating endotoxin may derive from bacteria causing either overt acute infections

or common chronic conditions. Additionally, endotoxin is believed to translocate from microbiota in the gut. In humans, energy-enriched diets increasing weight gain and insulin resistance associate with absorption of endotoxin from the

gastrointestinal track [2-4]. This “metabolic endotoxemia” resulting from the increased intestinal permeability/motility may lead to low grade inflammation. Severity of inflammation may depend on a complex interplay between specific proteins, receptors, and lipoproteins that mediate the endotoxin bioactivity and metabolic fate.

Many important disease pathologies are now being investigated under the lens of metabolic endotoxemia, including atherosclerosis, diabetes and insulin resistance, Parkinson’s disease, and cancer. Several hypotheses have been developed regarding the sources of this low-grade LPS exposure, including smoking, aging, chronic heavy alcohol consumption, high fat diet and periodontal disease [5].

Endotoxin induces multiple biological effects in vivo, for example, fever, leukocytosis, hypoferrinemia, platelet aggregation, thrombocytopenia, and coagulopathies [6-8]. These effects can be attributed to activation of various endogenous pathways or cascade mechanisms. For example, LPS triggers the complement, coagulation, fibrinolytic, and kinin pathways to release vasoactive peptides and also the release of, an array of cytokine mediators from macrophages and monocytes.

The major metabolic consequence of a high-fat diet is that insulin action and the regulatory mechanisms of body weight are impaired through a well-described lipotoxic Effect [9]. In addition, it has been recently determined that obesity and insulin resistance are associated with low-grade chronic systemic inflammation [10]

Elevated concentrations of circulating LPS correlate well with an increased atherosclerosis risk [11], whereas in vitro

studies have shown LPS to potently up-regulate atherogenic gene expression [12], cholesterol retention, and foam cell formation [13]. Moreover, LPS injection accelerates the formation of plaque in both mice [14] and rabbits [15], whereas genetic deletion of the LPS receptor Toll-like receptor 4 significantly reduces the development of plaque in apolipoprotein E-deficient mice [16].

Thus, endogenous LPS is continuously produced in the gut by the death of Gram-negative bacteria and physiologically translocated into intestinal capillaries through a TLR4-dependent mechanism [17] transported from the intestine toward target tissues by a mechanism facilitated by lipoproteins, notably chylomicrons freshly synthesized from epithelial intestinal cells in response to a high-fat diet [18]; and triggers the secretion of proinflammatory cytokines when it binds to the complex of mCD14 and the TLR4 at the surface of innate immune cells [19,20].

Therefore, we aimed to demonstrate that LPS would be an early factor in the triggering of high-fat diet-induced metabolic diseases. Very few data exist regarding the response of endotoxemia in male adolescents and male subjects above 50 years after a fat overload. Therefore, this study was undertaken with the following objectives:

- (1) To find the relationship of postprandial endotoxemia between male adolescents (MA18) and male subjects above 50 years (MA50) after a fat overload.
- (2) Whether male subjects above 50 years of age has got relationship with increased postprandial endotoxins and atherosclerosis

Materials and Methods

There are more than 20 assays for the detection of endotoxin [21], of which three have been used for the detection of endotoxin in clinical specimens: the rabbit pyrogen assay, the LAL bioassay, and immunoassays. The method of choice is the LAL assay.

Patients inclusion and exclusion criteria

This study was conducted in accordance with the ethical rules of the Helsinki Declaration. The study was approved by the Ethics Committee of the hospital, and all women gave written informed consent. Prior to the study, participants were informed that their confidentiality would be maintained and consent was obtained. 90 male adolescents below 18 years and 90 male subjects above 50 years were selected for the study. Patients were excluded if they had cardiovascular disease, arthritis, acute inflammatory disease, infectious disease, renal disease, were receiving treatment for hyperlipidemia or diabetes or were taking medications that could influence gastric emptying or the absorption time. Obese people were also excluded.

Preparation of patients and sample collection

On the morning of the visit, blood pressure, weight, and height were measured and compliance with dinner instructions was verified with a questionnaire. After that, each participant underwent a structured examination, which included an interview. Height, weight, waist circumference (WC) and hip measurements, a fasting venipuncture, and sequential determination of serum lipids were done. Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively. BMI was

calculated as weight (kilogram) divided by height (in meter) squared. WC was determined to the nearest 0.1 cm using a measuring tape positioned at the midpoint between the lowest rib and the iliac crest and hips were measured at the largest gluteal circumference. These measurements were used to calculate the waist-to-hip ratio (WHR).

Then, blood pressure was measured using a standard mercury sphygmomanometer. Blood samples were obtained from the antecubital vein and placed in vacutainer tubes. Postprandial blood samples were taken 1, 2, 3, and 4 hr after the end of the study meal. Samples were centrifuged; serum was collected and stored at 20 °C until analyzed. The diagnosis of DM was based on WHO criteria, i.e. a fasting plasma glucose level > 7.0 mmol/L or > 126 mg/dL, or a 2-h postprandial plasma glucose level > 11.1 mmol/L or > 200 mg/dL on more than one occasion, with symptoms of diabetes.

Serum LPS concentrations were measured by endotoxin assay, based on a Limulus amoebocyte extract with a chromogenic LAL assay (QCL-1000, Lonza Group Ltd.). Samples were diluted in pyrogen-free water and heated at 70°C for 10 min to inactivate endotoxin-neutralizing agents that inhibit the activity of endotoxin in the LAL assay. Internal control of recovery calculation was included in the assessment. All samples were tested in duplicate. The endotoxin content was expressed as endotoxin units (EU) per mL. Exhaustive care was taken to avoid environmental endotoxin contamination and all material used for sample preparation and the test was pyrogen-free. Plasma LBP levels were determined by a sandwich ELISA Technology. Plasma samples were diluted at least 200 times and assayed according to the manufacturer's instructions. The assay has a

sensitivity of 0.2 ng/ml. The intra-assay and interassay coefficients of variation were < 5 and < 10%, respectively.

Statistical analysis

All data were entered into an Excel spreadsheet, and were analyzed using standard statistical software such as SPSS. Chi-square test was used for categorical variables. All numerical data were presented as mean \pm standard deviation. A P value of less than 0.05 was considered statistically significant.

Result and Discussion

The mean BMI values were 20.0 ± 2.2 kg/m² in MA18 and 23.0 ± 3.1 kg/m² in MA50 (Table 1). The mean waist circumference was 69.9 ± 5.7 in MA18 and 72 ± 8.2 in MA50. The mean systolic blood pressure (mmHg) was 108.4 ± 9.3 in MA18 and 111.4 ± 12.3 in MA50, whereas the diastolic blood pressure (mmHg) was 71.9 ± 8.8 in MA18 and 75.9 ± 5.8 in MA50. Compared with MA18, MA50 were more likely to have higher values for waist circumference, blood pressure, glucose. Fasting plasma glucose and postprandial plasma glucose were in the range of normal in both categories (87 ± 6 mg/dL in MA18 and 89 ± 18 mg/dL in MA50).

The mean Plasma endotoxin (LPS) in MA18 in EU/mL was 0.30, 0.32, 0.37, 0.34 and 0.33 at fasting, 1, 2, 3, and 4 hr vs. 0.36, 0.39, 0.67, 0.63 and 0.56 in the MA50 during the same duration. The mean LPS binding protein (LBP) μ g/ml was 9.08, 10.3, 11.9, 11.8 and 11.5 at fasting, 1, 2, 3, and 4 h in the MA18 vs. 11.9, 13.4, 18.8, 15.7 and 13.8 in the MA50 during the same duration. Serum endotoxin activity had a significant positive correlation with MA50 ($P < 0.05$).

We have observed that endotoxemia (circulating endotoxin) associates more with MA50 than with MA18. The difference between the endotoxin circulating concentrations in MA50 suggest that the increased endotoxemia relates to age rather than only to the fat content or other conditions. Although age per se is a chronic low grade inflammatory condition even in lean subjects [22]. There is no information to date relating to endotoxin levels in aged.

Epidemiological studies have previously shown that increased endotoxin load, which can be a result of increased population s of endotoxin producing bacteria in the intestinal tract, is associated with certain obesity-related patient groups. Other studies have shown that purified endotoxin from *Escherichia coli* can induce obesity and insulin-resistance phenotypes when injected into germ-free mouse models. A more recent study has uncovered a potentially contributing role for *Enterobacter cloacae* B29 toward obesity and insulin resistance in a human patient. The presumed mechanism for the association of endotoxin with obesity is that endotoxin induces an inflammation-mediated pathway accounting for the observed obesity and insulin resistance. Interestingly, several reports have demonstrated clinical associations between circulating low levels of endotoxin and risk of cardiovascular disease. The first of these studies was published only twelve years ago from an Italian cohort and found that subjects with circulating endotoxin levels at 50 pg/ml or greater had a 3-fold greater risk of cardiovascular disease than those with circulating concentrations under 50 pg/ml LPS [23,24]. Further, a second major study demonstrated increased that circulating endotoxin levels among different ethnic groups correlated strongly with differences in risk factor for the development of cardiovascular disease amongst the different groups.

Table 1

	Male adolescents (MA18)	Male subjects above 50 years (MA50)
Age (years)	16-20	50+
BMI (kg/m ²)	20.0 ± 2.2 kg/m ²	23.0 ± 3.1 kg/m ²
Waist circumference (cm)	69.9 ± 5.7	72 ± 8.2
Systolic blood pressure (mmHg)	108.4 ± 9.3	111.4 ± 12.3
Diastolic blood pressure (mmHg)	71.9 ± 8.8	75.9 ± 5.8
Fasting Plasma Glucose (mg/dL)	87±6	89±18

Table 2

	Fasting	1 hr	2 hr	3 hr	4hr	P value
Plasma endotoxin (LPS) in MA18 (EU/mL)	0.30	0.32	0.37	0.34	0.33	NS
Plasma endotoxin (LPS) in MA50 (EU/mL)	0.36	0.39	0.67	0.63	0.56	P < 0.05
LPS binding protein (LBP) in MA18 (µg/mL)	9.08	10.3	11.9	11.8	11.5	NS
LPS binding protein (LBP) in MA50 (µg/ml)	11.9	13.4	18.8	15.7	13.8	P < 0.05

In addition to the modifications of inflammatory tone, several studies have also proposed that the gut microbiota participate in the control of food intake and fat mass development via several mechanisms including endogenous gut peptides production involved in food intake and energy homeostasis [25-28].

Most of the previous studies in this area have shown postprandial inflammation after a high-fat meal, and it was shown very recently that circulating endotoxin increases 2- to 3-fold in mice fed a high-fat diet [29].

A number of recent studies have documented that experimental endotoxemia mimicked by exposure to LPS in humans induces AT inflammation and insulin resistance through the activation of the AT secretome [30-32]. The activation of TLR4,

by the CD14–LPS complex is a proximal signalling step engaging the innate immune system into the fight against bacterial pathogens which is functional in adipocytes [33,34]. TLR4 signalling progresses toward the generation of an intracellular cytokine cascade through nuclear factor κ -light-chain-enhancer of activated B cells activation [35,36]. Accumulation of macrophages in AT of obese pregnant women was accompanied by the enhanced expression of TLR4-associated genes as well as genes for chemotactic and inflammatory cytokines.

A similar activation pattern was replicated in vitro by incubating isolated adipose stromal cells with LPS. Taken together, our data suggest that increased systemic LPS in the plasma of obese pregnant women may represent an exogenous stimulus to activate cellular signals leading to adipocytokines production. The recent observation that diet

induced modification of the gut flora with probiotics ameliorates the insulin resistance and glucose homeostasis of pregnant women brings support to our current hypothesis [37].

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