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Association between ferritin and hepcidin levels and inflammatory status in patients with type 2 diabetes mellitus and obesity

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ABSTRACT

Objective: The aim of this study was to determine the association between iron parameters and inflammation in obese individuals with and without type 2 diabetes mellitus (T2DM).
Methods: We studied 132 obese individuals (OB), 60 individuals with T2DM, 106 obese individuals with T2DM (T2DOB), and 146 controls (C). All of were men aged >30 y. Biochemical, iron nutrition, and oxidative stress parameters were determined. Peripheral mononuclear cells were isolated and total RNA was extracted to quantify tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB, interleukin (IL)-6, toll-like receptor (TLR)-2/4 and hepcidin by quantitative reverse transcription polymerase chain reaction.
Results: OB, T2DM, and T2DOB individuals had higher ferritin, retinol-binding protein 4, and thiobarbituric acid reactive substance (TBAR) levels than controls. T2DOB and T2DM individuals showed high high-sensitivity C-reactive protein (hsCRP) levels and OB with and without T2DM had elevated levels of serum hepcidin. Heme oxygenase activity was high in OB and T2DM and there were no differences observed in superoxide dismutase and glutathione parameters. A correlation between TBARS and ferritin in T2DOB was observed ($r = 0.31$; $P < 0.006$). Multiple linear regression analysis showed an association between diabetes and obesity with ferritin, TBARS, and hsCRP levels. The upper quartiles of ferritin, TBARS and hepcidin showed an adjusted odd ratio for T2DM of 1.782, 2.250, and 4.370, respectively. TNF-α, IL-6, hepcidin, NF-κB, TLR-2/4 mRNA abundances were increased in T2DM and T2DOB.
Conclusion: Elevated hsCRP and hepcidin levels, and increased gene expression of TNF-α, IL-6, NF-κB, and TLR-2/4 in patients with diabetes, obesity, or both exacerbate and perpetuate the insulin resistance and inflammatory state.

Introduction

Obesity and type 2 diabetes mellitus (T2DM) present mild chronic inflammation and insulin resistance (IR) in tissues such as adipose, muscle, pancreas, and liver [1,2]. Obesity generally precedes diabetes because visceral adipose tissue has particular cellular and metabolic characteristics. This condition generates IR and hyper-lipolytic effects [3]. Adipose tissue is an endocrine organ that secretes cytokines, adipokines, chemokines, and different growth factors [4–6].

Proinflammatory cytokines play a role in obesity and T2DM development. Immune tissues participate in IR and T2DM development [7,8], where glucose levels and macronutrients (saturated fatty acids) [9], oxidative stress (OS) [10], and micronutrients–specifically iron [9]–are key components. A clear association has been found between obesity, metabolic syndrome, T2DM and the immune system [11]. Toll-like receptors (TLRs) are proteins that participate in the development of inflammatory diseases. TLRs 2 and 4 are involved in IR and T2DM because they

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recognize fatty acids, glucose, advanced glycated end products, reactive oxygen species (ROS), and lipid peroxidation products [12]. Stimulation of TLR-2/4 activates nuclear factor (NF)-κB through nicotinamide adenine dinucleotide phosphate oxidase and monocyte chemoattractant protein-1, which stimulates macrophages, as well as proinflammatory cytokine synthesis [11, 13]. Also, adipocytes infiltrated for macrophages disrupt homeostasis by exacerbation of inflammatory response [14]. The activation of NF-κB by TLR-2/4 in adipocyte and others cellular types trigger the increasing of synthesis of tumor necrosis factor (TNF)-α [15], interleukin (IL)-6, and IL-1 [16–18].

Iron (Fe) has a potential role in diabetes [19], as it is a powerful prooxidant that causes an increase in ROS and OS, contributing directly to tissue damage, raising the risk for diabetes [20]. In addition to being the main marker of iron status, ferritin is an acute-phase protein whose levels increase during inflammatory processes, even in Fe-deficient conditions. Ferritin production is induced in macrophages, hepatocytes, and adipocytes through TNF-α and IL-1 action. High ferritin levels in T2DM have been associated to increased glucose and insulin levels, as well as hypertension, dyslipidemia, and obesity [21]. Hepcidin (Hpc), a 25 amino acid hormone, is synthesized in liver, adipose tissue, pancreas, and intestinal cells. Hpc synthesis is stimulated by iron stores, during infections and inflammations and downregulated during erythropoiesis and hypoxia [18]. Hpc negatively regulates Fe input into the bloodstream through the internalization and subsequent lysosomal degradation of the Fe output conveyor ferroportin (FPN; IREG1) in duodenum and macrophages [22]. Individuals with T2DM possess high levels of Hpc mRNA, and protein, which correlates positively with IL-6 and ferritin levels [23]. IL-6 regulates Hpc synthesis at a transcriptional level through STAT-3, inducing an increase in iron storage levels in macrophages [23].

Both OS and inflammation interact in chronic diseases development such as atherosclerosis, kidney failure, cancer, and T2DM [24]. Heme oxygenase (HO1), an inducible enzyme that catalyzes the degradation of heme to carbon monoxide (CO), biliverdin and Fe^{2+}, regulates cell proliferation, differentiation, and apoptosis as well as attenuates inflammation and modulates the immune response [25]. HO1 possesses binding sites for NF-κB and glucocorticoid response elements, where it might play a role in reducing inflammation during OS and glucose regulation [26].

The aim of this work was to study the association between general nutritional status, OS, Fe nutrition, and inflammation in obese individuals with or without T2DM.

Methods

Study participants

We studied 444 individuals: 106 obese with type 2 diabetes (T2DOB); 132 obese nondiabetic (OB) individuals; 60 (T2DM) patients with T2DM and a normal body mass index (BMI); and 146 healthy individuals (C group). All individuals were men aged >30 y. T2DOB and T2DM patients with insulin treatment were not included in the study. Patients with diabetes were undergoing treatment with metformin. None of the individuals were supplemented with minerals. The protocol was approved by the ethical committee of the Institute of Nutrition and Food Technology, University of Chile and a signed consent form was obtained from all participants.

Anthropometric examination and blood sampling

All patients were weighed, measured, and their BMI was calculated. Also, waist circumference and blood pressure was determined. Blood samples (30 mL) were obtained after overnight fasting. Eighteen mL of blood was used to measure biochemical indicators: glycemia (Dialab, Austria); insulin (radioimmunoassay, Siemens, Los Angeles, CA, USA); lipid profile (Dialab, Austria); high-sensitivity C-reactive protein (hsCRP; Orion Diagnostica, Espoo, Finland), retinol-binding protein 4 (RBP4; by enzyme-linked immunosorbent assay [ELISA]); thiobarbituric acid reactive substances (TBARS; OxiSelect™ TBARS Assay Kit, Cell Biolabs, San Diego, CA, USA); Glutathione (GSH) and total superoxide dismutase (SOD) activity (ELISA kit, Cayman Chemical Com, An Arbor, Michigan, USA). Hematologic and Fe nutrition status such as hemoglobin (Cell Dyn 3200 counter; Abbott Laboratories, Abbott Park, IL, USA), ferritin (measured by using ELISA; Dako Corp, Carpinteria, CA, USA), and Hpc (by ELISA, DRG Instruments GmBH, Germany); Fe and total iron-binding capacity (TIBC; by colorimetric method using tripyridyl-s-triazine [27]); soluble transferrin receptor (TfR; ELISA, Ramco, Stafford, TX, USA). Total body iron (TBI) was calculated according to previous work [28].

Peripheral mononuclear cell isolation and HO1 enzymatic activity

A 12-mL blood sample was collected with EDTA anticoagulant for peripheral mononuclear cell (PMC) isolation. PMCs were separated by Ficoll-Histopaque (1.119 density, Sigma, St. Louis, MO, USA). The mononuclear layer was removed and washed twice in phosphate-buffered saline, adjusted to 40×10^6 PMCs/mL using RPMI-1640 media with gentamicin, and stored until further procedure. HO1 enzymatic activity was measured according to previous work [29].

Quantitative real-time PCR

RNA was extracted from PMCs using Trizol Reagent (Invitrogen) and treated with RNase-Free DNase Set (Qiagen) according to product protocol. Total RNA (1.5 μg) was reverse transcribed using an AffinityScrip cDNA Synthesis Kit (Stratagene). Quantitative polymerase chain reaction (qPCR) was performed using Brilliant II SYBR™ Green QPCR Master Mix (Stratagene) in a Max Pro™ System 3000. Glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) and beta-2-microglobulin (B_2M) were used as housekeeping genes. The primers used were (5′ and 3′, respectively): **GADPH**: CCAGCAAGAGCACAAGAGGA and TCAAGGGGTCTAC ATGGCAA; B_2M: GATGCCGCATTTGGATTGGA and TGGAGCAA CCTGCTCAG ATA; **Hpc**: GACACCAGAGCAAGCTCAA and GAAAACAGAGCCA CTGGTCA; **NF-κB**: TGCATCCAAAGGTGCTCAGA and GCAGCTGGCAAAGCTTAGTA; **IL-6**: ATGTCTGAGGCTCATTCTGC and GCGGCTACATCTTTGGAATC; **TNF-α**: GTTCC TCAGCCTCTTCTCCT and ACAACATGGGCTACAGGCTT; **TLR-2**: AGATGCCTCC CTCTTACCCATGTT and AAGACTTTGGCCAGTGCTTGCT; **TLR-4**: AGGAACAGT GGGTACAGGATGCAA and TCACCCTTAGCATAAGGCCTGACA. PCR amplification efficiency of each primer pair was calculated from the standard curve slope. Final results were reported according to Pfaffl method [30].

Statistical analysis

All variables were checked for normality using Shapiro Wilk test. Anthropometric and biochemical results were expressed as means ± SEM. Anthropometric and biochemical parameter differences between groups were evaluated using one-way analysis of variance and Dunnett's as a post hoc test. Gene expression was analyzed using the Kruskal-Wallis test. For correlation analysis a Pearson's test was performed. STATA 11.0 software was used for linear and logistic regressions. For logistic regression, ferritin and Hpc mRNA expression levels were divided as quartiles. Statistical significance was assigned to $P < 0.05$.

Results

T2DOB and OB showed altered anthropometric parameters such as weight, BMI, and abdominal circumference. T2DM and T2DOB showed increased levels of glycemia and basal insulin, and OB individuals showed higher levels of insulin than C groups. Lipid profiles were similar between groups, except for high-density lipoprotein cholesterol, which remained higher in OB individuals compared with C (Table 1).

OB, T2DM, and T2DOB patients showed increased levels of ferritin. There was no difference in TfR levels. Nevertheless, TBI was higher in OB and T2DOB patients compared with the C group. RBP4 was increased in all studied groups compared with the C group. Hpc protein was elevated in all studied groups and a positive correlation was found between serum Hpc levels and TBI ($r = 0.45$; $P < 0.001$). hsCRP levels were increased mainly in T2DM and T2DOB patients. T2DM and OB patients displayed the highest HO activity and OB, T2DM, and T2DOB groups had higher TBARS levels than C subjects (Table 2). There were no significant