Plasma Thiobarbituric Acid-Reactive Substance Levels in Subclinical Hypothyroidism

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Key Words
Thiobarbituric acid-reactive substance · High-sensitive C-reactive protein · Subclinical hypothyroidism

Abstract
Objective: The purpose of this study was to determine thiobarbituric acid-reactive substance (TBARS) levels in subclinical hypothyroidism and to examine the effect of levothyroxine replacement on TBARS levels. Subjects and Methods: A cohort of 28 female patients with subclinical hypothyroidism and 24 healthy controls were enrolled in this study. The levels of plasma TBARS, serum lipids, and high-sensitive C-reactive protein (CRP) in patients with subclinical hypothyroidism at baseline and after achieving euthyroid state by levothyroxine were assessed. Results: TBARS levels of the patients were similar to those of the control group in the subclinical hypothyroid state and after restoration of euthyroidism by levothyroxine replacement. TBARS levels decreased after levothyroxine treatment, but did not reach statistical significance. There was no significant correlation between TBARS, lipid and CRP levels. Serum CRP levels were higher in subclinical hypothyroidism (4.28 ± 0.9 mg/l) than in the control group (1.95 ± 0.34 mg/l) and the difference was statistically significant (p = 0.03). After achieving euthyroid state, CRP levels decreased significantly in patients with subclinical hypothyroidism from 4.28 ± 0.9 to 2.32 ± 0.6 mg/l (p = 0.006). Conclusion: Our findings suggest that there is no significant alteration of plasma TBARS levels neither in subclinical hypothyroid state nor after achieving euthyroid state. Serum CRP level is higher in patients with subclinical hypothyroidism than in the control group. Normalization of thyroid state seems to effectively reduce serum CRP levels in subclinical hypothyroidism without any correlation with TBARS activity.

Introduction

The stages for treatment of subclinical hypothyroidism are progression to overt hypothyroidism, poor quality of life related to nonspecific symptoms, and suspected association with atherosclerosis [1]. It is not known whether or not subclinical hypothyroidism is related to risk for cardiovascular disease. One longitudinal 20-year study of a cohort of subclinical hypothyroid patients did not demonstrate any increases in cardiovascular deaths [2]. However, a number of studies suggest that there is an increased risk of cardiovascular disease in subclinical hypothyroidism [3, 4]. A study that focused on women over the age of 65 years with subclinical hypothyroidism
showed an increased prevalence of prior myocardial infarction that persisted after adjustment for other known risk factors [3]. Recently, Rodondi et al. [4] also showed that subclinical hypothyroidism is associated with an increased risk of cardiac heart failure.

Oxidative stress results from increased production of reactive oxygen radicals or impairment of the antioxidant system [5]. Thiobarbituric acid-reactive substance (TBARS) is a marker of oxidative stress, which is a simple, inexpensive but less specific method to evaluate oxidative stress [6]. It has been demonstrated that TBARS levels are elevated in coronary artery disease [7].

Elevated C-reactive protein (CRP) level has been found to be directly related to the risk of myocardial infarction [8]. Despite the possible relationship between subclinical hypothyroidism and atherosclerosis, there are no data on simultaneous measurement of the circulating CRP level and oxidative stress in subclinical hypothyroid patients. Therefore, the purpose of this study was to determine plasma TBARS levels in subclinical state, after achieving euthyroidism with levothyroxine replacement therapy, and simultaneously serum CRP and lipid levels.

Subjects and Methods

Subjects
The study was performed in 28 female subclinical hypothyroid patients and 24 age-matched healthy female controls. Subclinical hypothyroidism was defined as thyroid-stimulating hormone (TSH) levels of >5 mIU/l despite normal free thyroid hormone levels. Exclusion criteria were diabetes, cardiac, renal, hepatic, and other systemic diseases, morbid obesity, familial hyperlipidemias, and history of malignancy; patients taking drugs such as beta-blockers, antihypertensive, antihyperlipidemic agents, acetylsalicylic acid, antihistamines, multivitamin, hormone replacement, and corticosteroids, which could possibly be associated with acute-phase reaction and oxidative stress, alcoholic patients and current smokers were excluded from the study.

The control group consisted of age- and sex-matched euthyroid healthy hospital staff. Histories, physical examination, electrocardiography, and routine chemical analysis showed that the control subjects had no evidence for any disease. Written informed consent was obtained from all patients to participate in the study. The study was approved by the Ethics Committee of Dokuz Eylul University, Izmir, Turkey.

Body weight (kg) and height (cm) were measured without shoes and/or cap. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Waist circumference was measured as the minimum between the costal margin and iliac crest. Hip circumference was the maximal circumference over the buttocks as seen from the side. Waist-hip ratio (WHR) was calculated by dividing waist circumference by hip circumference.

Physical examination including systolic blood pressure and diastolic blood pressure was performed with a mercury sphygmomanometer after a 10-min rest.

Blood Sample Collection
Blood samples were collected at baseline and after the successful replacement therapy with levothyroxine (average daily dose being 0.05–0.15 mg). After baseline evaluation and blood sampling, all patients were treated with levothyroxine for 3–6 months. Treatment started with 25 μg/day and TSH was measured every 4 weeks to adjust the levothyroxine dose. All patients were reevaluated 4 weeks after restoration of euthyroidism.

After at least 12-hour overnight fasting, venous blood samples were drawn into two tubes: one to separate serum for CRP and lipid measurement and the other one containing ethylene diamine tetra-acetic acid for analysis of TBARS levels. These blood samples were placed in ice water and centrifuged at 4°C and 1,800 g for 15 min. Plasma samples were frozen at –80°C until analysis.

Methods
Serum glucose, cholesterol, triglyceride and high-density lipoprotein (HDL) cholesterol levels were measured using Rando enzymatic kits in a Roche Hitachi Modular system (Mannheim, Germany). Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald’s equation. Free 3,5,3’-triiodothyronine (FT3) and free thyroxine (FT4) levels were determined using an immunoassay (Immulite 2000, Diagnostic Products Corporation, Los Angeles, Calif., USA). TSH was measured with a chemiluminescence immunometric assay (Immulite 2000, Diagnostic Products Corporation). Antithyroid peroxidase, antithyroglobulin antibodies, and serum TSH levels were measured using a solid-phase chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation). CRP was measured by a Cobas Integra 400 autoanalyzer using a particle-enhanced turbidimetric assay (Cobas Integra C-Reactive Protein Latex, Roche Diagnostics, Indianapolis, Ind., USA). Plasma TBARS levels were measured according to the method of Buege and Aust [9] and Lapenna et al. [10]. Briefly, 0.5 ml of ethylene diamine tetra-acetic acid plasma was added to a reaction mixture (1.0 ml) formed by equal parts of 15% trichloroacetic acid, 0.25 N hydrochloric acid, and 0.375% thiobarbituric acid, plus 2.5 mM butylated hydroxytoluene and 0.1 ml of 8.1% sodium dodecyl sulfate, followed by 30 min heating at 95°C; pH value of the analytical reaction mixture was about 0.9. Butylated hydroxytoluene was used to prevent lipid peroxidation during heating. After cooling, the chromogen was extracted with n-butanol and read spectrophotometrically at 532 nm.

Statistical Analysis
Variable distributions were assessed by the Kolmogorov-Smirnov normality test. According to the variable distribution, independent samples t test or Mann-Whitney U test were used. In regard to variable distribution, paired samples t test or Wilcoxon signed-ranks test were used to assess the differences between pre- and post-treatment variables. Correlations between different parameters were tested by Pearson correlation analysis; p < 0.05 was accepted as statistically significant. Statistical analysis was performed using the SPSS version 11.0 package for Windows. Data are expressed as mean ± standard error.
Table 1. Laboratory and demographic characteristics of the control group and patient group (pre- and post-treatment levels) and comparisons of parameters between groups

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>After</th>
<th>Control</th>
<th>p&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>47.21 ± 3.02</td>
<td>46.08 ± 2.12</td>
<td>0.761</td>
<td>0.01*</td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>67.82 ± 1.88</td>
<td>66.88 ± 2.01</td>
<td>67.56 ± 2.1</td>
<td>0.927</td>
<td>0.01*</td>
</tr>
<tr>
<td>BMI</td>
<td>26.39 ± 0.77</td>
<td>26.02 ± 0.81</td>
<td>26.53 ± 0.92</td>
<td>0.908</td>
<td>0.013*</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>78.25 ± 2.33</td>
<td>76.82 ± 2.2</td>
<td>77.88 ± 2.27</td>
<td>0.909</td>
<td>0.001*</td>
</tr>
<tr>
<td>Hip, cm</td>
<td>99.79 ± 1.99</td>
<td>98.75 ± 1.92</td>
<td>98.46 ± 2.07</td>
<td>0.647</td>
<td>0.003*</td>
</tr>
<tr>
<td>WHR</td>
<td>0.782 ± 0.013</td>
<td>0.776 ± 0.013</td>
<td>0.788 ± 0.011</td>
<td>0.683</td>
<td>0.068</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>116.79 ± 2.06</td>
<td>117.50 ± 2.27</td>
<td>0.815</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>73.92 ± 1.57</td>
<td>76.25 ± 1.98</td>
<td>0.416</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FT&lt;sub&gt;4&lt;/sub&gt;, ng/dl</td>
<td>1.18 ± 0.03</td>
<td>1.42 ± 0.04</td>
<td>1.34 ± 0.03</td>
<td>0.001*</td>
<td>0.000*</td>
</tr>
<tr>
<td>TSH, mIU/l</td>
<td>18.4 ± 2.93</td>
<td>2.43 ± 0.28</td>
<td>1.4 ± 0.17</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>191.75 ± 6.95</td>
<td>185.36 ± 6.42</td>
<td>190.25 ± 5.06</td>
<td>0.866</td>
<td>0.11</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>109.18 ± 8.18</td>
<td>125.79 ± 10.49</td>
<td>120.08 ± 12.73</td>
<td>0.811</td>
<td>0.033</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>57.82 ± 2.67</td>
<td>54.64 ± 2.53</td>
<td>55.29 ± 2.43</td>
<td>0.492</td>
<td>0.009*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>112.99 ± 5.86</td>
<td>104.66 ± 5.31</td>
<td>110.94 ± 4.46</td>
<td>0.788</td>
<td>0.039*</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>4.28 ± 0.9</td>
<td>2.32 ± 0.6</td>
<td>1.95 ± 0.34</td>
<td>0.033*</td>
<td>0.006*</td>
</tr>
<tr>
<td>TBARS, μmol/l</td>
<td>0.81 ± 0.06</td>
<td>0.72 ± 0.05</td>
<td>0.79 ± 0.07</td>
<td>0.719</td>
<td>0.154</td>
</tr>
</tbody>
</table>

SBP = Systolic blood pressure; DBP = diastolic blood pressure. * p < 0.05.

<sup>a</sup> Subclinical hypothyroid group versus control group.

<sup>b</sup> Before levothyroxine versus after levothyroxine.

Results

The etiology of subclinical hypothyroidism was chronic autoimmune thyroiditis in all cases. There was no significant difference in age, weight, BMI, waist and hip circumference, lipid levels, WHR, and blood pressures between both groups (table 1). Mean levels of thyroid hormones and TSH are also shown in table 1. At the baseline evaluation, TBARS level in the subclinical hypothyroid group (0.81 ± 0.06 μmol/l) was similar to that in the control group (0.79 ± 0.07 μmol/l). TBARS levels decreased in patients with subclinical hypothyroidism after levothyroxine treatment, but this reduction was not statistically significant. Patients with subclinical hypothyroidism had a higher CRP level (4.28 ± 0.9 mg/l) than controls (1.95 ± 0.34 mg/l) before levothyroxine replacement. After achieving euthyroid state with levothyroxine replacement, CRP levels decreased significantly in patients with subclinical hypothyroidism. Serum total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride levels were similar in the subclinical hypothyroid patients prior to levothyroxine replacement, when compared with the subjects in the control group (table 1). A nonsignificant reduction in serum total cholesterol was observed after levothyroxine treatment; however, serum tri-glyceride levels increased significantly when euthyroidism was restored. Although HDL and LDL cholesterol levels decreased significantly, LDL/HDL ratios before and after levothyroxine treatment were similar. After treatment with levothyroxine, weight, waist and hip circumference, and BMI values decreased significantly. However, reduction of WHR did not reveal statistical significance.

There was no correlation between pretreatment TBARS levels and other variables including serum CRP values. TBARS levels of subclinical hypothyroid patients, before and after restoration of euthyroidism, were similar to the control group. High levels of CRP were negatively correlated with free thyroid hormone levels (r = –0.333, p = 0.016 for FT<sub>3</sub>; r = –0.452, p = 0.001 for FT<sub>4</sub>; fig. 1). Suppressed FT<sub>3</sub> level was an independent predictor of serum CRP levels after adjusting for age, BMI, total and LDL cholesterol (model r<sup>2</sup> = 0.23, beta = –0.456, p = 0.01).

Discussion

Our data showed that the TBARS level was not higher in subclinical hypothyroid patients than in controls, although increased production of oxidative stress has been
shown in subclinical hypothyroidism [11]. Probable explanations include the fact that TBARS is a nonspecific marker of oxidative stress [7]; the association of oxidative stress and atherosclerosis is present in case of elevated serum cholesterol, and that there is a reduction of HDL levels in our patients after levothyroxine replacement. LDL could be protected against oxidation by the action of HDL [12]. Antioxidant action of HDL during copper-mediated LDL oxidation has been demonstrated in various studies [13, 14], which showed that serum paraoxonase activity, which has the capacity to retard the accumulation of lipid peroxides in LDL under oxidizing conditions, is correlated with serum HDL concentration [15, 16]. Similarly, Milionis et al. [17] have found no alteration on plasma paraoxonase-1 activity in subclinical hypothyroid patients before and after levothyroxine therapy. It has been shown in several studies that serum concentration of HDL could be increased in subclinical hypothyroidism [18, 19]. The underlying mechanism of the elevated HDL concentration in hypothyroidism is not clear. Decreased adrenergic regulation of lipolysis [19], reduced hepatic triglyceride lipase [20], and the impairment of peripheral lipoprotein lipase activity [21] are considered to be responsible for high HDL levels. CRP has been shown as a predictor of cardiovascular events [8]. It is a mediator of inflammation and it might be a key molecule linking inflammation to oxidative stress in atherosclerosis. Thomas et al. [22] have shown that oxidative stress is associated with CRP levels in coronary artery disease. Our findings suggest that CRP values increase in patients with subclinical hypothyroidism, and may count as an additional risk factor for the development of atherosclerosis; similar findings are shown in various studies [23–26]. Normalization of thyroid state by levothyroxine replacement seems to effectively reduce serum CRP levels in subclinical hypothyroidism without any correlation with TBARS activity and lipid levels. Ozcan et al. [23] found significantly elevated CRP levels in the high-normal range in subclinical hypothyroidism, similar to our study. Furthermore, they showed that the elevated CRP levels were reduced after thyroxine replacement. Although Ozcan et al. [23] did not observe any significant correlation between thyroid hormone levels and CRP, we found that free thyroxine levels were negatively correlated with CRP. In another study, Tuzcu et al. [24] observed that CRP levels are significantly increased and positively correlated with serum TSH in subclinical hypothyroidism. Christ-Crain et al. [25] also found elevated CRP levels in subclinical hypothyroid patients, but there was no improvement of elevated CRP levels after levothyroxine replacement in their study. On the other hand, Hueston et al. [26] reported that in the large population from the National Health and Nutrition Examination Survey, CRP levels did not differ for patients with subclinical hypothyroidism compared to euthyroid subjects. However, most of the patients in their study had elevations of TSH level below 10 IU/l. In contrast, in our study, most of the patients (64.2%) had TSH levels higher than 10 IU/l, and the mean TSH level of the patients was 18.4 IU/l.

**Conclusion**

This study demonstrated an elevation of CRP levels in patients with subclinical hypothyroidism, which may be associated with increased risk for cardiovascular disease. Restoration of euthyroidism by levothyroxine replacement therapy seems to be effective in reducing serum CRP levels in patients with subclinical hypothyroidism. Altered levels of TBARS were not observed in the subclinical hypothyroid state or after achieving euthyroid state by levothyroxine.
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