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Oxidation Products and Antioxidant Markers in Plasma of Patients with Graves' Disease and Toxic Multinodular Goiter: Effect of Methimazole Treatment

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Oxidative stress plays an important role in hyperthyroidism-induced tissue damage, as well as in development of autoimmune disorders. To clarify influence of thyroid metabolic status and autoimmune factors on blood extracellular indices of reactive oxygen species (ROS) generation and free radical scavenging in hyperthyroidism, we studied patients with newly diagnosed and untreated Graves' disease without infiltrative ophthalmopathy (17 female and 8 male, aged 41.8 ± 8.9) and toxic multinodular goiter (15 female and 9 male, aged 48.4 ± 10.1) under the same antithyroid treatment protocol. Initially and after achievement of stable euthyroidism with methimazole, plasma levels of hydrogen peroxide (H_2O_2), lipid hydroperoxides (ROOH) and ceruloplasmin (CP) and serum concentrations of thiobarbituric acid-reacting substances (TBARS) were determined. Similarly, activities of plasma superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) were assayed. The results were compared to those of age- and sex-matched controls. Average duration of hyperthyroidism and treatment period were similar in both patients groups. H_2O_2, ROOH and TBARS concentrations were significantly higher in hyperthyroid patients compared to controls. Hyperthyroidism caused an evident increase in SOD and CAT activities and CP level, as well as a decrease in GPx and GR activities. Achievement of euthyroidism resulted in normalization of all analyzed parameters in both hyperthyroid patients groups. These findings suggest that the changes in blood extracellular indices of oxidative stress and free radical scavenging in hyperthyroid patients are influenced by thyroid metabolic status, and are not directly dependent on autoimmune factors present in Graves' disease.

Keywords: Oxidative stress; Antioxidants; Plasma; Graves' disease; Toxic multinodular goiter; Methimazole

INTRODUCTION

Thyroid hormones accelerate the basal metabolic rate and oxidative metabolism by induction of mitochondrial enzymes. Accelerated electron transport results in the increased generation of superoxide anion at the site of ubiquinone.[1] Enhanced reactive oxygen species (ROS) production and changes in antioxidant protective systems of various tissues participate in development of hyperthyroidism-induced tissue damage.[2,3] From clinical point of view we can distinguish two main forms of hyperthyroidism, basing on immune (Graves' disease) or non-immune (toxic multinodular goiter) background.[4] Graves' disease is an autoimmune disorder of the thyroid gland characterized by production of TSH receptor stimulating autoantibodies,[5] which leads to development of the hyperthyroid state. In toxic multinodular goiter, proliferation of thyroid cells causes formation of hyperfunctioning thyroid nodules, secreting excess amounts of thyroid hormones independently from TSH regulation. There is growing evidence that oxidative stress plays an important role in development of autoimmune disorders.[6] Some experimental data suggest close relation between ROS generation and the initiation of the immune response in Graves' disease.[7] In contrast, others conclude that ROS production in hyperthyroidism is connected with altered thyroid function, but not with the presence of

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autoimmune process.[8] There are also conflicting data on the free radical scavenging properties of different antithyroid drugs, as their postulated beneficial feature in the treatment of hyperthyroidism.[7,9,10,11,12]

In earlier studies, various blood parameters of oxidative stress and antioxidant defence were analyzed in hyperthyroidism due to Graves' disease[13] or toxic multinodular goiter.[10,14] However, the antithyroid drugs belonging to different pharmacological groups were used in these reports. Also, little is known about the extracellular antioxidant enzymes activity in hyperthyroid patients. To clarify influence of thyroid metabolic status and autoimmune factors on blood extracellular indices of ROS generation and free radical scavenging in hyperthyroidism, we performed a complex estimation of patients with Graves' disease and toxic multinodular goiter under the same antithyroid treatment protocol.

MATERIALS AND METHODS

Subjects

The study was performed on 49 patients with newly diagnosed and untreated hyperthyroidism due to Graves' disease without infiltrative ophthalmopathy (17 female and 8 male, aged 41.8 ± 8.9) or toxic multinodular goiter (15 female and 9 male, aged 48.4 ± 10.1). Hyperthyroidism has been present for 1–3 months before treatment (mean 1.6 months for Graves' disease and 1.8 months for toxic multinodular goiter). In all cases the diagnosis was based on clinical symptoms and signs, serum hormones levels, concentrations of anti TSH receptor antibodies, as well as ultrasonographic and isotopic thyroid scans. Age- and sex-matched healthy volunteers were used as controls (15 female and 9 male, aged 44.9 ± 10.2). Excluding criteria for all individuals were smoking, alcohol drinking, presence of diabetes mellitus, hypertension, liver or kidney disorders, severe vascular diseases, other endocrine, immunological or inflammatory disorders, hyperlipaemia, regular drug ingestion or antioxidant use. Informed consent was obtained from the subjects prior to commencement of the study.

The hyperthyroid patients were treated with methimazole (an initial dose of 60 mg/day). After the first month of therapy, the methimazole dose started to be gradually reduced and L-thyroxine was added to maintain euthyroidism. During treatment the patients were regulary checked-up to record a moment of hormonal normalization.

Venous blood samples were drawn on fasting into test tubes (heparinized and without anticoagulant) before treatment and after achievement of stable euthyroidism (after 2–4 months, mean 2.9 months for Graves' disease and 3.2 months for toxic multinodular goiter). After centrifugation at 1500*g* for 5 min, serum or plasma were separated and stored at −80°C until biochemical tests.

Biochemical Analysis

All the chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Serum fT_4, fT_3 and TSH concentrations were determined by immunofluorometric assay (Delfia, Pharmacia, Sweden). Levels of anti TSH receptor antibodies were measured by radioimmunoassay (TRAK, Brahms, Germany).

Hydrogen peroxide (H_2O_2) concentration was assayed following the method described by Gallati and Pracht.[15] Briefly, 1 ml of plasma was mixed with 0.9 ml, 1 μmol/l 3.3′.5.5′-tetramethylbenzidine and 0.1 ml horseradish peroxidase (25 U) in 0.2 M potassium citrate buffer (pH 3.95). After 30 min incubation in room temperature, 2 N sulphuric acid was added and the supernatant was read at 450 nm. A standard curve was prepared with known amounts of H_2O_2, using the same procedure.

Plasma lipid hydroperoxides (ROOH) levels were determined by the FOX II assay (ferrous oxidation in xylenol orange),[16] with a reagent PeroXOquant (lipid compatibile formulation) commercially available from Pierce (Rockford, IL, USA). This reagent was obtained by mixing of reagent A (25 mM ammonium ferrous(II) sulfate, 2.5 M H_2SO_4) with reagent C (4 mM butylated hydroxytoluen, 125 μM xylenol orange in methanol) in the proportion 1:100. Aliquots of plasma (90 μl) were transferred to vials and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) in methanol (10 μl of a 10 mM solution) was added to some vials, to remove hydroperoxides. Methanol (10 μl) was added to the remaining tubes to generate test samples. After incubation at 20°C for 30 min, 900 μl of the PeroXOquant reagent was added and the vials were left for a further 30 min. After centrifugation at 12,000*g* for 10 min, the supernatant absorbance was measured at 560 nm. The hydroperoxides were determined by the difference between the absorbance of samples with, and without, the elimination of ROOH by TCEP. A standard curve was produced with a working reagent, using known amounts of hydrogen peroxide.

Thiobarbituric acid-reacting substances (TBARS) concentration was evaluated by the use of thiobarbituric acid assay, according to Buege and Aust.[17] Briefly, 0.2 ml of serum was mixed with thiobarbituric acid reagent (consisting of 0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl). The reaction mixture was placed in a boiling water bath for 15 min, cooled and centrifuged.

Absorbance of the supernatant was evaluated at 532 nm. A standard curve was prepared with known amounts of 1.1.3.3.-tetraethoxypropane.

Superoxide dismutase (SOD) activity was determined by the method of Roos *et al.*,[18] based on inhibition by plasma of O_2^- catalysed reduction of ferricytochrome C in comparison to known standards. For a standard curve, mixture consisting of 0.675 ml (20 mmol/l Na_2CO_3, 0.1 mmol/l EDTA, 1 mmol/l NaN_3, pH 10.0), 0.1 ml of xantine (50 μmol/l), 0.1 ml of ferricytochrome C (5 μmol/l) and 0.1 ml of a SOD standard was incubated for 5 min and absorbance was determined at 550 nm. The reaction was started by adding 0.025 ml of xantine oxidase (0.025 U/ml). The samples were read again after 5 min. For tested samples, the SOD standard was substituted with 0.1 ml of plasma.

Catalase (CAT) activity was determined spectrophotometrically by the method of Goth.[19] Briefly, 0.2 ml of plasma was incubated with 1 ml of substrate (65 μmol/ml hydrogen peroxide in 60 mmol/l sodium phosphate/potassium phosphate buffer, pH 7.4), in 37°C for 60 s. The enzymatic reaction was stopped with 1 ml of 32.4 mmol/l ammonium molybdate and yellow complex of molybdate and hydrogen peroxide was read at 405 nm.

Glutathione peroxidase (GPx) activity was assessed by the assay available from OXIS International (Portland, OR, USA). The principle of this method is decrease in absorbance at 340 nm, as the result of the oxidation of NADPH to $NADP^+$. A plasma sample was added to a solution containing glutathione, glutathione reductase (GR) and NADPH. The enzyme reaction was initiated by adding the substrate, tert-butyl hydroperoxide. The rate of decrease in the absorbance at 340 nm was directly proportional to the GPx activity in the sample.

Plasma glutathion reductase (GR) activity was evaluated by the commercially available assay (OXIS International, Portland, OR), based on the oxidation of NADPH to $NADP^+$ catalysed by glutathione reductase. The reduction of GSSG was determined indirectly by the measurement of the consumption of NADPH, as demonstrated by decrease in absorbance at 340 nm, as a function of time.

Ceruloplasmin (CP) was determined by oxidation of *p*-phenylodiamine, according to method of Ravin.[20] Briefly, 0.05 ml of plasma was added to 0.7 ml of substrate (7.95 mmol/l *p*-phenylodiamine in 0.43 M acetic buffer, pH 5.6) and absorbance was read at 546 nm, after 15 min incubation at 37°C. Control samples contained additionally 0.2 ml of sodium azide (460 mmol/l).

Statistical Methods

Statistical analysis was carried out using the Statistica 6.0 for Windows PL package. Results obtained were expressed as mean ± SD. Because not all data showed normal distribution (when checked by Shapiro – Wilk W test), differences among groups were tested by nonparametric tests (with $p < 0.05$ selected as a significance level). In each hyperthyroid group, mean group values of patients before and after treatment were compared with paired Wilcoxon test. Comparison between each of the hyperthyroid groups and the control group was made using unpaired Kruskal–Wallis test, followed by Dunn test in case of significant difference.

RESULTS

Control subjects fT_4, fT_3 and TSH concentrations were within the normal population range. Graves' disease patients revealed high level of anti TSH receptor antibodies, while toxic multinodular goiter group was not discrimined from controls. In all hyperthyroid patients before therapy fT_4 and fT_3 levels were significantly higher, and TSH level significantly lower in comparison to controls (Table I). Achievement of stable euthyroidism during methimazole treatment was confirmed by normalization of fT_4, fT_3 and TSH concentrations.

The changes of free radical activity markers are shown in Table II. H_2O_2, ROOH and TBARS concentrations were significantly higher in hyperthyroid patients compared to controls. Table III summarizes the changes of extracellular antioxidant system components. Hyperthyroidism

TABLE I Thyroid hormones, TSH and anti-TSH receptor antibodies (Anti-TSHR Ab) levels in serum from patients with Graves' disease and toxic multinodular goiter before and after methimazole treatment

		Graves' disease ($n = 25$)		Toxic multinodular goiter ($n = 24$)	
	Control ($n = 24$)	Before treatment	After treatment	Before treatment	Afterss treatment
FT4 (pmol/l)	12.6 ± 2.6	69.3 ± 18.1*	13.1 ± 3.9**	57.5 ± 14.5*	14.8 ± 3.9**
FT3 (pmol/l)	7.4 ± 2.1	32.3 ± 12.2*	6.5 ± 1.8**	25.9 ± 7.2*	7.7 ± 2.4**
TSH (mU/l)	1.58 ± 0.43	0.04 ± 0.04*	1.72 ± 0.51**	0.05 ± 0.03*	1.49 ± 0.45**
Anti-TSHR Ab	5.2 ± 3.4	99.8 ± 76.1*		4.9 ± 2.2	

Results are expressed as mean ± SD. *Significantly different from the control group ($p < 0.05$), **significantly different from the pretreatment value ($p < 0.05$).

TABLE II Hydrogen peroxide (H_2O_2) and lipid hydroperoxides (ROOH) levels in plasma and thiobarbituric acid-reacting substances (TBARS) levels in serum from patients with Graves' disease and toxic multinodular goiter before and after methimazole treatment

		Graves' disease ($n = 25$)		Toxic multinodular goiter ($n = 24$)	
	Control ($n = 24$)	Before treatment	After treatment	Before treatment	After treatment
H_2O_2 (nmol/ml)	6.92 ± 1.85	9.48 ± 2.03*	7.20 ± 1.88**	10.13 ± 2.05*	7.36 ± 1.92**
ROOH (μmol/l)	11.83 ± 3.41	21.68 ± 5.73*	13.22 ± 3.49**	19.29 ± 6.17*	11.38 ± 3.53**
TBARS (nmol/ml)	6.23 ± 1.54	8.27 ± 1.61*	6.87 ± 1.88**	7.63 ± 2.14*	6.45 ± 1.48**

Results are expressed as mean ± SD. *Significantly different from the control group ($p < 0.05$), **significantly different from the pretreatment value ($p < 0.05$).

caused an evident increase in SOD and CAT activities and CP level, as well as a decrease in GPx and GR activities. Methimazole treatment resulted in normalization of all analyzed parameters in both hyperthyroid patients groups.

DISCUSSION

Graves' disease and toxic multinodular goiter are two principal, etiologically different, clinical forms of hyperthyroidism.[4] Graves' disease is caused by the immune system alterations, resulting in production of TSH receptor stimulating antibodies, while toxic multinodular goiter reveals no autoimmune background.[5] Nowadays, oxidative stress is accounted as an important factor in development of autoimmune disorders.[6] Experiments concerning influence of methimazole on formation of oxygen radicals by monocytes suggest that the presence of oxidative stress and the initiation of the immune response in Graves' disease may be closely related.[7] On the other hand, Szabo *et al.*[8] found that enhanced superoxide anion generation by polymorphonuclear granulocytes is present in Graves' disease as well as in patients with nodular goiter.

Conflicting data on the free radical scavenging properties of different antithyroid drugs were presented. Several *in vitro* studies have shown that methimazole acts as a scavenger of free radicals, what may explain the immunosuppressive action of this drug in Graves' disease.[7,21,22,23] A possible explanation can be a close structural similarity of methimazole to thiourea, a known scavenger.[24] Wilson *et al.*[12] have found that both methimazole and propylthiouracil exert *in vitro* direct effect on the immune system and reveal some ROS scavenging ability. However, the same author in a clinical study suggested that carbimazole (a methimazole precursor) does not act as a free radical scavenger.[9] Also Bianchi *et al.* have shown that oxidative stress indices in hyperthyroid patients are corrected after achievement of euthyroidism, without any influence of thyrostatic (methimazole or propylthiouracil) *per se*.[11] Previously, various blood parameters of oxidative stress and antioxidant defence were analyzed in hyperthyroidism due to Graves' disease [13] or toxic multinodular goiter.[10,14] The antithyroid drugs belonging to different pharmacological groups were used in these reports. We decided to use for all patients a standard treatment protocol, employing methimazole in a high initial dose (60 mg/day), which has been later gradually reduced and L-thyroxine has been added to maintain euthyroidism.

Average duration of hyperthyroidism before treatment and average period of antithyroid therapy were similar in the patients with Graves' disease and toxic multinodular goiter.

In this study, extracellular oxidative stress parameters (H_2O_2, ROOH and TBARS) levels were significantly increased in both hyperthyroid patients groups. Methimazole treatment produced normalization of all analyzed indices of increased oxidation in both hyperthyroid patients groups. Our findings correspond with those of other investigators,[11,13,25] who found an increased plasma level of lipid peroxidation products in immune hyperthyroidism. Two of these authors[11,13] noted normalization of TBARS after achievement of euthyroidism with

TABLE III Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities and ceruloplasmin (CP) concentration in plasma from patients with Graves' disease and toxic multinodular goiter before and after methimazole treatment

		Graves' disease ($n = 25$)		Toxic multinodular goiter ($n = 24$)	
	Control ($n = 24$)	Before treatment	After treatment	Before treatment	After treatment
SOD (U/ml)	17.35 ± 4.76	97.75 ± 26.34*	18.56 ± 4.96**	119.10 ± 28.01*	20.12 ± 4.43**
CAT (U/ml)	21.85 ± 5.99	51.22 ± 9.26*	23.01 ± 6.22**	44.04 ± 10.13*	25.12 ± 6.68**
GPx (mU/ml)	5.84 ± 1.60	3.33 ± 0.94*	5.86 ± 1.65**	2.92 ± 0.53*	4.98 ± 1.68**
GR (mU/ml)	12.58 ± 2.96	8.96 ± 2.59*	11.65 ± 3.09**	9.64 ± 2.22*	11.96 ± 2.52**
CP (mg/dl)	15.86 ± 3.99	20.31 ± 5.44*	16.26 ± 4.22**	19.98 ± 5.79*	15.72 ± 3.61**

Results are expressed as mean ± SD. *Significantly different from the control group ($p < 0.05$), **significantly different from the pretreatment value ($p < 0.05$).

different thyrostatic drugs. Instead, Seven *et al.*[25] observed still elevated TBARS concentrations after propylthiouracil treatment. A possible interpretation can be a different treatment period between the consecutive blood samples drawing from the patients. For Bianchi *et al.*[11] and Komosińska *et al.*[13] studies these periods were approximately 13,9 and 18 weeks, respectively, we treated the Graves' patients for approximately 11.6 weeks and the toxic multinodular goiter patients for approximately 12.8 weeks, while for Seven *et al.*[25] the treatment period was 4 weeks. Thus, probably oxidation stress was still more intense since shorter time of the thyrostatic therapy. Plasma MDA was reported to be increased in hyperthyroidism due to Graves' disease[26] and toxic multinodular goiter,[10,14] with a decrease after treatment for 8[26] and 12[10] weeks with different antithyroid drugs.

A controversy exists if hyperthyroid state is associated with increase or decrease in the activities of antioxidant defence enzymes.[3,27,28] In our study, plasma SOD activity was evidently higher in all hyperthyroid patients, presumably secondary to increased ROS generation. Similarly, SOD activity was reported to be increased in serum[29] and erythrocytes[13,25,26] of Graves' disease patients, as well as in erythrocytes of individuals with toxic multinodular goiter.[10,14] Methimazole treatment caused a reversal of SOD activity in both our patients groups. According to other authors, therapy with various thyrostatic drugs resulted in increasing,[25] partial reversal[10,26] or normalization[13] of SOD activity. On the other hand, a few papers reported a decrease of intracellular SOD activity in hyperthyroid Graves' patients.[9,30] After antithyroid therapy SOD activity was partially reversed[9] or normalized.[30] Since SOD activity should increase H_2O_2 production, protection from ROS would be conferred by coordinated increase in CAT activity.[31] We found CAT activity was increased in both hyperthyroid patients groups, with a partial reversal in Graves' disease patients after methimazole treatment. In earlier papers, higher level of CAT activity was described in immune hyperthyroidism[13] and toxic multinodular goiter.[10,14] However, Guerra *et al.*[26] found CAT activity significantly decreased in Graves' disease. Thyrostatic therapy led to partial reversal[10,26] or normalization[13] of CAT activities. It is possible that the contradictory data observed regarding the state of antioxidant defence in human hyperthyroidism may be due to methodological differences of measurements or—more probably—to the different evolution period of the disease before antithyroid treatment.[30] In recent hyperthyroidism, an initial increment of the enzymatic antioxidant defenses indicates that the system is reacting to accelerated ROS generation.[32] In longer periods of evolution, there may be consumption of the antioxidant system elements, necessary for neutralizing the oxidative damage, leading to situation of oxidative stress. Unfortunately, most of authors do not describe the length of thyrotoxicosis development period of patients studied. However, above mentioned hypothesis could be support by comparison of data presented by Abalovich *et al.*[30] and our results. For patients observed by us hyperthyroidism has been present for 1–3 months (mean 1.6 months for Graves' disease and 1.8 months for toxic multinodular goiter) and SOD and CAT activity were found to be increased. In the Abalovich *et al.*[30] study, the patients have had Graves' disease for more than 6 months and evidently decreased erythrocyte SOD and CAT activity.

Experimental investigations of ROS-scavenging enzymes in erythrocytes of several species showed SOD may be constitutively present only at low levels, but highly inducible under oxidative stress, while GPx is normally abundant and less inducible.[33] Similar observations was made for erythrocyte enzymes in Graves' disease patients,[25] revealing significant induction of SOD activity, whereas GPx activity was not significantly different from controls despite of propylthiouracil therapy. On the other hand, others found erythrocyte GPx activity significantly increased in immune[13] or non-immune[10,14] hyperthyroidism, with correction after antithyroid treatment.[10,13] Activity of plasma GPx was showed to be decreased in hyperthyroid patients not selected due to disease background, rising to normal values after therapy with methimazole.[34] Similarly, serum GR and total antioxidant status were decreased in Graves' disease, with improvement after achievement of euthyroidism,[13] while erythrocyte GR activity were found to be higher in toxic multinodular goiter patients,[10,14] with partial reversal after thyrostatic therapy.[10] In this study, plasma GPx and GR activities were significantly decreased in all hyperthyroid patients, with normalization after antithyroid therapy. The decrease in the part of extracellular antioxidant potential, represented by reduced GPx and GR activities in hyperthyroid patients, may indicate that plasma antioxidants are the agents involved in the defence against ROS that are used up most rapidly.[13] It can be suggested by comparison of described above Komsinska *et al.*[13] and our results, regarding extra- and intracellular GPx and GR activities. What else, the contradictory results of GPx and GR activities presented in different studies could be probably also explained by different time of thyrotoxicosis evolution. In case of longer evolution period (above 6 months,[30]) even intracellular GPx and GR activities are clearly decreased. CP level was higher in both hyperthyroid patients groups, with a decrease to euthyroid values after therapy of methimazole. These observations are consistent with those of other authors, which showed elevated CP levels in hyperthyroidism[35] and particularly in toxic

multinodular goiter patients,[10,14,36] with partial reversal after antithyroid drugs.[10]

Our results suggest that changes in blood extracellular indices of ROS generation and free radical scavenging in hyperthyroid patients are influenced by thyroid metabolic status, and are not directly dependent on autoimmune factors present in Graves' disease.

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