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Original Contribution

CHANGES IN PARAMETERS OF OXIDATIVE STRESS IN PATIENTS WITH GRAVES' DISEASE

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ABSTRACT

Graves' disease (GD) is an autoimmune thyroid disorder. It accounts for 70-80 % of all cases of hyperthyroidism. The aim of this study is to examine the change of oxidative stress and antioxidant enzyme activities in 23 Graves' disease patients before treatment with methimazole and in 31 healthy controls. The levels of malondialdehyde (MDA) in the plasma, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities in erythrocytes were measured. Serum levels of TSH and free thyroxin (fT4) were also estimated. We observed increased concentrations of fT4 and suppressed TSH levels before treatment. Significantly higher MDA concentrations were found in hyperthyroid GD patients compared to controls (1.96±0.10 μmol/l vs. 1.71±0.05 μmol/l, $p<0.05$). Activity of GPX in erythrocytes in hyperthyroidism was higher compared to controls (9.3±1.2 U/gHb vs. 6.6±0.5 U/gHb, $p<0.05$). Our data confirm the presence of increased lipid peroxidation in hyperthyroid patients. We suppose that in hyperthyroidism the enhanced activity of GPX was induced by oxidative stress and, as well, was connected with TSH receptor hyperstimulation.

Keywords: Graves' disease, oxidative stress, lipid peroxidation

INTRODUCTION

Graves' disease (GD) is an autoimmune thyroid disorder. It accounts for 70-80 % of all cases of hyperthyroidism Hyperthyroidism is associated with increased concentration of thyroid hormones and increased speed of the basal metabolism, accompanied by an increase in the total consumption of oxygen. In untreated patients, or in animal models, the hyperthyroidism increases the formation of reactive oxygen species (ROS) leading to oxidative damage to biomembrane lipids (1-7). Cells have developed a comprehensive set of antioxidant defence mechanisms to limit their effects. The presence of the following antioxidative enzymes in the thyroid gland has been documented: superoxide dismutase (SOD) (8, 9), catalase (CAT) (10), glutathione peroxidase (GPX) (11). However, clinical investigations of the disturbed antioxidative defence system in hyperthyroidism are scarce and conflicting (2, 4, 6, 12, 13). Alterations in the free radical scavenger systems in highly selected groups of hyperthyroid patients such as Graves' disease patients are insufficiently investigated and explained.

Thus, the aim of the present study was to investigate the possible induction of oxidative stress as a consequence of hyperthyroidism in Graves' disease and the changes in antioxidant protective system. For this purpose we investigated the levels of lipid peroxidation products - malondialdehyde (MDA) in plasma and the activities of antioxidant defence enzymes SOD, CAT and GPX in erythrocytes obtained from newly diagnosed hyperthyroid patients with Graves' disease before therapy and in healthy controls.

MATERIALS AND METHODS

Subjects

Twenty tree out-patients (5 males, 18 females, of mean age 42.6 ± 10.3 years) from the Department of Internal Medicine, Stara Zagora University Hospital (Bulgaria) with recently diagnosed and untreated Graves' disease were recruited and investigated in this study. In all patients the clinical diagnosis of Graves' disease was confirmed on the basis of elevated free thyroid hormone levels, suppressed TSH levels and diffuse

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hypoechoic patterns of thyroid gland. Biomarkers for free radical damage and antioxidant activity were assessed prior to commencement of treatment with methimazole.

Blood samples obtained from 31 healthy individuals (4 males, 27 females, of mean age 43.8 ± 12.1 years) who had no family history of autoimmune disease were used as controls. To eliminate the factors, which might affect parameters of oxidative stress, we excluded from Graves' disease patients and from healthy controls, all smoking and alcohol drinking subjects, as well as individuals suffering from acute or chronic diseases.

Informed consent was obtained from all participants in the study according to the Ethical Guidelines of the Helsinki Declaration.

Biochemical analysis

Fasting samples of venous blood were collected in the morning between 8.00 and 10.00 h.

FT4 was measured by competitive immunoassay on the ACS180 (Chiron Diagnostics USA). TSH was measured by a third generation two-site chemiluminometric assay on the ACS180. The reference range was 11.5-22.7 pmol/l for FT4 and 0.35-5.5 μIU/ml for TSH.

Blood for determining the parameters of oxidative stress was collected in tubes containing ethylenediamine-tetraacetic acid (EDTA), centrifuged for 15 min at 3000 rpm, and plasma was carefully separated. The erythrocyte pellets were washed three times with saline, and 0.5 ml of the cell suspension was diluted with 2 ml cold water to lyse the erythrocytes. To 0.2 ml lysate 1.8 ml water and ethanol/chloroform (3:5/v:v) were then added to precipitate haemoglobin. The tubes were shaken vigorously for 5 min. The supernatant was used for determination of enzyme activity.

Total amount of lipid peroxidation products in the plasma of healthy volunteers and patients was estimated using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products (14). In brief, 1.0 ml of plasma, 1.0 ml of normal saline and 1.0 ml of 25% trichloroacetic acid (TCA) were mixed and centrifuged for 20 min at 2000 rpm. One ml of protein-free supernatant was taken, mixed with 0.25 ml of 1% TBA and boiled for 1h at 95° C. After cooling, the intensity of the pink colour of the product obtained was read at 532 nm. Results were expressed as μM/l.

Superoxide dismutase activity was determined as described by Sun et al., (15) with minor modifications. The xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion reduces nitroblue tetrazolium (NBT) to formazan, which is monitored at 560 nm. SOD of the sample removes the superoxide anion and inhibits the reduction. The level of this reduction was used as a measure of SOD activity. The final concentrations of xanthine, xanthine oxidase and nitroblue tetrazolium in the assay were 50 μM, 10 units/ml and 0.125 mM. One unit of enzymatic activity was defined as an amount that causes 50% inhibition of NBT reduction to formazin. The results were expressed as U/gHb.

Catalase activity in the erythrocyte lysates was assessed by the method described by Beers and Sizer (16). Briefly, hydrogen peroxide (30mM) was used as a substrate and the decrease in its concentration at 22° C in phosphate buffer (50 mM, pH 7.0) was followed spectrophotometrically at 240 nm. One unit of CAT activity was defined as enzyme amount that degrades 1 μM H2O2 per min. Results were presented as units per g haemoglobin (U/g Hb). The haemoglobin concentration of lysate was determined by the cyanmethemoglobin method (17).

Glutathione peroxidase activity was measured by the method of Paglia et al (18). The enzymatic reaction was initiated by addition of H_2O_2 to the reaction mixture containing reduced glutathione, NADPH and glutathione reductase and the change in the absorbance at 340 nm was monitored by spectrophotometer. Activity was given in units per g haemoglobin (U/g Hb).

Statistical methods

Statistical analysis was carried out using the Statistica 5.5 for Windows. The results were reported as means ± SD (SE). Student's *t*-test was used to determine whether differences between means were significant. $p<0.05$ was considered statistically significant.

RESULTS

Serum levels of fT4 and TSH in newly diagnosed Graves' disease patients and in controls are presented on **Table 1**.

The results of studied parameters of oxidative stress are listed on **Table 2**.

The levels of MDA in patients in

hyperthyroidism were detected to be significantly higher than that of the healthy volunteers (1.96±0.1 μmol/l vs. 1.71±0.05 μmol/l, $p<0.05$).

***Table 1**. The results of levels of fT4 and TSH in controls and in hyperthyroid Graves' disease's patients – before thyrostatic drug treatment*

Groups / Parameters	Controls	Graves' disease patients – hyperthyroid
N	31	23
FT4 (pmol/l)	14.86 ± 2.08 **	64.32 ± 14.48 **
TSH (μIU/ml)	1.77 ± 0.66 **	0.05 ± 0.07 **

*The results are expressed as mean ± S.D. Statistical significance (Student's t-test): ** p <0.01*

***Table 2.** The results of levels of lipid peroxidation products - malondialdehyde (MDA) (μmol/l) in the plasma and the activities of antioxidative enzymes: superoxide dismutase (SOD) (U/gHb), catalase (CAT) (U/gHb) and glutathione peroxidase (GPX) (U/gHb) in erythrocytes of control group and hyperthyroid Graves' disease's patients*

Groups / Parameters	Controls	Graves' disease patients – hyperthyroid
N	31	23
MDA (μmol/l)	1.71 ± 0.05*	1.96 ± 0.1*
SOD (U/gHb)	2594.4 ± 151.2	2911.9 ± 237.7
CAT (U/gHb)	26626.37 ± 2209.6	23650.6 ± 3329.0
GPX (U/gHb)	6.6 ± 0.5*	9.3 ± 1.2*

*The results are expressed as mean ± S.E. Statistical significance (Student's t-test): * p<0.05*

SOD activity tended to be higher in patients in hyperthyroid state than that of controls, but not significantly (2911.9±237.7 U/gHb vs. 2594.4±151.2 U/gHb, $p=0.24$). Our results showed that catalase activity in patients in hyperthyroid state was lowered but not significantly, compared to that of control group (23650.6±3329.0 U/gHb vs. 26626.4±2209.6 U/gHb; $p<0.44$).

Activity of GPX in erythrocytes in hyperthyroidism was higher compared to controls (9.3±1.2 U/gHb vs. 6.6±0.5 U/gHb, $p<0.05$).

DISCUSSION

Lipid peroxidation is probably the most extensively investigated process induced by free radicals. In our study we studied the most widely used index of lipid peroxidation, which is MDA formation, assayed with the thiobarbituric acid assay. We found that hyperthyroid Graves' disease was related to increases in MDA levels in plasma. Experimental studies confirmed that oxidative muscular injury was caused by thyroid hormone excess (19,20). The major complications of hyperthyroidism – thyrotoxic myopathy and cardiomiopathy were related to the oxidative muscular injury.

An imbalance in antioxidant systems might also be related to cellular and tissue injury. In experimental hyperthyroidism, alterations in the levels of the most important members of the enzymatic defence systems against oxygen radicals - SOD, CAT and GPX have been distinguished (21).

However, clinical data concerning the changes of antioxidant enzymes in Graves' disease are in small number and with contradictory results (4, 6, 12, 13). Wilson et al found reduced levels of SOD in untreated Graves' patients compared to controls (12), while other investigations observed increased activities (4, 6). The results of different studies give conflicting information on GPX activity in red blood cells in patients with Graves' disease as well. Komosinska –Vassev et al reported elevated activity of GPX in hyperthyroid Graves' disease patients compared to healthy individuals (4). In recent study Vrca et al demonstrated that after 30 days of the commencement of methimazole treatment of Graves' disease patients, the activity of GPX increased, but after 60 days the values were the same as those in second measurement (13). A weakness in this work was the failure in evaluating GPX activity in control healthy subjects.

In our study, we found in untreated patients a tendency for increased activity of SOD. It appears that this change was induced

by excessive production of free radicals in a state of oxidative stress.

We suggest that in hyperthyroid state the reduced catalase activity in our patients was compensatory and related to the enhanced activity of GPX. In accordance with experimental studies (22, 23) we suppose that the high activity of this enzyme was induced by oxidative stress and increased H_2O_2 production, as well as connected with the stimulation of TSH receptor by TSH receptor antibodies.

Our data illustrates that hyperthyroidism in Graves' disease is related to the increased free radical formation and disturbances in antioxidant protective systems.

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