Free Radical Activity and Antioxidant Defense Mechanisms in Patients with Hypothyroidism

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Abstract. Free radical-mediated oxidative stress has been implicated in the etiopathogenesis of several autoimmune disorders. Hypothyroidism in humans is widely believed to impair health. The biochemical factors mediating decline in health, however, are poorly elucidated. Pathological consequences of hypothyroidism point to a high potential for antioxidant imbalance. The study population consisted of 60 subjects divided into two groups: 30 people with hypothyroidism and 30 age-matched healthy participants. We examined the levels of total triiodothyronine (T_3), free triiodothyronine (FT_3), total thyroxine (T_4), free thyroxine (FT_4), thyroid stimulating hormone (TSH), thiobarbituric acid reactive substances (TBARS), and enzymatic and non-enzymatic antioxidant status. The mean TSH level was significantly higher in hypothyroid patients than in control subjects. On the other hand, the levels of FT_4, T_4, FT_3, and T_3 were significantly lower in hypothyroid patients compared to control subjects. The mean level of plasma thiobarbituric acid reactive substances (TBARS) was significantly higher in hypothyroid patients than in healthy controls. However, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and reduced glutathione (GSH) were significantly lower in hypothyroid patients than in control subjects. Our preliminary results confirm the hypothesis that people with hypothyroidism have reduced antioxidative defense.

Keywords. Antioxidant status • Hypothyroidism • Lipid peroxidation • Oxidative stress

Introduction

Thyroid hormones are among the most important humoral factors involved in setting the basal metabolic rate on a long-term basis in target tissues such as liver, heart, kidney and brain.[1] Oxygen free radicals can develop during several steps of normal metabolic events. Although free radicals have the potential to damage the organism, their generation is inevitable for some metabolic processes. The main endogenous sources of free radicals are the mitochondrial electron transport chain, the microsomal membrane electron transport chain, reactions of oxidant enzymes, and auto-oxidation reactions.[2][3][4]

Both hydrogen peroxide and superoxide anion produce highly reactive hydroxyl radicals through the Haber-Weiss reaction. The hydroxyl radical can initiate lipid peroxidation, which is a free radical chain reaction leading to damage of membrane structure and function.[5] Variations in the levels of thyroid hormones can be one of the main physiological modulators of *in vivo* cellular oxidative stress due to their known effects on mitochondrial respiration. In particular, it has been suggested that the increase in reactive oxygen species induced by a deficiency of thyroid hormones can lead to an oxidative stress condition in the liver and in the heart and some skeletal muscles with a consequent lipid peroxidative response.[4]

Reactive oxygen species (ROS) including partially reduced forms of oxygen; i.e. superoxide anion, hydrogen peroxide, and hydroxyl radical, as well as organic counterparts such as lipid peroxides; are produced as natural consequences of oxidative cell metabolism.[6] Under physiological conditions, ROS generation is controlled by a large number of anti-free radical systems which act as protective mechanisms. These systems consist of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase as well as

non-enzymatic antioxidants, among which the most important are vitamins C and E, carotenoids, and glutathione. Disturbance of the prooxidant/antioxidant balance results from the increased production of ROS, inactivation of detoxification systems, or excessive consumption of antioxidants. The disturbance is a causative factor in the oxidative damage of cellular structures and molecules such as lipids, proteins, and nucleic acids.[7]

Subclinical hypothyroidism is defined as a serum thyroid stimulating hormone (TSH) concentration above the statistically defined upper limit of the reference range when serum free T_4 concentration is within its reference range. Greater sensitivity of assays and more frequent assessment of serum TSH levels have resulted in more patients requiring interpretation of abnormal thyroid function test results. However, controversy surrounds the definition, clinical importance, and necessity for prompt diagnosis and treatment of hypothyroid disease. Previous review articles and position statements differ in their conclusions and recommendations, often a consequence of difficulties in interpreting inadequate and conflicting data.[8] In the midst of this uncertainty, clinicians still desire expert guidance for the diagnosis and management of hypothyroid disease.

Hypothyroidism-associated oxidative stress is the consequence of both increased production of free radicals and reduced capacity of the antioxidative defense.[9][10] Hypothyroidism-induced dysfunction of the respiratory chain in the mitochondria leads to accelerated production of free radicals (i.e., superoxide anion, hydrogen peroxide, and hydroxyl radical as well as lipid peroxides), which consequently leads to oxidative stress (OS).[4][11] Metabolic disorder from autoimmune-based hypothyroidism can also increase oxidative stress.[13]

Methods

Study Population

The study population consisted of 60 subjects (age- and sex-matched) divided into two groups: hypothyroid patients (n=30) and healthy control subjects (n=30). All the patients and controls were recruited from K.G. Hospital and Post Graduate Medical Institute, Coimbatore, Tamil Nadu, India, during January of 2007-to-March of 2008. General health characteristics such as age, sex, smoking status, menopausal status, alcohol consumption, and dietary habits (particularly as related to preference) were investigated by a self-administered questionnaire. We classified values into high, low, or normal thyroid hormone levels by the following criteria. Subjects classified as having a high level of thyroid hormones had FT_4 values > 1.6 ng/mL or TSH < 0.4 μIU/mL, or both. Those classified as having a low level had FT_4 values < 0.68 ng/mL or TSH values > 5.0 μIU/mL, or both. Subjects grouped as normal had FT_4 and TSH values within the reference ranges > 0.68-to-1.6 ng/mL and 0.4-to-5.0 μIU/mL, respectively.

Blood Collection and Hemolysate Preparation

Blood samples were collected by venous puncture in heparinized tubes and the plasma was separated by centrifugation at 1000 g for 15 minutes After centrifugation, the buffy coat was removed and the packed cells were washed three times with physiological saline. A known volume of the erythrocytes was lysed with hypotonic phosphate buffer (pH 7.4). The hemolysate was separated by centrifugation at 2500 g for 15 minutes at 2˚C.

Biochemical Investigation

The level of serum lipids was determined using a fully automated clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany). The levels of serum thyroid stimulating hormone (TSH), total triiodothyronine (T_3), free thyroxine (FT_4), and free triiodothyronine (FT_3) were measured by a Microparticle Enzyme Immunoassay (MEIA) on the AXSYM System (Abbott Laboratories, Abbott Park, USA), while the serum total thyroxine (T_4) was measured by the Fluorescence Polarization Immunoassay (FPIA) method on the AXSYM System using the standard laboratory methodologies.

Estimation of Lipid Peroxidation

Lipid peroxides were estimated by measurement of thiobarbituric acid reactive substances in plasma by the method of Yagi.[14] The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde (a secondary product of lipid peroxidation) was estimated. The absorbance of clear supernatant was measured against reference blank at 535 nm.

Assay of Superoxide Dismutase and Catalase

Superoxide dismutase (SOD) was assayed using the technique of Kakkar et al.[15] based on inhibition of the formation of nicotinamide adenine dinucleotide, phenazine methosulfate, and amino blue tetrazolium formazan. A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction min/mg/Hb. Catalase (CAT) was assayed colorimetrically at 620 nm and expressed as µmole of H_2O_2 consumed min/mg/Hb as described by Sinha.[16] The reaction mixture (1.5 mL) contained 1.0 mL of 0.01 mole pH 7.0-phosphate buffer, 0.1 mL of hemolysate, and 0.4 mL of 2 mole H_2O_2. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

Estimation of Reduced Glutathione

Reduced glutathione (GSH) content was determined by the method of Ellman.[17] Plasma,1.0 mL, was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5,5'-dithiobisnitro-benzoic acid [DTNB] in 100 mL of 0.1% sodium nitrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

Assay of Glutathione Peroxidase

Glutathione peroxidase (GPx) activity was measured using the method described by Rotruck et al.[18] with modifications. Briefly, the reaction mixture contained 0.2 mL of 0.4 mole Tris-HCl buffer pH 7.0, 0.1 mL of 10 mmole sodium azide, 0.2 mL of hemolysate, 0.2 mL glutathione, and 0.1 mL of 0.2 mmole hydrogen peroxide. The contents were incubated at 37˚C for 10 minutes. The reaction was arrested by 0.4 mL of 10% TCA, and the contents centrifuged. The supernatant was assayed for glutathione content using Ellman's reagent. GPx activity was expressed as µmole of GSH consumed min/mg/Hb.

Assay of Glutathione-S-transferase

Glutathione-S-transferase (GST) activity was determined spectrophotometrically using the method of Habig et al.[19] The reaction mixture contained 1.0 mL of 0.3 mmole phosphate buffer (pH 6.5), 0.1 mL of 30 mmole 1-chloro-2, 4-dinitrobenzene (CDNB), and 1.7 mL of double distilled water. After preincubating the reaction mixture at 37˚C for 5 minutes, the reaction was started by the addition of 0.1 mL of hemolysate and 0.1 mL of glutathione as substrate. The absorbance was followed for 5 minutes at 340 nm. Reaction mixture without the enzyme was used as blank. The activity of GST is expressed as µmole of CDNB-GSH conjugate formed/min/mg/Hb using an extinction coefficient of 9.6 mmole-1 cm-1.

Statistical Analysis

All data were expressed as mean ± SD of number of experiments. The statistical significance was evaluated by Student's t-test using SPSS version 10.0 (SPSS, Cary, NC, USA).

Results

Demographic characteristics of the study population are shown in Table 1. The mean age of hypothyroid patients was 43±14 years and of control subjects, 47±13 years. Hypothyroid patients had a significantly increased body mass index (31±5.1kg/m^2) compared to control subjects (27±4.5kg/m^2). However, the levels of serum lipids in hypothyroid patients and control subjects did not differ significantly.

Table 1. Demographic characteristics and lipids in control subjects and hypothyroidism.

Parameter	Control subjects	Hypothyroid patients
No. of subjects	30	30
Age (years)	47±13	43±14
Duration of HPO (years)	—	4.9±2.1
Body mass index (kg/m^2)	27±4.5	31±5.1*
Total cholesterol (mg/dl)	187±21	190±19 NS
Triglyceride (mg/dl)	142±23	140±26 NS
HDL-C (mg/dl)	40±9	38±10 NS
LDL-C (mg/dl)	62±11	59±12 NS

Values are given as mean ± SD from 30 subjects in each group.

Hypothyroid patients compared with control subjects: *p<0.05; NS=not significant.

Table 2 shows the serum TSH and thyroid hormone levels of hypothyroid patients and control subjects. Compared to control subjects, the hypothyroid patients' mean TSH level was significantly higher.

Hypothyroid patients also had significantly lower levels of T_4, FT_4, FT_3, and T_3.

Table 3 illustrates the level of circulatory lipid peroxidation and antioxidant status in control subjects and hypothyroid patients. The extent of lipid peroxidation TBARS was significantly increased in hypothyroid patients when compared to healthy controls. For studying the deleterious consequence of diabetes on antioxidant status, the activities of the enzymatic antioxidants SOD, CAT, GPx, GST, and the non-enzymatic antioxidant GSH were measured. The activities of the enzymatic antioxidants and the level of GSH were significantly lower in hypothyroid patients when compared to healthy subjects.

Table 2. Serum thyroid hormone status in control subjects and hypothyroidism.

Parameter	Control subjects	Hypothyroid patients
FT_4 (ng/ml)	1.16±0.71	0.53±0.10**
FT_3 (pg/ml)	2.87±0.85	2.21±0.62 *
T_4 (µg/dl)	8.21±2.10	1.86±0.91***
T_3 (ng/ml)	0.95±0.25	0.51±0.11**
TSH (mIU/ml)	2.24±1.17	15.83±4.18***

Values are given as mean ± SD from 30 subjects in each group.

Hypothyroid patients compared with control subjects: *p<0.05, **p<0.01, ***p<0.001.

Discussion

Resch et al.[20] found that hypothyroidism was associated with enhanced oxidative stress and lipid peroxidation, and supposed that this might lead to the development and progression of atherosclerosis. In our study, hypothyroid patients' level of TBARS was higher than that of healthy subjects. Our data showed a significantly decreased SOD activity in hypothyroid patients compared to controls. In addition, the activities of the enzymes GPx and CAT were also clearly significantly lower in patients than in healthy subjects.

ROS have been reported to induce oxidative damage to membrane lipids, proteins, and DNA, and might result in cell death by necrosis or apoptosis.[21] Both GPX and CAT are major defenses against harmful effects of ROS in cells, and in cultured thyrocytes, both have a high capacity to degrade exogenous hydrogen peroxide (H_2O_2).[22] Specifically, observations indicate that GPx is involved in the degradation of fairly low H_2O_2 levels, whereas CAT is required to degrade H_2O_2 at higher concentrations. It is thus possible that the lower activities of GPx and CAT lead to H_2O_2-induced apoptosis of thyroid cells in Hashimoto's thyroiditis patients. In an *in vitro* study by Demelash et al.,[23] impaired capacity of GPx in degrading H_2O_2 in cultured thyroid pig cells aggravated the apoptic response. This data and our results suggest the possibility that reduced GPx and CAT activities in hypothyroid patients might participate in the initiation of the autoimmune process and might lead to H_2O_2-induced damage of thyroid cells related to cytosolic oxidative stress.

The mechanism linking hypothyroidism with oxidative stress and antioxidants is unknown. The effects of hypothyroidism on antioxidant parameters have been investigated in hypothyroid patients with intellectual disability.[4] The study showed that despite good medical care, patients had a decrease in the activity of three antioxidant defenses. Changes were more prominent among male subjects.

Table 3. Circulatory lipid peroxide and antioxidant status in control subjects and hypothyroid patients.

Parameter	Control subjects	Hypothyroid patients
TBARS (nmole/ml)	2.62±0.42	6.32±0.45***
SOD (Unit a mg/Hb)	2.91±0.46	1.83±0.27***
CAT (Unit b mg/Hb)	70.5±10.61	48.5±9.30***
GPx (Unit c mg/Hb)	8.75±2.32	6.21±1.55***
GST (Unit d mg/Hb)	2.52±0.45	1.05±0.40***
GSH (mg/dl)	41.11±7.52	25.36±4.91**

Values are given as mean ± SD from 30 subjects in each group.

Hypothyroid patients compared with control subjects: **p<0.01,***p<0.001.

Unit a- One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute.

Unit b - µmole of H_2O_2 consumed/minute.

Unit c - µmole of GSH consumed/min.

Unit d - µmole of CDNB–GSH conjugate formed/min.

Antioxidant deficiencies may lead to a failure to effectively combat extrinsic factors (i.e., weather, diet, drugs, and physical exercise) and intrinsic factors

(i.e., injuries, weakness, and fatigue) involved in oxidative stress. An extensive body of evidence now exists confirming that antioxidants are involved in the cellular defense against oxidative stress in a variety of pathological conditions. It has been suggested that hypothyroidism leads to oxidative stress and to a reduction of antioxidant defenses. In addition, previous experimental studies have reported that hypothyroidism is characterized by endothelial dysfunction of blood vessels.[24] To determine the status of the antioxidant system in relation to thyroid gland dysfunction, we used a complex of different diagnostic indicators related to damage of various biological tissues.

Although the pathophysiological consequences of the decelerated antioxidant levels are not yet elucidated, this biochemical change is thought to be a physiological adaptation and response to hypothyroidism. In agreement with previous findings, thyroid hormones are involve in combating the toxicity of oxidative stress in animals[25] and in humans.[12] Thus, under normal conditions, the protective effect of thyroid hormone against oxidative stress can be explained by the function of antioxidants as a defense system.

However, a chronic state of hypothyroidism is characterized by impairments in the redox potential. This leads to free radical chain reactions and to metabolic suppression of antioxidant capacity. Our results from this study support the suggestion that the hypothyroidism of patients with intellectual disability in some way is linked to the low levels of the major antioxidant molecules found in these patients. The depletion of antioxidants observed in hypothyroid individuals may reflect the increased free radical production in the electron transport chain in the mitochondrial inner membrane. The increase of free radicals is not compensated, as one would expect, by a decrease of antioxidants. A high oxidative state in hypothyroid people has metabolic and biochemical characteristics such as increased mitochondrial enzyme activity. Thus, it is likely that patients' cells are damaged by prolonged oxidative stress that far exceeds the capacity of the patients' organs to synthesize antioxidant molecules or to synthesize them from extracellular sources.[6]

Hypothyroidism is generally associated with decreased content of tissue protein. Hypothyroidism also specifically reduces most tissues' cellular thiol reserve and alters glutathione/GSH-Px content. Our hypothyroid patients had significantly decreased SOD activity. Importantly, SOD is the first line of enzymatic defense against intracellular free radicals. Because of that, a decrease of SOD activity would expose the cell membrane and other components to oxidative damage. Catalase shares with GSH-Px its function of catalyzing the decomposition of H_2O_2 to water. A low level of catalase activity, then, could primarily damage the endoplasmic reticulum in the cells. Glutathione reductase was little affected by the presence of hypothyroidism.[11]

In conclusion, our present study suggests a very high production of ROS and oxidative stress in patients with hypothyroidism, with enhanced lipid peroxidation and concomitant failure of antioxidant defense mechanisms. Physical signs and symptoms in people with hypothyroidism are less reliable and there is a need for serum testing to determine the appropriate dosage of replacement thyroid hormones. Our purpose in this study was to provide evidence for, and to recommend, blood testing for hypothyroid patients' antioxidant system in order to monitor the progression of pathology and to prompt the consideration of medical care.

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