Original Article

Investigation of Zinc and Copper Levels in Methimazole-Induced Hypothyroidism: Relation with the Oxidant-Antioxidant Status

(experimental hypothyroidism / oxidant-antioxidant status / copper / zinc)

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Abstract. Thyroid hormones are associated with the oxidative and antioxidative status of the organism. Depression of metabolism by hypothyroidism has been reported to decrease oxidant production and thus protect tissues against oxidant damage. The purpose of the present study was to investigate Zn and Cu levels in MMI-induced hypothyroidism and to show whether there is a connection between these trace elements and the oxidant-antioxidant status in experimental hypothyroidism. 3-Nitrotyrosine was measured as a marker of nitro-oxidative stress. In order to examine the antioxidant status of MMI-induced hypothyroidism in rats, GSH and SOD levels were determined as well. Significantly decreased 3-nitrotyrosine, Cu and Zn levels were observed in our experimental model when compared with the controls. On the other hand, GSH and SOD levels remained constant. It may be suggested that Cu and Zn serve as antioxidant molecules and exert their effects in an indirect manner to reduce oxidative stress in experimental hypothyroidism.

Introduction

Hypothyroidism is a clinical entity resulting from deficiency of thyroid hormones or, more rarely, from their impaired activity at the tissue level. In hypothyroidism, the basal metabolic rate is decreased, as are other processes dependent upon thyroid hormones (Hallengren, 1998; Laurberg et al., 2005).

Oxidative stress, characterized by an elevation in the steady-state concentration of reactive oxygen species (ROS), has been implicated in a wide range of biological and pathological conditions (Suntres and Lui, 2006). Thyroid hormones are associated with the oxidative and antioxidative status of the organism. Depression of metabolism by hypothyroidism has been reported to decrease oxidant production and thus protect tissues against oxidant damage. However, data on the oxidative status of hypothyroidism are limited and controversial (Isman et al., 2003; Sarandol et al., 2006).

Trace metals have been shown to influence hormones at several levels, including hormone secretion and activity, and binding to the target tissue (Aihara et al., 1984). Trace elements such as zinc (Zn) and copper (Cu) possess a very significant role in the regulation of many physiological processes (Hughes and Saman, 2006). Cu enters cells by membrane transporters and soluble transport proteins called metallochaperones, and members of the cation diffusion facilitator (CDF) family of transport proteins transport Zn into and outside the cell (Gaither and Eide 2001; Rosenzweig, 2001). Human thyroid tissue contains approximately 54.9 mg/kg Cu and 61 ppm Zn in it (Boulyga et al., 1999; Yaman and Akdeniz 2004). Zn is referred to as cofactor of many enzymes and has been shown to be important in maintaining membrane function and integrity; it plays a major role in cellular signalling. Moreover, zinc has been shown to have antioxidant properties by protecting sulphydryl groups against oxidation and inhibiting the production of ROS by transition metals (Bray and Bettger, 1990; Oteiza and Mackenzie, 2005; Rubio et al., 2007). On the other hand, Cu

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Abbreviations: CAT – catalase(s), Cu – copper, GSH – glutathione, MMI – methimazole, RNS – reactive nitrogen species, ROS – reactive oxygen species, SOD – superoxide dismutase, T3 – triiodothyroxin, T4 – thyroxin, TSH – thyroid-stimulating hormone, Zn – zinc.

undergoes redox-cycling reactions, constituents of oxidase enzyme iron absorption. Furthermore, dietary Cu supplementation may have beneficial effects by improving endogenous antioxidant defences as well. Deficiency of Zn and Cu has been observed to affect the endocrine system adversely (Bordin et al., 1993; Galhardi et al., 2005; Valko et al., 2005; Jones et al., 2006; Leach et al., 2006). There are no conclusive references regarding Cu and Zn influence on experimental hypothyroidism. In our current study we hypothesized that Zn and Cu may support the antioxidant system in experimental hypothyroidism.

Peroxynitrite ($ONOO^-$) is a reactive nitrogen species (RNS) that has been found to cause lipid peroxidation and cytotoxicity. $ONOO^-$ is a powerful oxidant which is highly reactive toward biological molecules including protein and non-protein sulphydryls, DNA, and membrane phospholipids (Genovese et al., 2007). $ONOO^-$ is formed by the rapid reaction of nitric oxide radical ($NO^{\cdot}$) with superoxide radical ($O_2^{\cdot -}$) (Whiteman et al., 2003; Radi, 2004). Nonetheless, addition of peroxynitrite to biological fluids leads to nitration of aminoacid residues, and the presence of these has been widely used as a marker of peroxynitrite-mediated (NO-dependent) damage *in vivo (*Kaur and Halliwell, 1994). 3-Nitrotyrosine is generated when $ONOO^-$ is added to tyrosine itself, or to proteins containing tyrosine residues (Daiber et al., 2004). Detection of protein 3-nitrotyrosine is regarded as a marker of nitro-oxidative stress and is observed especially in inflammatory processes (Naviliat et al., 2005).

In aerobic cells, active oxygen species, e.g. $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2) are generated as by-products of oxidative metabolism in mitochondria. These species are toxic to biomembranes and eventually lead to peroxidation of lipids unless they are removed by free radical-scavenging enzymes. Antioxidant enzymes act to scavenge free radicals by converting them to less harmful molecules (Yilmaz et al., 2003). These might be referred to as enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT) or metabolic antioxidants like glutathione (GSH) (Hermans et al., 2007). CAT represent the most important endogenous antioxidant defence against ROS-induced damage of the cell membrane. SOD protects tissues from oxygen free radicals by catalyzing the removal of $O_2^{\cdot -}$. Besides, CAT were shown to be responsible for the detoxification of significant amounts of H_2O_2 (Vergani et al., 2004; Narvaez-Mastache et al., 2007). In blood plasma, these chain-breaking antioxidants can trap free radicals directly, thereby interrupting chain-propagating reactions (Koracevic et al., 2001). Furthermore, selenoproteins are proteins that contain the selenocysteine form of selenium. Although selenium has a variety of functions, its antioxidant role has been the primary focus of research. Glutathione peroxidase (GPX) and thioredoxin reductase (TR) play a major role in controlling two major redox systems in the cell, i.e., the glutathione and thioredoxin systems. Selenium, therefore, as part of glutathione peroxidase, is considered one of the antioxidant nutrients and has interdependent roles with vitamin E, iron (as catalase), and Zn and Cu (as superoxide dismutase). Moreover, TR reduces thioredoxin, which in turn is capable of controlling various cellular redox-related processes such as transcription (e.g., activation of transcription factors), protein-DNA interactions, growth control, and DNA synthesis. These antioxidants help prevent the generation of free radicals, capable of removing toxic hydroperoxides and decrease the risk of oxidative damage to tissues. Additionally, other selenoproteins such as selenoprotein P and selenoprotein W appear to play a role in oxidant defence as well (Gladyshev et al., 1998; Holben and Smith, 1999).

The influence of thyroid failure on protein oxidation, antioxidant status and trace elements have been studied; however, not yet well fully understood. These findings led us to explore Zn and Cu levels togerther with oxidant-antioxidant system parameters in MMI-induced hypothyroidism and to elucidate whether there is a connection between these trace elements and the oxidant-antioxidant status in experimental hypothyroidism.

Material and Methods

In this study, 22 female Wistar Albino rats, 3 months of age, weighing 151–174 g were used. Animals were obtained from Istanbul University Animal Laboratory. All experimental protocols were approved by the Animal Care and Use Committee.

The control group was fed *ad libitum*, while the experimental group received tap water plus MMI-added fodder (75 mg/100 g) for 30 days, in order to induce hypothyroidism (Dariyerli et al., 2004). MMI was given according to the weight of the rats. Under urethane anaesthesia, blood samples were drawn from the heart into plastic syringes between 08:00 and 10:00 am. Samples were centrifuged at 2000 *g* for 10 min to obtain plasma.

Triiodothyroxin (T_3), thyroxin (T_4) and thyroid-stimulating hormone (TSH) levels were measured by the RIA method (Diagnostic Products Corporation, Los Angeles, CA). The coat-A-count procedure is a solid-phase radioimmunoassay where ^{125}I-labelled T_3, T_4 and TSH compete for a fixed time with T_3, T_4 and TSH in the sample for antibody sites. Radioactivity counting was performed in a gamma counter (model 1185, Searle, Nuclear Chicago Division, Chicago, IL).

CuZn SOD activity measurement

CuZn SOD activity was determined by the method of Sun et al. (1998) based on the inhibition of nitroblue tetrazolium (NBT) reduction. The absorbance of the reduction product was read at 560 nm in a spectrophotometer. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50 %.

Table 1. The results of the experimental procedure on rats

	Controls (N = 11)	Rats with hypothyroidism (N = 11)	P value
Weight (g)	161 ± 10	163 ± 11	P > 0.05
T3 (ng/100 ml)	53.58 ± 5.69	23.58 ± 2.79	P < 0.001
T4 (ng/100 ml)	4.31 ± 0.72	1.15 ± 0.28	P < 0.001
TSH (μIU/ml)	0.41 ±0.07	1.28 ± 0.18	P < 0.001

Values are means ± S.E.M. (N = 11 in all groups) * P ≤ 0.05 was considered significant

GSH measurement

GSH levels were determined by the method of Beutler et al. (1963).

3-Nitrotyrosine measurement

The 3-nitrotyrosine quantification in plasma was measured using an ELISA kit (ZID 7500 A, TCS Biological nitrotyrosine ELISA kit, Buckingham, UK). A standard curve was constructed using serial dilutions of nitrated BSA, which competed with immobilized nitrated proteins for the polyclonal anti-nitrotyrosine antibody. This method uses a rabbit anti-nitrotyrosine primary antibody and a donkey anti-rabbit IgG antibody coupled to horseradish peroxidase as secondary antibody. The assay was performed as per manufacturers' instructions (Bekpinar et al., 2005).

Cu and Zn measurement

One ml of serum was diluted to 10 ml with deionized water. The test tube was placed in the auto-sampler carousel after mild shaking. Serum samples were analysed for Cu and Zn contents using an atomic absorption spectrophotometer. The measurement was carried out automatically and the results are the average of two replicates according to the standard addition calibration method (Balkıs and Cagatay, 2001).

Statistical methods

Differences between experimental groups were assessed by the Mann-Whitney U test. The statistical software package SPSS 10.0 (SPSS, Inc., Chicago, IL) was used and P < 0.05 was considered significant.

Results

The thyroid state of the animals is shown in Table 1. A statistically significant increase in TSH and significant decrease in T_3 and T_4 (P < 0.001) levels of the experimental group are evidence of induced hypothyroidism.

At the end of the experimental period, the groups were checked for the differences in weight and no significant difference in weights was found (Table 1).

Significantly decreased plasma 3-nitrotyrosine, Cu and Zn levels were observed in rats with hypothyroidism when compared with the control group [(P < 0.01), (P < 0.01) and (P < 0.01), respectively]. Furthermore, no statistical significance was observed in the levels of SOD and GSH levels [(P > 0.05), (P > 0.05)] when compared with the control group.

Discussion

One of the major effects of thyroid hormone is the increment of mitochondrial respiration which results in increased generation of ROS, leading to oxidative damage to membrane lipids. There is a good deal of evidence to indicate that metabolic depression brought about by hypothyroidism is associated with a decrease in free radical production and subsequent protection against lipid peroxidation. This supports the notion that the reduced demand for oxygen in hypothyroidism might serve as a protective factor in tissue injury due to reactive oxygen metabolites (Isman et al., 2003; Sener et al., 2006a,b; Venditti and Meo, 2006). However, the mechanism involved in this protective effect has not been completely elucidated. Furthermore, MMI is suggested to have antioxidant properties and act as a radical scavenger, maintaining the GSH pool in the kidney of rats (Sausen et al., 1992; Braunlich et al., 1997).

In our current work, the level of 3-nitrotyrosine which reflects peroxynitrite-mediated oxidative damage in experimental hypothyroidism was apparently diminished. Nitrosative stress occurs when the generation of RNS in a system exceeds the system's ability to neutralize and eliminate them. Nitrosative stress may lead to nitrosyl-

Table 2. Plasma 3-nitrotyrosine, SOD, GSH, Cu and Zn levels of hypothyroid rats and the control group

	3-nitrotyrosine (μM/L)	SOD (U/ml)	GSH (%mg)	Cu (μg/dl)	Zn (μg/dl)
Control (N = 11)	0.21 ± 0.02	19.54 ± 2.48	27.01 ± 2.47	131.56 ± 8.41	66.22 ± 8.73
Hypothyroid (N = 11)	0.18 ± 0.02*	21.06 ± 2.62	24.63 ± 1.90	103.0 ± 10.27*	53.89 ± 4.94*

Values are means ± S.E.M. (N = 11 in all groups) * P ≤ 0.05 was considered significant

ation reactions that can alter protein structure, thus inhibiting normal function. Tyrosine nitration is induced by reactive nitrogen oxide species including $ONOO^{-}$, which is synthesized by the reaction between superoxide anion $O_2^{\cdot}$ and $NO^{\cdot}$. In hypothyroid rats, the decreased nitrosative stress is explained by the diminution in oxygen consumption and $O_2^{\cdot}$ production (Tenorio-Velazquez et al., 2005; Rubio et al., 2007). This supports the idea that the reduced demand for oxygen in hypothyroidism might serve as a protective factor in tissue injury due to reactive oxygen metabolites. As far as we are concerned, one of the reasons for the diminution of protein oxidation in MMI-induced experimental hypothyroidism might root from the low level of secreted T_3 and T_4 from the thyroid gland to the bloodstream. Another protective mechanism might be due to the antioxidative activity of MMI since sulphur-containing compounds have been mentioned in the literature as antioxidants in recent years (Nishimura et al., 2006). MMI was used as an agent to induce hypothyroidsm; therefore, a possible antioxidant effect of MMI should be taken into consideration.

In a normal cell, there is an appropriate prooxidant: antioxidant balance. However, this balance can be shifted towards the prooxidants when the production of oxygen species is increased greatly or when the levels of antioxidants are diminished. In the literature, this state is called "oxidative stress" and can result in serious cell damage if the stress is massive or prolonged (Leach et al., 2006).

The mechanism of antioxidant system in MMI-induced hypothyroid rats might be different. In our current work, we hypothesized that Cu and Zn might be somehow related with the oxidant-antioxidant status of the organism in experimental hypothyroidism and this might be reflected in the levels of these trace elements. We found significantly decreased Cu and Zn levels in hypothyroidism-induced rats compared with the controls.

Trace elements are known to influence many physiological processes; however, evaluation of Zn and Cu as antioxidants in experimental hypothyroidism is a novelty of this study. Zn and Cu have not been shown to interact directly with an oxidant species but rather prefer to exert their effects in an indirect manner. In the present study, GSH and SOD levels remained unchanged and seem not to play a protective role in this experimental model. Consequently, we may speculate that, in case of oxidative stress, the first defence line could be Cu and Zn elements in bloodstream. There are a few suggestions on this matter. Gibbs et al. (1985) and Bray et al. (1990) have suggested two mechanisms for antioxidant properties of zinc. The first mechanism is about the protection of sulphydryl groups against oxidation. The second mechanism by which Zn may function as an antioxidant molecule involves prevention of hydroxyl radical ($OH^{\cdot}$) and $O_2^{\cdot -}$ production by transition metals. Very few suggestions were made about the antioxidant properties of Cu. Galhardi et al. (2005) investigated the effects of dietary Cu supplementation on the lipid profile and antioxidant defences in serum of rats and reported that the markers of oxidative stress, lipid hydroperoxide and lipoperoxide, were decreased with Cu supplementation. However, we should consider the role of low-molecular forms of Cu in oxidative stress as well. $OH^{\cdot}$ radicals have attracted more interest than superoxide and H_2O_2 in biological systems, because they cause oxidative damage to biomolecules. Although $OH^{\cdot}$ generation requires redox active transition metals such as Cu and Fe, almost all cellular transition metal ions are tightly bound to proteins. Cu-mediated oxidation of bio-molecules such as DNA has been documented in detail. The possibility has been raised that the accumulated Cu metallothioneins in Long-Evans rat (LEC) livers may participate in the copper Haber-Weiss reaction (Nakamura et al., 1997). Zhang et al (2004) suggested that the homeostasis of metal ions in both serum and erythrocytes could be more or less influenced by the altered thyroid hormones, and they reported that serum Cu and Zn exhibited significantly positive correlation with T_3 and T_4. We also found decreased T_3, T_4 and Cu, Zn levels in MMI-induced hyperthroid rats. These results may illustrate the protective effect of these trace elements as cofactors of antioxidant enzymes in limiting oxidative stress. The utilization of Cu and Zn by the CuZn SOD enzyme may lower the levels of these trace elements in plasma. In this regard, any significant modification of the trace element status would lead to changes in the activity of antioxidants and have important consequences on the susceptibility of tissues to oxidative stress.

In conclusion, our work suggests that there is equilibrium between oxidant and antioxidant systems in experimental hypothyroidism, where Cu and Zn have important roles in maintaining this equilibrium and supporting the antioxidant functions. It is our belief that this study will contribute positively to the studies dealing with antioxidants, oxidative stress and experimental hypothyroidism. Therefore, our current work will bring different insight into the formation of new approaches in prevention of pathologies resulting from oxidative damage in hypothyroidism.

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