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EFFECT OF THYROXINE ON ANTIOXIDANT DEFENSE ENZYME ACTIVITIES AND GLUTATHIONE CONTENT IN BRAIN OF DIFFERENT MATURATED RATS

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Effect of thyroxine on antioxidant enzyme activities and GSH content in brain (cortex and rest of the brain) of different maturated rats were studied. Male Mill Hill hybrid hooded rats aged 15, 45 and 75 days were treated with L-thyroxine, T_4 (40 μg/100 g body mass), s.c., one dose per day, 14 days (finally aged 30, 60 and 90 days, respectively). Effect of T_4 on antioxidant defense (AD) enzyme activities and GSH content in brain differs with respect to age. T_4 treatment decrease activities of Mn SOD and CuZn SOD in the rest of the brain of 60 days old rats in comparison to controls and CAT activity in cortex of 30 and 60 day old rats. GSH-Px activity in cortex of 90 days old rats was decreased in T_4 treated group in respect to controls, while activity remains unchanged in 30 and 60 day old rats. The activity of GSH-Px in the rest of the brain

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was unaffected by the treatment in all groups. GSH content in cortex of animals of 30 and 60 days old rats were higher in comparison with orresponding controls, while in 90 days old rats effects were opposite. L- thyroxine treatment significantly decrease GSH content in rest of the brain of 30 days old rats in respect with corresponding controls, while in 60 and 90 days old animals such changes were not detected.

Key words: antioxidant defense enzymes, glutathione, cerebral cortex, brain, rats, thyroxine

INTRODUCTION

Thyroid hormones (TH): 3,5,3'-L-triiodothyronine, T_3 and 3,5,3'5'-L tetraiodothyronine, T_4) play crucial roles in the growth and differentiation of many organs, including brain development (PORTERFIELD and HENDERSON, 1993; OPPENHEIMER and SCHWARTZ, 1997). The importance of TH in brain development is most often illustrated by the severe neurological deficits observed in humans and animals deprived of TH during development. Deficiency or excess of TH during the perinatal period results in severe mental and physical retardation in humans.

Developing rodent cerebellum might be an excellent model for studying the molecular mechanisms of TH action in the brain, because perinatal hyperthyroidism greatly affects its ontogeny and metabolism. Brain development in the rodent occurs early relative to humans. For example, the rat brain at birth is at the same stage as the human brain at five to six months of gestation and the rat brain at ten days of postnatal age is equivalent to the human brain at birth. The development of the cerebellum occurs at a later stage of brain development.

Oxidative stress is believed to increase the concentration of reactive oxygen free radicals *in vivo*, which are responsible, at least in part, for a wide variety of degenerative processes (STADTMAN, 1992; HALIWELL and GUTTERRIDGE, 1989). Oxy radicals (reactive oxygen species - ROS) are generated continuously in all parts of brain during normal metabolism and brain activity. Whether damage occurs or further develops depends mainly on the balance between the generation of ROS and the activity of cellular antioxidant defense. When the generation of ROS exceeds the capacity of intracellular defense and repair mechanisms or the function of antioxidant defenses is impaired, the cell dies (ALLEN and TRESINI, 2000). Oxidative stress is cytotoxic consequence of ROS and oxidant formation. The superoxide anion radical ($O_2^{\cdot-}$) is generated by multiple enzymatic and non-enzymatic pathways and is often at the start of oxidative stress cascade (ALLEN, 1991).

Several enzymes are important in the antioxidative defense system, because they are involved in metabolism of either free radicals or reactive oxygen intermediates to non radical products. These enzymes include: superoxide dismutases, SOD (manganese containing -Mn and copper zinc containing -CuZn) which scavenge $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2) and oxygen (O_2), catalase (CAT) who removes H_2O_2 and family of GSH-dependant enzymes known as glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glutathione-S-transferase (GST).

Besides the above mentioned enzymes in detoxifications of ROS are included a non-enzymatic components, namely low molecular mass antioxidants. Among the many non-enzymatic antioxidants, the most important is glutathione (GSH). Glutathione represents a major non-enzymatic antioxidant and the most abundant non-protein thiol source in the cell (WARNER, 1994; DRÖGE, 2002). GSH serves as a substrate for GSH-Px and GST and under physiological conditions GR will rapidly reduce any oxidized glutathione (GSSG) to reduced form (GSH). Glutathione has several major functions: it detoxifies ROS under normal and impaired homeostasis, detoxifies drugs and maintains an essential thiol status of proteins and other molecules and provides the main molecular form in which cysteine can be stored within the organism and used for transfer between tissues (KANNAN *et al.*, 1992; JULIUS *et al.*, 1994).

In the present work, we studied the influence of TH on the antioxidative enzyme activities: SOD, EC 1.15.1.1. (Mn and CuZn), CAT (EC 1.11.1.6.), GSH-Px (EC 1.11.1.9.), GR (1.6.4.2.) and GST (C 2.5.1.18.), as well as, GSH content in the brain (cerebral cortex and the rest of the brain) of different maturated rats.

MATERIALS AND METHODS

The experiments were carried out with the male *Mill Hill hybrid hooded* rats. Animals were housed from birth to 30th day of age with their mothers. After 30 days, they were transfered to individual cages (four animals per cage). All animals were held under controlled conditions of illumination (lights on:5 a.m.-5 p.m.) and temperature (23°C) and were allowed free access to water and food. Animals at the 15th, 45th and 75th day of age were treated with L-thyroxine, T_4 (40 μg dissolved in 9 mM NaOH/100 g body mass), s.c., one dose per day, during the next 14 days before sacrificing (finally aged 30, 60 and 90 days, n=31) as performed earlier by WOOTEN and CASCARINO (1980). Control group was consisted of non-treated (intact) animals (n=29).

All animals were sacrificed by decapitation always between 8 and 10 a.m. to avoid any possible rhytmic variations in the antioxidant enzyme level. Fresh whole brains were dissected out immidiately after the decapitation and cerebral cortex was cutted off from the rest of the brain (GLOWINSKI and IVERSEN, 1966). Homogenization was performed with Janke and Kunkel (Staufen, Germany) Ika-Werk Ultra-Turrax homogenizer at 0-4 °C in 0.25 M sucrose, 1 mM EDTA and 0.05 M TRIS-HCl solution, pH 7.4 (ROSSI *et al.*,1987; DE WAZIERS and ALBRECHT, 1987). The homogenates were sonicated for 30s at 10 kHz on ice to release enzymes (TAKADA *et al.*, 1982) and used to determine the content of total glutathione (GSH+GSSG). The remaining sonicates were centrifuged (90 min, 85000 x g, 4 °C) and the supernatant was used for GSH-dependent antioxidant enzyme activity assays and total protein determination. All chemicals were Sigma (St. Louis, MO, U.S.A.) products.

GSH-Px activity was assayed using t-butyl hydroperoxide as substrate (PAGLIA and VALENTINE, 1967; TAMURA *et al.*, 1982) and the activity was

expressed in nanomoles of NADPH oxidized/min/mg protein. For the determination of GST activity, 1-chloro-2,4-dinitro benzene (CDNB) was used as a substrate (HABIG *et al.*, 1974) and the activity was expressed in nmol GSH used/min/mg protein. GR activity was measured as suggested by GLATZLE *et al.* (1974) and expressed in nmol oxidized NADPH/min/mg protein. For the GSH assay sonicated samples were deproteinized by 10% sulfosalycilic acid (2:1, v/v) and centrifuged 10 min on 3020 x g. Content of total GSH (GSH, reduced+GSSG, oxidized) was determined by enzymatic method suggested by TIETZE (1969) as modified by GRIFFITH (1980) and expressed as nmol GSH/g wet mass. All antioxidant enzyme assays were performed at 25°C and expressed as specific activity (units per mg protein). Protein content was measured by the method of LOWRY *et al.* (1951) using bovine serum albumin as a reference.

Statistical analysis was performed using protocols suggested by HINKLE *et al.* (1997). In experimental design here applied treatment was performed on different maturated rats, thereby the effects were statisticaly analyzed considering two factors: treatment and age using two-way analysis of variance (two-way ANOVA).

RESULTS

Effects of T_4 treatment on Mn SOD and CuZn SOD activities in cortex and rest of the brain

Mn SOD and CuZn SOD activities in cortex homogenates of 30, 60 and 90 days old rats were not affected by the T_4 treatment. The corresponding values for enzyme activities are shown in Table 1.

Table 1. - The activities of Mn SOD and CuZn SOD in cortex of 30, 60 and 90 days old rats treated with T_4 and controls (C). Enzyme activities are expressed in units per mg protein (as specific activity). The results are presented as Mean ± SEM

	30 days		60 days		90 days	
	C	T_4	C	T_4	C	T_4
Mn SOD	3.7 ± 0.2	4.1 ± 0.2	3.3 ± 0.2	3.3 ± 0.3	2.2 ± 0.2	2.3 ± 0.1
CuZn SOD	18.6 ± 2.0	16.9 ± 1.0	23.0 ± 2.7	18.9 ± 2.4	13.0 ± 1.3	16.0 ± 0.9

On the other side, T_4 treatment induce decrease in Mn SOD activity in 60 days old rats (2.44 ± 0.13 vs. 1.82 ± 0.13, $p<0.001$) and increase in 90 days old rats (1.55 ± 0.09 vs. 2.05 ± 0.10, $p<0.01$),(Fig.1), and decrease in CuZn SOD activity in 60 days old animals (28.04 ± 2.58 vs. 19.80 ± 1.88, $p< 0.01$), (Fig. 2).

Effects of T_4 treatment on CAT activity in cortex and rest of the brain

The change in CAT activity was observed only in cortex of 30 and 60 days old rats (Fig. 3). CAT activity in 30 and 60 days aged rats were lower in T_4 treated animals than corresponding controls (1.41 ± 0.11 vs. 0.85 ± 0.07, $p<0.001$; 2.06 ± 0.24 vs.1.50 ± 0.10, $p<0.001$). 90 days old rats were not affected by the T_4 treatment.

On the other hand, in the rest of the brain, we couldn't detect any changes in CAT activity. The interesting observations from our work is that CAT activity in brain homogenates of both tissues (cortex and rest of the brain) was exceptionally low in comparison to other rat tissues, indicating that brain is poor in CAT content.

Effects of T_4 treatment on GSH-dependant antioxidant enzyme activities in cortex and rest of the brain

GSH-dependant antioxidant enzyme activities were different in respect to treatment and age. The results of activities of GSH-dependant enzyme are presented in Table 2. In cortex of 30 days old rats there were no changes in enzyme activities between compared groups, as well as in 60 day old rats. In 90 day old rats T_4 treatment induce decrease in GSH-Px activity (19.99 ± 3.68 vs 8.48 ± 1.11, $p<0.001$) and increase in GR activity (37.36 ± 2.20 vs. 50.98 ± 2.31, $p<0.01$). GST activity was unaffected by the T_4 treatment.

*Table 2. - The activities of GSH-Px, GST and GR in cortex and rest of the brain of 30, 60 and 90 days old rats treated with L-thyroxine (T_4) and controls (C). Enzyme activities are expressed in units per mg protein (as specific activity). The results are presented as Mean ± SEM. **p<0.01 ***p<0.001*

	30 days		60 days		90 days	
	C	T_4	C	T_4	C	T_4
			CORTEX			
GSH-Px	18.6 ± 1.4	19.4 ± 1.0	2.6 ± 0.7	4.0 ± 1.1	20 ± 4***	8.5 ± 1.1
GST	128 ± 8	151 ± 10	162 ± 9	151 ± 9	155 ± 12	174 ± 8
GR	69.8 ± 5.1	66.6 ± 4.4	37.8 ± 2.1	37.0 ± 1.8	37 ± 2**	51.0 ± 2.3
			REST OF THE BRAIN			
GSH-Px	19.2 ± 1.2	19.3 ± 1.7	14.4 ± 2.0	12.9 ± 1.0	30.1 ± 1.2	25.1 ± 2.8
GST	171 ± 10	189 ± 9	193 ± 8	179 ± 10	214 ± 8	209 ± 14
GR	58.8 ± 2.8	64.4 ± 3.1	41.4 ± 1.8	37.4 ± 2.2	46.8 ± 1.0	46.1 ± 2.3

On the other side, in the rest of the brain, T_4 treatment had no effects on GSH-dependant enzyme activities in all compared groups and age.

Effects of T_4 treatment on GSH content in cortex and rest of the brain

Our results showed that GSH content after T_4 treatment were statisticaly higher in animals of 30 (197 ± 19 vs. 309 ± 20, $p<0.001$) and 60 days (33 ± 8 vs. 147 ± 9, $p<0.05$) old rats in respect with corresponding controls, while in 90 days old rats, effects were opposite (311 ± 29 vs. 80 ± 1, $p<0.001$), (Fig. 4).

L-thyroxine treatment significantly decrease GSH content in rest of the brain (Fig 5) of 30 days old rats (301 ± 21 vs. 74 ± 14, $p<0.001$) in respect with controls, while in 60 and 90 days old animals such changes were not detected. From Fig. 4 and Fig. 5 we can concluded that T_4 treatment had opposite effects in cortex than in the rest of the brain.

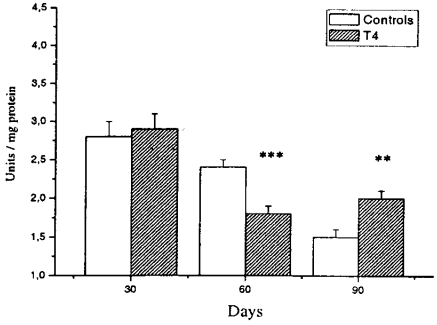

Fig. 1. - Mn SOD activity in the rest of the brain of 30, 60 and 90 days old rats treated with L-thyroxine (T_4) and controls (C) expressed in Units per mg protein. Columns represent mean values and vertical bars are S.E.M.
** $p < 0.01$ * $p < 0.001$

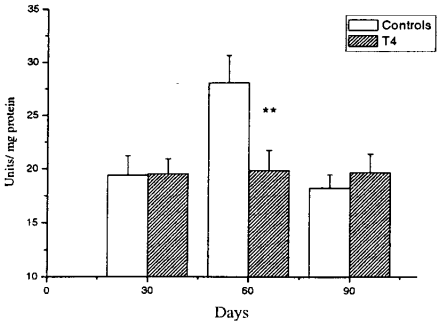

Fig. 2. - CuZn SOD activity in the rest of the brain of 30, 60 and 90 days old rats treated with L-thyroxine (T_4) and controls (K) expressed in Units per mg protein. Columns represent mean values and vertical bars are S.E.M.
** $p < 0.01$

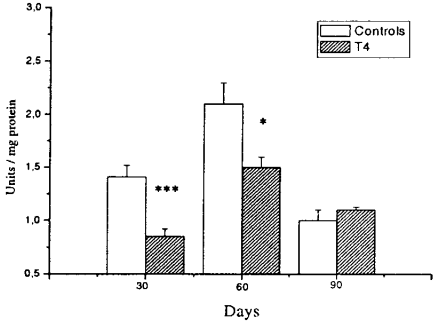

Fig. 3. - CAT activity in the cortex of 30, 60 and 90 days old rats treated with L-thyroxine (T_4) and controls (C) expressed in Units per mg protein. Columns represent mean values and vertical bars are S.E.M.
*$p < 0.05$ *** $p < 0.001$

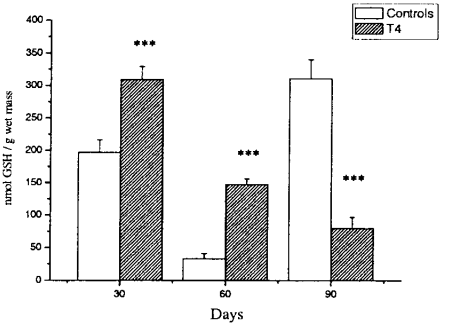

Fig. 4. - GSH content in the cortex of 30, 60 and 90 days old rats treated with L-thyroxine (T_4) and controls (C) expressed in nmol GSH per g wet mass. Columns represent mean values and vertical bars are S.E.M.
*** $p < 0.001$

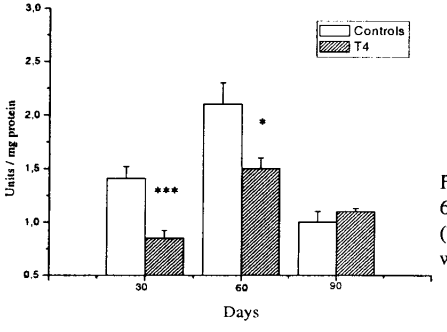

Fig. 5. - GSH content in the rest of the brain of 30, 60 and 90 days old rats treated with L-thyroxine (T_4) and controls (C) expressed in nmol GSH per g wet mass. Columns represent mean values and vertical bars are S.E.M.
*** $p < 0.001$

DISCUSSION

Thyroid hormones are physiologic modulators of both tissue oxidative stress and protein degradation. TH can affect oxidative stress through their effects on mitochondrial respiration (VIDELA, 2000), with hypothyroidism known to decrease and hyperthyroidism to increase respiratory activity (FERNANDEZ and VIDELA, 1993; GIAVAROTTI *et al.*, 1998). On the other side, great controversy exists as to whether hyperthyroidism is associated with an increase or decrease in the activity of antioxidant defense enzymes (ASAYAMA *et al.* 1987, FERNANDEZ *et al.* 1985). Mitochondria are considered a major source of free radicals, such as superoxide in healthy tissues (TURRENS and BOVERIS, 1980) because they are responsible for more than 95% of cellular oxygen consumption and these highly reactive oxidants can modify lipids, carbohydrates, proteins, and DNA.

The inherent biochemical and physiological characteristics of the brain with its high lipid concentrations and high energy requirements, makes this tissue particularly susceptible to free radical-mediated damage. The brain, like many other tissues has a various antioxidative defense, which help to maintain a balanced redox status (DEL MONTE, 2005; PETROVIĆ *et al.*, 1982; PETROVIĆ *et al.*, 1991; SAIČIĆ *et al.* 2001; 2003; 2004; VENDITTI *et al.*, 2004). From experimental data published elsewhere (HUH *et al.* 1998) it was shown that male rats have a higher extent of lipid peroxidation than females, which makes them more susceptible for oxidative stress. On the basis of these observations, we investigated the effects of thyroid hormone on antioxidative defense system in different aged male rats rendered hyperthyroid versus intact controls.

Results obtained from our experiments indicate that oxidative stress is present in different parts of rat brain in various extents. We concluded according the presented data, that oxidative stress is more present in rat cortex than rest of the brain. Such conclusion is based on results gathered from activities of CAT and GSH-Px which is decreased in T_4 treated animals. These enzymes are responsible for scavenging of H_2O_2 which might be very toxic for brain, especially if we consider the ability of this toxic by-product of superoxide anion detoxification to penetrate cells without carriers, thanks to its nonpolar nature. GSH-Px activity is decreased in most tissues of hyperthyroid rats and there was a parallel decrease in CAT in most tissues. Other GSH-dependant enzymes were not affected by T_4 treatment. These results suggest that the enhanced oxidative metabolism and decreased GSH-Px and CAT activity in hyperthyroidism result in an increased lipid peroxidation and possible organ damage.

The diminished levels of tissue GSH have been generally seen in the rest of the brain of 30 days old rats and in the cortex of 90 days old animals, while exceptional increase in the cortex of 30 and 60 old animals indicating protective role of GSH during the maturation and assembling of cortex. The occurrence of lower concentrations of GSH in older animals can be explained by the following possibilities. Firstly, the actual loss of GSH may be a result of increased rate of oxidation due to higher consumption of oxygen and concomitant higher generation

of H_2O_2 and hydroperoxides. Secondly, the diminished GSH concentration may be due to either increase degradation or decreased synthesis of GSH. This distinct fall in GSH content is age and treatment dependent. Thirdly, the lower concentration of GSH may be due to increased utilization of GSH in the removal of lipid and other peroxides. This suggests, increased turnover between GSSG and GSH and maintaining of stable redox environment. It has been postulated that redox environment obtained by redox couples is one of developmental determinant (SCHAFER and BUETTNER, 2001).

The cytosolic CuZn SOD did not change in 30 and 90 day old rats, but changed in 60 day old animals (decreased activity, $p<0.01$). On the other side, mitochondrial Mn SOD showed different changes in 60 day old animals (decrease activity, $p<0.001$) and 90 day old rats (increased activity, $p<0.01$) indicates adaptation on the hyperthyroid state and increased oxidative stress.

In this investigation, we have demonstrated that during maturation of rats, T_4 treated animals exhibit a diminished reducing potential especially in the cortex. This observations suggests, that during the period of lower GSH concentrations, hyperthyroid rat brain becomes susceptible to oxidative damage due to higher generation of oxidative molecules such as H_2O_2, hydroperoxides etc. Thus, older animals could be at risk and more vulnerable to deleterious effects of hyperthyroid state.

CONCLUSIONS

The large volume of circumstantial evidence presented here indicates that hyperthyroid brain tissue undergoes several biochemical changes that predispose them to free radical-mediated injury. Thyroid hormones have a profound effect on mitochondrial oxidative activity, synthesis and degradation of proteins and vitamin E, the sensitivity of the tissues to catecholamine and the levels of antioxidant defense enzymes. Detailed analysis of grand means reveiled, that T_4 direct effects should be viewed as clear change of T_4 treated animals in comparison with controls. Therefore, direct effects of T_4 on mature rats might be summarized as part of its overall catabolic role. Furthermore, animals at 30 days of age responded different to treatment in respect to old animals. These results suggest endogenous developmental pathern of antioxidant enzyme expression which could be modified by external factors. Different response of non-mature rats to thyroxine comparing to older rats observed in this study could be attributed to the difference in thyroxine metabolism and developmental phase of regulatory physiological systems including antioxidative defense system.

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EFEKAT TIROKSINA NA AKTIVNOST ANTIOKSIDACIONIH ZAŠTITNIH ENZIMA I KOLIČINU GLUTATIONA U MOZGU PACOVA RAZLIČITE STAROSTI

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Izvod

U ovom radu ispitivan je efekat tiroksina na aktivnost antioksidacionih zaštitnih enzima i količinu glutatationa (GSH) u mozgu (moždana kora i ostatak mozga) pacova različite starosti. Mužjaci pacova soja Mill Hill hybrid hooded starosti 15, 45 and 75 dana tretirani su sa L-tiroksinom, T_4 (40 μg/100 g telesne mase), s.c., jedna doza dnevno, 14 dana (do finalne starosti 30, 60 i 90 dana). Efekat T_4 na na aktivnost antioksidacionih zaštitnih enzima i količinu GSH u mozgu razlikuje se sa starošću pacova. Tretman sa T_4 smanjuje aktivnost Mn SOD i CuZn SOD u ostatku mozga pacova starih 60 dana u poredjenju sa kontrolama i aktivnost CAT u moždanoj kori pacova starih 30 i 60 dana. GSH-Px aktivnost u moždanoj kori pacova starih 90 dana je smanjena kod pacova tretiranih sa T_4 u odnosu na kontrole, dok je aktivnost nepromenjena kod pacova starih 30 i 60 dana. Aktivnost GSH-Px u moždanoj kori pacova je nepromenjena u svim ispitivanim grupama. Količina GSH u moždanoj kori pacova starih 30 i 60 dana je povećana u poredjenju sa kontrolama, dok je kod pacova starih 90 dana smanjena. Tretman sa L-tiroksinom značajno smanjuje količinu GSH u ostatku mozga pacova starih 30 dana u poredjenju sa kontrolama, dok kod pacova starosti 60 i 90 dana promene nisu detektovane.

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