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EFFECT OF THYROXINE ON GLUTATHIONE-DEPENDENT ANTIOXIDANT ENZYME ACTIVITIES AND GLUTATHIONE CONTENT IN THE INTERSCAPULAR BROWN ADIPOSE TISSUE OF DIFFERENT MATURATED RATS

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Summary: Effect of thyroxine on glutathione-dependent antioxidant enzyme activities and glutathione (GSH) content in the interscapular brown adipose tissue (IBAT) of different aged 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 GSH-dependent antioxidant enzyme activities in the IBAT differs with respect to age. T_4 treatment gradually decrease activities of all GSH-dependent antioxidant enzymes in 60 and 90 days old rats in comparison to young ones. GSH content in animals of 30 and 60 days old rats are lower in comparison with 90 days old rats, but the effects are oppposite. L-thyroxine treatment significantly increase GSH content in 30 days old rats ($p<0.001$) in respect with coresponding controls, while decrease in 60 and 90 days old animals were detected ($p<0.01$). Different response of non-mature rats to thyroxine comparing to older rats could be attributed to the difference in thyroxine metabolism and developmental phase of regulatory physiological systems maturation including antioxidative.

Key words: thyroxine, glutathione-dependent antioxidant enzymes, glutathione, interscapular brown adipose tissue, rats

Introduction

Thermogenesis is the major function of interscapular brown adipose tissue (IBAT) which is found in small mammals. Thyroid hormone (TH) is essential for normal development in vertebrate species. Normal thyroid gland activity is concerned mainly with energy metabolism in nearly all tissues of the body. TH is a major regulator of energy homeostasis with hyperthyroidism increasing basal metabolic rate and body temperature. The development of a hyperthyroid state in vertebrates elevates basal metabolic rate due to increments in the rate of O_2 consumption in target tissues (1) an effect accomplished by both (a) short-term mechanism activating mitochondrial cytochrome *c* oxidase and (b) long-term pathway involving changes in nuclear and mitochondrial gene expression through 3,3,5-triiodothyronine signaling. In the latter mechanism, respiratory genes may be upregulated through liganded TH receptor which binds to a TH-responsive element in the promoter regions (2). In IBAT, as well as in other aerobic tissues acceleration of aerobic metabolism by thyroxine enhances the generation of reactive oxygen species (ROS) in mitochondrial and microsomal sites (3). These conditions determine a higher consumption of cellular antioxidants (4, 5) and inactivation of antioxidant enzymes (6) thus inducing oxidative stress (7) with the concomitant increase in lipid peroxides and protein oxidation (1).

The data concerning changes in the amount of low molecular antioxidants such as glutathione (GSH), (8, 9), as well as the activity of antioxidative enzymes in different hyperthyroid rat tissues were investigated by Petrović et al. (10), Asayama et al. (8), Mijalković (11), Saičić et al. (12, 13).

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GSH represents a major non-enzymatic antioxidant and the most abundant non-protein thiol source in the cell (14, 15). GSH serves as a substrate for glutathione peroxidase (GSH-Px) and glutathione-S-transferase (GST) and under physiological conditions, glutathione reductase (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 (16, 17).

TH are known to act directly on multiple sites in vertebrate cells, but it seems that mitochondria are the most striking target. IBAT is very rich with mitochondria and potentially important source of free radicals.

In the present work we examine the effect of L-thyroxine, T_4 on total glutathione content (GSH, reduced + GSSG,oxidized) and glutathione-dependent antioxidant enzyme activities: glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione-S-transferase (GST, EC 2.5.1.18) and glutathione reductase (GR, EC 1.6.4.2.) in IBAT of different aged rats, 30, 60 and 90 days.

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 near by 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 *mg* dissolved in 9 mmol/L 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 (18). The study was performed using double control group protocol. One control group was consisted of non-treated (intact) animals (n=29). The second control group received 9 mmol/L NaOH/100 g body mass, the same way as T_4 treated animals (n=26).

All animals were sacrificed by decapitation always between 8 and 10 a.m. to avoid any possible rhytmic variations in the antioxidant enzyme level. Immidiately after the decapitation IBAT were extracted and washed out with saline solutions (154 mmol/L NaCl). Homogenization was performed with a Janke and Kunkel (Staufen,Germany) Ika-Werk Ultra-Turrax homogenizer at 0–4 °C in 0.25 mol/L sucrose, 1 mmol/L EDTA and 0.05 mol/L TRIS-HCl solution, pH 7.4 (19, 20). The homogenates were sonicated for 30s at 10 kHz on ice to release enzymes (21) and used to determine the content of total glutathione (GSH + GSSG). The remaining sonicates were centrifuged (90 min, 85000 × 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 (22, 23) 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 (24) and the activity was expressed in nmol GSH used/min/mg protein. GR activity was measured as suggested by Glatzle et al. (25) 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 × g. Content of total GSH (GSH,reduced + GSSG,oxidized) was determined by enzymatic method suggested by Tietze (26) as modified by Griffith (27) and expressed as nmol GSH/g wet mass. All GSH-dependent antioxidant enzyme assays were performed at 25 °C and expressed as specific activity (units per mg protein) and as total activity (units per g wet mass). Protein content was measured by the method of Lowry et al. (28) using bovine serum albumin as a reference.

Statistical analysis was performed using protocols suggested by Hinkle et al. (29). 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

The GSH-dependent antioxidant enzyme activities after T_4 treatment were presented in *Table I* (activity expressed both per mg protein – as specific and per g wet mass – as total). Statistical data are presented in *Tables II* and *III*.

GSH-Px specific activity was significantly inreased ($p<0.01$) in T_4 treated 90 days aged rats (33.3 ± 3.2) in comparison with corresponding controls (22.4 ± 2.0). At the same time, the activity of GSH-Px in 30 and 60 days aged rats did not show any differences between compared groups (*Table I*). On the other hand, when activity of GSH-Px expressed both as specific and as total (*Table I*) decrease during development in rats (significant age effect- (A); $p<0.001$; *Tables II* and *III*). This effect is evident in 30 days aged rats in respect to old ones.

We observed the similar effect in GST activity which is not statistically significant, but shows the

Table I The activities of glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST) and glutathione reductase (GR) in the IBAT of 30, 60 and 90 days old rats treated with L-thyroxine (T_4), internal controls (Ki) and controls (K). Enzyme activities are expressed in units per mg protein as specific and in units per g wet mass as total. The results are presented as Mean ± SD.

	30 days			60 days			90 days		
	Controls (K)	Internal Controls (Ki)	Treatment (T_4)	Controls (K)	Internal Controls (Ki)	Treatment (T_4)	Controls (K)	Internal Controls (Ki)	Treatment (T_4)
Specific activity									
GSH-Px	34.4 ± 16.4	44.0 ± 14.3	44.2 ± 14.5	12.8 ± 4.2	22.4 ± 7.7	14.0 ± 2.9	22.4 ± 4.4	23.4 ± 6.9	33.3 ± 6.5
GST	53.9 ± 20.9	54.3 ± 15.3	53.5 ± 13.1	31.3 ± 7.7	41.1 ± 11.2	37.3 ± 7.2	36.6 ± 3.4	36.5 ± 7.7	43.7 ± 11.3
GR	113.5 ± 38.3	98.9 ± 29.0	91.7 ± 34.8	50.5 ± 5.9	51.8 ± 16.3	51.1 ± 9.4	61.1 ± 8.5	69.4 ± 5.9	60.4 ± 8.7
Total activity									
GSH-Px	548 ± 305	880 ± 625	873 ± 313	576 ± 223	940 ± 181	424 ± 91	807 ± 178	798 ± 201	1244 ± 264
GST	966 ± 532	951 ± 603	1072 ± 338	1380 ± 255	1586 ± 345	1133 ± 257	1314 ± 32	1304 ± 239	1681 ± 607
GR	1749 ± 714	1740 ± 748	1632 ± 311	2156 ± 252	2078 ± 720	1523 ± 135	2281 ± 152	2392 ± 236	2240 ± 260

Table II Two-way analysis of variance (ANOVA) for GSH-dependent antioxidant enzyme activities in rats of different age treated with T_4 or corresponding controls. Results are presented as specific activity and expressed in units per mg protein. Df – degree of freedom, MS – mean square. *** $p < 0.001$

		(A)	(T)	A × T	Error
GSH-Px	Df	2	2	4	65
	MS	3935	353	138	139
	F	28.4***	2.54	1.00	
GST	Df	2	2	4	62
	MS	2304	106	78.9	175
	F	13.2***	0.61	0.45	
GR	Df	2	2	4	71
	MS	20656	329	512	692
	F	29.9***	0.48	0.74	

Table III Two-way analysis of variance (ANOVA) for GSH-dependent antioxidant enzyme activities and GSH content in rats of different age treated with T_4 or corresponding controls. Results are presented as total activity and expressed in units per g wet tissue. Df – degree of freedom, MS – mean square. *** $p < 0.001$ * $p < 0.05$

		Age (A)	Treatment (T)	A × T	Error
GSH-Px	Df	2	2	4	65
	MS	403970	335015	375831	119516
	F	3.38*	2.8	3.14*	
GST	Df	2	2	4	62
	MS	1392245	33731	295041	179740
	F	7.75***	0.19	1.64	
GR	Df	2	2	4	74
	MS	2072760	542947	233355	260631
	F	7.95***	2.08	0.9	
GSH	Df	2	2	4	36
	MS	44867	10605	44686	1194
	F	37.6***	8.88***	37.4**	

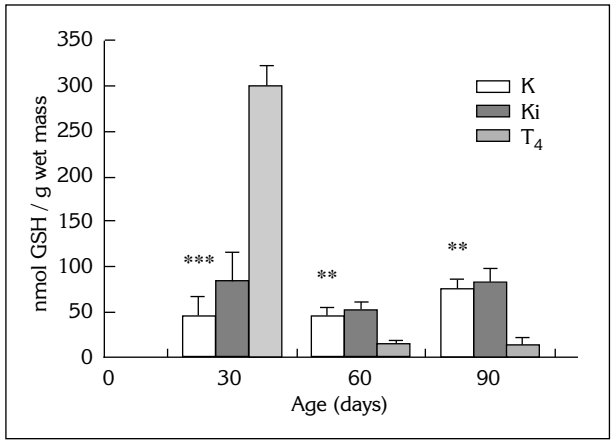

Figure 1. Glutathione content (GSH) in the IBAT of 30, 60 and 90 days old rats treated with L-thyroxine (T_4), internal controls (Ki) and controls (K) expressed in nmol GSH per g wet mass. Columns represent mean values and vertical bars are S.E.M.
** $p < 0.01$ *** $p < 0.001$

same trend, e.g. decreasing during development (significant age effect – (A); $p<0.001$; *Tables II* and *III*).

At the same time, specific GR activity appeared to be lower ($p<0.05$) in T_4 treated 30 days aged rats (91.7 ± 9.3) in comparison with the corresponding controls (113.5 ± 10.6). GR activity also decrease ($p<0.001$) during development in rats (significant age effect - (A), *Tables II* and *III*).

The effect of treatment is presented in *Tables II* and *III* as (T) effect. Treatment is strongly age dependent (interaction A × T, in *Tables*) only in GSH content (*Tables II* and *III*; *Figure 1*).

Our results showed, that T_4 treatment had opposite effects on GSH content. While in 30 days old animals we found a significant increase ($p<0.001$) in

GSH content between T_4 treated (300.7 ± 20) and corresponding controls (45.7 ± 5.9) in 60 and 90 days old rats we found decrease in GSH content ($p<0.01$). 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 control and T_4 disolving buffer treated animals. 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.

Discussion

From experimental studies, as well as an epidemiological data, it can be inferred that hyperthyroidism is associated with a general increase in tissue oxidative stress. On the other side, great controversy exists as to whether hyperthyroidism is associated with an increase or decrease in the activity of antioxidant enzymes (8, 30). It was shown, that antioxidative defence system is endogenous dynamic system incorporated in homeostatic regulation lead by internal regulatory signals (31).

ROS have been related with many physiological and pathophysiological processes. Under physiological conditions, it is estimated that approximately 80% of stationary oxygen uptake depends on mitochondrial respiratory chain activity (32). However, it's been shown that mitochondrial respiratory chain generates superoxide anion radical ($O_2^{\cdot -}$) at two different places, such as in the proximity of NADH dehydrogenase and of ubiqinon-cytochrome b. Superoxide anion radical is free radical species which is hydrogen peroxide precursor during its generation in mitochondria (33). In that regard, treatment with TH increase activity of several enzymes coupled with mitochondrial respiratory chain, content of cytochrome c, as well as the size and number of mitochondria. Therefore we may conclude, that TH influence on respiration in mitochondria by changing the concentration of some components in electron-transport chain, as well as redox state of its components (34–36).

BAT is anatomically distinct from white adipose tissue and is located in a number of regions of the body. It is particularly abundant in the interscapular, axillary and perirenal regions. The brown adipocyte contains several lipid droplets and the fat-free cytoplasm is occupied almost exclusively with mitochondria packed with cristae (37). Proliferation and hypertrophy of BAT occurs in response to increased thermic need (i.e. cold exposure). This growth is accompanied by increases in total protein, amount of mitochondria (37–39) and alteration in mitochondrial ultrastructure (40). The factors controlling brown fat proliferation are not clearly defined. Norepinephrine (or an intact sympathetic nervous system) is required, but is not a sufficient agent alone. Thyroid hormone is required as a permissive synergistic agent (41–43) for the BAT adaptive thermic response, but only at low concentrations (44, 45).

Therefore, we choose to examine the effects of induced hyperthyroidism on GSH-dependent antioxidant enzymes, as well as GSH content in the IBAT of different maturated rats, since this tissue, as we mentioned before, are rich with mitochondria and therefore might be significant source of ROS generation under T_4 stimulation.

Changes in GSH-Px and GST activity in 90 day old rats suggest that in maturated rats enzymatic antioxidant activities in the IBAT depend on age ($p<0.001$, *Tables II* and *III*) more than treatment itself. The slight rise in activity of GSH-Px and GST in 90 day old rats is followed with statistically significant decrease in total amount of GSH in T_4 treated animals compared with corresponding controls. This distinct fall in GSH content is age and treatment dependent ($p<0.001$, *Table III*).

There were no changes in GSH-Px and GST activity in 30 day old rats. On the other side, GSH content in 30 day old rats in T_4 treated animals were significantly higher than corresponding controls ($p<0.001$, *Table III*). The diminished levels of tissue GSH have generally been correlated with the covalent binding of xenobiotics to tissue macromolecules (46). 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 an concomitant higher generation of hydrogen peroxide and hydroperoxides. Secondly, the diminished GSH concentration may be due to either increase degradation or decreased synthesis of GSH. In fact, activity of GSH-dependent antioxidant enzymes have been found to be higher in the T_4 treated animals in comparison to controls, which implies higher consumption of reduced GSH. In the same time, GR activity was not changed. Thirdly, the lower concentration of GSH may be due to increased utilization of GSH in the removal of lipid and other peroxides.

Also, we must take in consideration that during maturation there may be an accumulation of toxic substances which would elevate the activity of enzymes such as GSH-Px and GST, resulting in the intracellular depletion of reduced GSH. Higher concentrations of TH itself in T_4 treated animals, may induce their own removal by GSH-dependent antioxidant enzymes, particularly with GST.

On the other side, quite opposite effects were observed in 30 day old T_4 maturated animals. Statistically significant rise in GSH content ($p<0.001$, *Table III*) may be explained different in comparison to old rats. Elevated GR activity in T_4 treated 30 day

old rats is probably adaptive response to overall biochemical and physiological processes within the cells, rather than direct effect of antioxidaive regulatory elements. 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 (47). One of cellular redox couples is GSH/GSSG and its optimal ratio is considered as developmental causal.

In this investigation, we have demonstrated that during maturation of rats T_4 treated animals exhibit a diminshed reducing potential. This observations suggests, that during the period of lower GSH concentrations in hyperthyroid rats IBAT 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.

It can be concluded from presented results, which under normal conditions there are a delicate balance between the rate of formation and the breakdown of ROS in the IBAT which is partially under the control of TH. Alteration in the thyroid state of the body influences the antioxidative defence in the IBAT and can lead to a pathophysiological state. Different response of non-mature rats to thyroxine comparing to older rats, could be attributed to the difference in thyroxine metabolism and developmental phase of regulatory systems maturation including antioxidative. Direct effects of T_4 on mature rats might be summarized as part of its overall catabolic role.

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EFEKAT TIROKSINA NA AKTIVNOST ANTIOKSIDACIONIH GLUTATION-ZAVISNIH ENZIMA I KOLIČINU GLUTATIONA U INTERSKAPULARNOM MRKOM MASNOM TKIVU PACOVA RAZLIČITE STAROSTI

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Kratak sadržaj: Ispitivan je efekat tiroksina na aktivnost antioksidacionih glutation-zavisnih enzima i količinu glutationa (GSH) u interskapularnom mrkom masnom tkivu (IBAT) pacova različite starosti. Mužjaci Mill Hill hybrid hooded pacova starih 15, 45 i 75 dana tretirani su sa L-tiroksinom, T_4 (40 mg/100 g telesne mase), s.c., jedna doza dnevno, tokom 14 dana (do finalne starosti 30, 60 i 90 dana). Efekat T_4 na aktivnost GSH-zavisnih antioksidacionih enzima u IBAT-u se razlikuje u odnosu na starost.Tretman sa T_4 smanjuje aktivnost svih GSH-zavisnih antioksidacionih enzima kod pacova 60 i 90 dana starosti u poređenju sa mladim jedinkama. Količina GSH kod životinja starih 30 i 60 dana je niža u poređenju sa pacovima starih 90 dana. Tretman tiroksinom značajno povećava količinu GSH kod pacova starih 30 dana ($p<0,001$) u odnosu na odgovarajuće kontrole, dok kod pacova starosti 60 i 90 dana izaziva smanjenje ($p<0,01$). Različit odgovor ne-maturiranih pacova na tiroksin u odnosu na maturirane životinje može se pripisati razlikama u metabolizmu tiroksina i razvojnoj fazi regulatornih fizioloških sistema uključujući i antioksidacioni.

Ključne reči: tiroksin, glutation-zavisni antiksidacioni enzimi, glutation, interskapularno mrko masno tkivo, pacovi

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