Original Article

The Effects of Hypo- and Hyperthyroidism on Nuclear, Cytosolic, Endoplasmic and Mitochondrial Fractions of Sialoglycoproteins in Rabbit Hepatocytes

(thyroid hormone / hyperthyroidism / hypothyroidism / sialoglycoproteins / hepatocytes / rabbits)

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Abstract. Enhanced sialylation of glycoproteins occurs during hypo- and hyperthyroidism. The role of sialic acid (SA) in cell membranes is well-standing, but its role in intracellular structures is still under analysis. We wanted to evaluate the influence of thyroid hormones on the sialylation ratio of intracellular proteins presented in cytosolic, mitochondrial, endoplasmic and nuclear fractions of rabbit hepatocytes. Twenty-one New Zealand male rabbits were divided into three groups. Hypothyroidism (N = 7) or hyperthyroidism (N = 7) was induced by adding propylthiouracyl (2 mg/l) or L-thyroxine (12 mg/l), respectively, to the drinking water for four weeks. Results were compared with healthy (euthyroid) control animals (N = 7). After isolation of intracellular fractions, standard SDS-PAGE electrophoresis and transfer onto nitrocellulose membrane were performed. Identification of SA residue was carried out with digoxigenin-labelled lectins: *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA). We noticed significantly higher level of SNA than MAA linkage sialoglycoproteins in all evaluated fractions. The sialylation ratio was significantly lower ($P < 0.05$) in the nuclear fraction in case of hyperthyroidism (detected with both agglutinins). In contrast to the nuclear fraction the content of SNA-detected sialoglycoproteins was significantly reduced in mitochondrial fraction of hyperthyroid hepatocytes ($P < 0.05$). Non-significant augmentation of MAA-detected sialoglycoproteins was observed in the mitochondrial fractions in both hypo- and hyperthyroidism. The fluctuations of sialoglycoproteins in endoplasmic fraction were not significant. Our work showed that the subcellular structures are rich in SA residues. Differing effects of thyroid hormones on sialylation ratio suggest an important role for hypo- and hyperthyroidism in sialoglycoprotein metabolism.

Introduction

The biological role of thyroid hormones is to maintain the normal energy level, growth, temperature and development. Disorders of thyroid gland are among the most frequent endocrine disorders (Nishio et al., 2005). The major target organ for thyroid hormones is liver. Approximately 8 % of the hepatic genes are regulated by thyroid hormones *in vivo*; therefore, the liver is an ideal tissue to study the effect of thyroid hormones on gene expression (Feng et al., 2000). Enhanced sialylation represents frequently occurring alterations of the carbohydrate structures in numerous cancers (Krześlak et al., 2003), and pathological disturbances of sialylation occur in hypo- and hyperthyroidism (Uniyal et al., 1998; Janega et al., 2002; Winsz-Szczotka et al., 2006).

Sialic acid (SA) is one of the most abundant terminal monosaccharides on glycoproteins of the eukaryotic cells. On their surface SA plays an important role mainly in the cell-cell interaction. It is also present inside the cells, where it is linked to glycoproteins occurring in the cytosol, nucleus (Bork et al., 2005) and mitochondria (Bosmann et al., 1972). The role of intracellular SA is not clear but previous study suggested its role e.g. in the regulation of the polysialylation of the neural cell adhesion molecule (Bork et al., 2005). Our purpose was to analyse the sialylation ratio of intracellular fractions obtained from liver of experimental hypo- and hyperthyroid rabbits.

Material and Methods

All experimental protocols were approved by the Ethical Committee for Animal Studies, Medical University of Lublin. Twenty-one male New Zealand, 8-week-old rabbits, housed in a conventional room in 12-h light/

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Abbreviations: Gal – galactose, MAA – *Maackia amurensis* agglutinin, PMSF – phenylmethanesulphonylfluoride, SA – sialic acid, SNA – *Sambucus nigra* agglutinin.

dark cycles with full access to water and food were divided into three groups. Hyperthyroidism (N = 7) was evoked by intramuscular injection of L-thyroxine (Sigma Chemical Co., St. Louis, MO) in a dose of 200 µg/kg body mass for four subsequent days, and 24 h after the last treatment the rabbits were sacrificed (Das and Chainy, 2001). Hypothyroidism (N = 7) was induced by the administration of 0.05% propylthiouracil in drinking water for 21 days in accordance with previously described methods (Nishio et al., 2005; Farwell et al., 2006).

Results were compared with the healthy (euthyroid) control group (N = 7). The occurrence of hypo- and hyperthyroidism as well as euthyroidism was confirmed by the measuring of free thyroxine (T_4) and triiodothyronine (T_3) in rabbits' serum obtained before sacrifice. The evaluation of the fT_4 and fT_3 levels was commercially performed by radioimmunoassay in Children Hospital Laboratory, Lublin, Poland. After the time of study rabbits were sacrificed and a piece of liver tissue of each animal was immediately frozen and stored at –70 °C. The isolation of nuclear, mitochondrial and cytosolic fractions was performed according to the previously described method of Blobel and Potter (1966) with minor modifications. The liver tissues were minced, washed three times with the isotonic sucrose buffer and subsequently homogenized at 4 °C using the Potter homogenizer for 3–5 min with buffer No. 1 containing 5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM PMSF, 50 mM Tris-HCl, pH 7.4, and 0.25 M sucrose. After centrifugation (800 × *g* for 7 min) postnuclear supernatant (No. 1) was prepared from the pellet. The pellet was suspended in buffer No. 1 with 2.2 M sucrose. After centrifugation (40,000 × *g* for 60 min) the pellet was washed with isotonic sucrose buffer and next the nuclei were layered on sucrose cushions and centrifuged at 800 × *g* for 10 min. The cytosolic and mitochondrial fractions were obtained by differential centrifugation of supernatant No. 1. After the first centrifugation at 800 × g for 10 min the pellet was removed and the supernatant was assigned for the second centrifugation at 10,000 × *g* for 60 min. After centrifugation the pellet containing the mitochondrial fraction was stored for the analysis and the supernatant was next centrifuged (100,000 × *g* for 60 min). After centrifugation the supernatant contained the cytosolic fraction and the pellet contained the endoplasmic reticulum fraction (with Golgi apparatus).

Subcellular protein fractions were separated (each sample contained equal, 80 µg/line, protein level) on standard 10% polyacrylamide SDS-PAGE electrophoresis and blotted onto nitrocellulose membrane. Total protein was analysed with commercially available Biorad Protein Assay (Biorad Lab. GmbH, Munchen, Germany) according to producer's instructions. The integrity of proteins was tightly controlled in two ways. Firstly, all electrophoretic gels were examined with Commassie Blue staining. Secondly, the efficiency of electrotransfer and proteins integrity was detected directly on the nitrocellulose membrane with Ponceau S. Identification of sialoglycoproteins was carried out by means of digoxigenin-labelled lectins: *Sambucus nigra* agglutinin (SNA), which stains SA-α2,6-Galactose (Gal) linkage, and *Maackia amurensis* agglutinin (MAA), which stains SA-α2,3-Gal linkage (DIG Glycan Differentation Kit, Roche Diag., Mannheim, Germany). Quantification of sialoglycoproteins was done using a computer scanner and ZERO-Dscan Image Analysis System (Scanalytics, Billerica, MA). Values of sialoglycoproteins in hypo- and hyperthyroidism were expressed as a percent of optical density with regard to sialoglycoproteins occurring in normal (euthyroid) livers. Molecular weight of sialoglycoproteins was evaluated by comparison to the molecular mass standard (Rainbow markers, Amersham Life Sciences, Little Chalfont, UK). Student's *t*-test was applied to perform the statistical analysis. Statistically significant values were considered when $P < 0.05$.

Results

We noticed significantly higher fT_4 and fT_3 levels after thyroxin injection in comparison to the control group (mean fT_3: 6.58 ± 1.01 ng/ml and 1.06 ± 0.23 ng/ml in hyperthyroidism and control, respectively, $P < 0.05$; mean fT_4: 6.26 ± 0.97 ng/dl and 1.09 ± 0.14 ng/dl in hyperthyroidism and control respectively, $P < 0.05$). Significantly lower ($P < 0.05$) fT_4 and fT_3 levels were observed after propylthiouracil treatment in comparison to the control group (mean fT_3: 0.44 ± 0.03 ng/ml; mean fT_4: 0.48 ± 0.04 ng/dl in hypothyroidism).

The presence of sialoglycoproteins detected with SNA was noticed in most cases of evaluated samples (Fig. 1a). Contrary to SNA, clear bands corresponding to MAA sialoglycoproteins were observed only in the nuclear fraction but the fuzzy bands were also observed in the mitochondrial, endoplasmic and cytosolic fractions of hepatocytes (Fig. 2b).

The highest amount of SA residue with α2,6 linkage was observed in sialoglycoproteins at molecular weight between 14–110 kDa in the nucleus and approximately 35–110 kDa in the cytosol, endoplasmic reticulum and mitochondria. Therefore, the sialoglycoproteins at above-mentioned molecular masses were taken for further analysis. The content of SA residue was increased non-significantly in nuclear and cytosolic fractions, but it was significantly decreased in the mitochondrial fraction obtained from hyperthyroid rabbits in comparison to the control. The character of sialylation of the cytosolic fraction in hypo- and hyperthyroidism was ambiguous; some band intensities were augmented (e.g. the band corresponding to approximately 90 kDa) but other band intensities were reduced (e.g. the band at 97 kDa) in hyperthyroidism in comparison to the control (see Fig. 1a). A similar finding was noticed in the cytosolic fraction of hypothyroidism cases where some bands representing the SA residue were increased (e.g. at 90 kDa) and others were decreased (e.g. at 46 or 97 kDa, see Fig. 1a, arrows). In a lesser degree, the above observation was present in the nuclear fraction, where slightly increased SNA sialoglycoprotein was no-

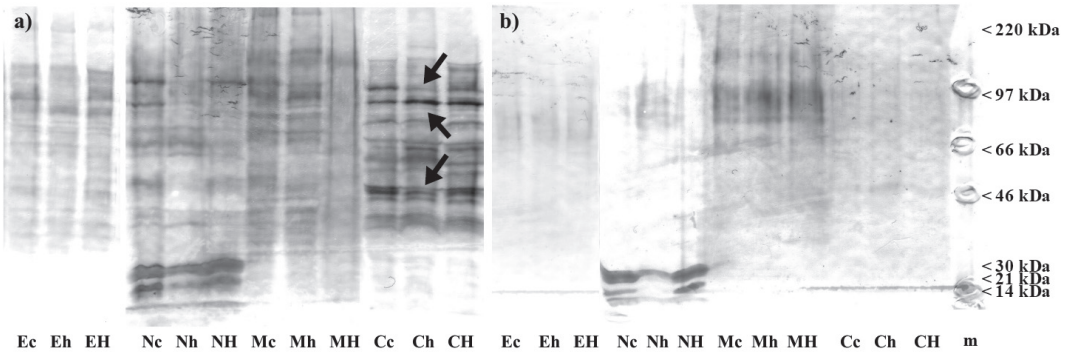

Fig. 1. Representative pictures of sialoglycoproteins detected with SNA (a) or MAA (b) on nitrocellulose membrane in endoplasmic (E), nuclear (N), mitochondrial (M) and cytosolic (C) fractions obtained from hypothyroidism (h), hyperthyroidism (H) and control (c) hepatocytes. m – molecular mass standard. The opposite effects of hypothyroidism on cytosolic sialoglycoproteins (see arrows, the description is in the text).

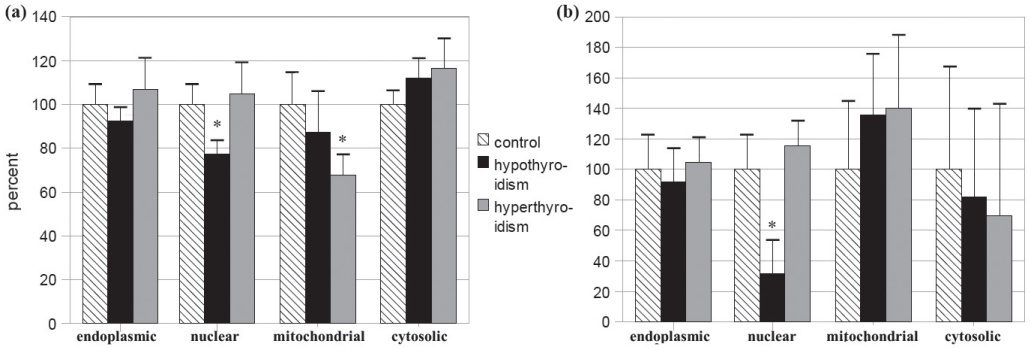

Fig. 2. The total content of sialoglycoproteins detected with SNA (a) or MAA (b) agglutinins obtained from hepatocytes (subcellular fractions: endoplasmic, nuclear, mitochondrial and cytosolic) of hypo-, hyper- and euthyroid (control) rabbits. A statistically significant decrease of sialylation was noticed in the nuclear fraction in case of hypothyroidism. A significant decrease of SNA-detected sialoglycoproteins was shown in the mitochondrial fraction of hyperthyroid hepatocytes. Asterisks indicate the differences of sialylation ratio in comparison to control hepatocytes. Means and standard deviations were expressed as percent of values presented in the control group (control values were assigned to 100 %). Student's *t*-test, * $P < 0.05$

ticed in hyperthyroidism at 30 kDa but decreased at 80 kDa in hyperthyroidism. The sialylation ratio was reduced in the nuclear fraction in case of hypothyroidism at all analysed molecular masses. The pattern of SA residues observed in the endoplasmic fraction was similar to that in the cytosolic fraction, but the intensity of bands was considerably lower. As opposed to nuclear and cytosolic fractions the content of SA residues with α2,6 linkage was abolished in the mitochondrial fraction in case of hyperthyroidism. In hypothyroid hepatocytes the content of SNA sialoglycoproteins in the mitochondrial fraction was slightly decreased in comparison with the control, but the result did not reach statistical significance.

MAA sialoglycoproteins were less numerous than SNA in rabbit hepatocytes and the bands were more fuzzy. A significant augmentation of MAA-detected sialoglycoprotein was noticed at approximately 20 kDa molecular mass in the nuclear fraction in case of hyperthyroidism. In hypothyroidism the content of SA α2,3 linkage was significantly lower in comparison to the control. We did not detect the SA moiety with α2,3 linkage at 20 kDa molecular mass in hypothyroidism. The remaining fractions did not reveal changes in the content of MAA-detected sialoglycoproteins in hypo- or hyperthyroidism (Fig. 2).

Discussion

Sambucus nigra agglutinin and *Maackia amurenisis* agglutinin are widely used for detection of different SA linkages. These lecithins detect SA residues linked to Gal of glycoproteins or glycolipids with α2,6 (SNA) or α2,3 (MAA) linkage. The sialylation process is catalysed by α2,6 and α2,3 sialyltransferases which transfer SA from cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) to terminal positions on sugar chains (Okajima et al., 1999). Previous studies indicated that hypothyroidism induced the α2,3 sialyltransferase expression in pituitary thyrotrophs (Helton and Magner, 1995) and thyroxine treatment rapidly decreased the synthesis of sialyltransferase mRNA in mouse hepato-

cytes (Feng et al., 2000). These findings suggest that the sialylation ratio decreases in hyperthyroidism (negatively regulated expression of sialyltransferase by thyroxine). Moreover, another study showed that the increase in the protein-bound SA in hypothyroid rats is probably due to intense disruptions of membranes, but accelerated secretion of glycoproteins with urine can be the reason for the decrease of SA content in serum glycoproteins of hyperthyroid rats (Uniyal et al., 1998).

According to our knowledge this study was the first to focus on the influence of thyroid hormones on SA residues linked to the intracellular glycoproteins obtained from rabbit hepatocytes. This work did not evaluate the effect of thyroid hormone on the definite, specific proteins but it analysed the overall influence of hypo- and hyperthyroidism on sialoglycoproteins in different subcellular fractions. The well-standing method described by Blobel and Potter (1966) was used to extract nuclear, cytosolic, endoplasmic and mitochondrial fractions. Our study showed the presence of SA-α2,6-Gal linkage in all evaluated fractions. Moreover, we noticed that α2,3-Gal is a rarely present intracellular linkage of SA. The above finding confirmed that beside cellular membranes, also intracellular proteins are rich in SA residues. The differential levels of SNA-detected sialoglycoproteins in all subcellular fractions of hypo- and hyperthyroidism cases indicate that thyroid hormones play an important role in the sialylation process. An interesting finding were the opposite effects of hypo- and hyperthyroidism on various glycoproteins at nuclear, cytosolic and, to a lesser degree, in endoplasmic fractions. In some of them the sialylation ratio was higher in hyperthyroidism in comparison to the control group, but on the other hand, some glycoproteins displayed significantly lower sialylation in the same conditions compared to the euthyroid fraction. The observed change of the sialylation ratio could be due not only to the reduction or augmentation of sialyltransferase activity, but also to the alteration of either protein or SA metabolism during hypo- and hyperthyroidism.

This effect was not observed in the mitochondrial fraction. The independent, autonomous synthesis of the carbohydrate portion of glycoproteins as well as proteins by mitochondria can be the reason of various effects of thyroid hormones on this fraction in comparison to the nuclear and cytosolic fractions. The presence of the SA-α2,6-Gal sialoglycoprotein bands in the mitochondrial fraction of hyperthyroidic hepatocytes was completely abolished. The above finding is in accordance with the theory about the negatively regulated expression of sialyltransferase by thyroxine.

In conclusion, our results indicate that intracellular proteins obtained from hepatocytes have a large number of sialoglycoproteins that can be detected with *Sambucus nigra* and *Maackia amurenisis* agglutinins. Moreover, the effect of thyroid hormones can significantly change the content of SA in mitochondrial and nuclear fractions of hepatocytes.

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