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EFFECT OF THYROTROPIN AND ADENOSINE ON INOSITOL PHOSPHOLIPIDS PATHWAY IN HUMAN THYROID IN GRAVES' DISEASE

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The present study demonstrates that exposure of human thyroid slices obtained during planned surgery of patients with Graves' disease to thyrotropin stimulates the phospholipid metabolism as measured by an increase incorporation of 2-myo-[^{3}H]-inositol into phosphatidylinositol and poliphosphatidylinositides and the generation of $InsP_3$. The results indicate that adenosine, probably via the A1 type of P1 receptor, modulates these actions, both incorporation of labelled substrate into thyroid slices as well as its metabolism to active compounds which could play a role in a cell signalling system. These observations indicate the significance of the phosphatidilinositol pathway in signal transmission of both, thyrotropin as well as P1 purinergic receptors agonists in human thyroid.

In Graves' disease thyroid hyperfunction and growth is ascribed to the thyrotropin-mimicking effect of Thyroid Stimulating Antibodies (TSAb) directed against the TSH receptor on the surface of the thyroid cells.

Zakarija et al (1) using FRTL-5 cells indicated an action of immunoglobulins present in Graves' Disease through adenylate cyclase system. Laurent et al (2) suggested the action of TSAb on thyroid cells in Graves' disease through the adenylate cyclase system and insensitivity of the inositol cascade system to TSAb in thyrocytes. However they did not use Graves' human thyroid tissue in their experiments. On the other hand claims have

ABBREVIATIONS:

TSH - thyrotropin, PIP_2 - phosphatidylinositol-4,5-bisphosphate, $InsP_3$ - inositol 1,4,5-trisphosphate, $InsP_2$ - inosistol 4,5-bisphosphate, InsP - inositol 5-monophosphate, GInsP - glycerophosphoinositol, PIPs -polyphosphoinositides, cAMP - cyclic AMP PIP - phospatidylinositol-4-phosphate, PIP_2 - phosphatidylinositol-4,5-bisphosphate PI - phosphatidylinositol.

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been made that the existence of a growth stimulating antibodies different from TSAb, might cause thyroid growth in Graves' disease and some euthyroid goiters through a pathway distinct from adenylate cyclase. (3,4).

Experiments with the thyroid tissue from animals (5-7) and humans (8) suggest the physiological role of the phosphatidylinositol pathway in the signal transduction after thyrotropin. Recently reciprocal influences of agonists of TSH and P1 receptor on this pathway has been proven (9). The aim of the present study was to answer the question, if TSH acts on the inositol phospholipid pathway in thyroid obtained from patients with Graves' disease and if this action could be modified through adenosine, a stimulator of P1 receptor.

MATERIAL AND METHODS

Human thyroid tissue was obtained after planned lobectomies of 10 female patients with Graves' disease aged 37 $\pm$ 13 yrs (19 - 49 yrs). All patients were in clinical and laboratory euthyroidism.

The resected tissue immediately after surgery was weighted and cut during 10 minuts into slices and placed in a centrifuge tube á 50 ml. The slices were preincubated for 1 h at 37°C in Tris-HCl buffer pH 7,4 (1ml/g of slices) containing glucose (5 mM), NaCl (0,1 mM), K_2HPO_4 (1 mM), $CaCl_2$ (1 mM), $MgCl_2$ (0,8 mM), KCl (5 mM), unlabelled myo-inositol (1 mM) and labelled myo-2-[^{3}H]-inositol (0,25 μCi/g of slices) obtained from Amersham. After preincubation slices were washed 5 times in the same medium without labelled myo-inositol enriched with LiCl (10 mM). Next the slices were divided into 0,5 g portions and transferred to 2 ml of medium (as above) and incubated for 1 h at 37°C with TSH (100 mU/ml), adenosine (1mM) or TSH and adenosine (the same concentrations) as well as without these substances according to Mośko et al (10). During incubation the slices were shaken constantly. Incubations were terminated after 1 hr by addition of 0,1 ml of conc. $HClO_4$. The content of each test tube was homogenized, transferred to centrifuge tubes á 10 ml, mixed with 0,5 ml of 1 M $HClO_4$ used for washing the homogenizer, and centrifuged at 1000 r.p.m. for 10 min. Supernatant was decanted and the pellet was suspended in 0,5 ml of 1 M $HClO_4$. After centrifugation (1000 r.p.m. for 10 min) the pellet was used for phospholipids preparations and supernatant was combined with that, obtained before and neutralized to pH 7,4 with 1 M KOH, cooled and centrifuged at 3000 r.p.m. for 5 min.

The supernatant was diluted with water to a volume of 10-15 ml and the glycerophosphoinositol (GInsP) and inositol phosphates (InsPs) were separated using Dowex 1 x 8 resin column (formate form) (11). Free [^{3}H]-inositol was washed through with water. GInsP was eluted with 5 mM sodium tetraborate in 60 mM sodium formate. InsPs were eluted with the increasing concentrations of ammonium formate in 0,1 M formic acid: InsP - 0,2 M ammonium formate; $InsP_2$ - 0,4 M ammonium formate; $InsP_3$ - 1,0 M ammonium formate. Fractions (3 ml) were collected and counted for radioactivity.

The pellet used for phospholipids preparations was suspended in 3,8 ml of acidified mixture of chloroform - methanol - water (5:10:4 v/v). After 10 min. 1ml of chloroform and 1 ml of 0,1 M HCl were added. Test tubes were shaken and centrifuged (3000 r.p.m. for 5 min) to separate the two phases. Protein in the interphase was measured by the method of Lowry et al. (12). Organic phase was evaporated until dryness and lipid residue was dissolved in 1,0 ml of mixture benzene - ethanol (4:1 v/v). Phospholipids were separated using thin layer chromatography. Quantity of phospholipids was determined by measurments of organic phosphorus (13).

Incorporation of labelled myo-2-[^{3}H]-inositol into phosphatidylinositides was determined by radioactivity measurments. Bray's scintillator liquid (naphthalene, 1,4-bis-(5-phenyl-2-oxasolyl)-benzene, 2,5-diphenyloxazole, ethylene glycol, dioxane) in a volume 10 ml was added to each probe obtained from column chromatography and 5 ml for phospholipids probes. The samples were counted for radioactivity by a liquid scintillator spectrometer LKB Wallac 1209 Rack Beta.

Statistical analysis were performed using non-parametric Wilcoxon rank test.

RESULTS

Tab 1. presents the results of experiments where the thyroid slices were divided after preincubation into the following groups: the control group without agonists, stimulated groups by TSH in a dose of 100 mU/ml and by adenosine in a concentration of 1 mM and the group with both, adenosine and TSH in these same concentrations together. Thyrotropin insignificantly increased the levels of GInsP and inositol phosphate in comparison to the control group. The rise of inositol bisphosphate and trisphosphate as well as total radioactivity connected with inositols were significant. Adenosine significantly stimulated GInsP and InsP and significantly decreased $InsP_2$ and $InsP_3$. Adenosine and TSH used together moderately increased GInsP and InsP. Ins P_2 decreased significantly, but the quantity of $InsP_3$ increased comparably to stimulation after TSH.

The results of incorporation of labelled myo-2-[^{3}H]-inositol into inositol phospholipids in the investigated tissue are presented in table 2. TSH significantly increased the quantity of radiolabelled total phospholipids, phosphatidylinositol and PIPs. Adenosine increased the contents of PI and diminished the quantity of PIPs. Adenosine

Tab 1. Distribution of [^{3}H]-glicerophosphoinositol (GInsP) and [^{3}H]-inositol phosphates (InsP, $InsP_2$ and $InsP_3$) in the thyroid slices incubated with TSH and adenosine (n=10)

	GInsP	InsP	$InsP_2$	$InsP_3$	TOTAL
CONTROL	1501 ± 1173	1053 ± 755	344 ± 516	458 ± 449	3185 ± 1958
TSH	1682 ± 1107	1106 ± 451	406 ± 481*	809 ±519*	3842 ± 1941*
ADENOSINE	2513 ± 678*,**	1288 ± 332	139 ± 188*,**	229 ± 174 **	4108 ± 812**
ADENOSINE AND TSH	2151 ± 737*,**	1411 ± 312	108 ± 80**	797 ± 747***	4368 ± 1706**

TSH was added in the 100 mU/ml, adenosine in concentration 1 mM. In the flasks with both stimulus adenosine was added 10 min previously to TSH. Control group was the parallel incubated without stimulants. The samples were measusred in duplicates. The results are presented in cpm/100 mg of protein as means and SD.

* $p < 0.05$ in comparison to control.

** $P < 0.05$ in comparison to TSH.

*** $p < 0.05$ in comparison to adenosine.

Tab 2. Effect of TSH and adenosine on the incorporation of myo-2-[3H]-inositol into the inositol phospholipids in the thyroid tissue obtained from patients with Graves' disease (n=10)

	TOTAL	PI	PIPs
CONTROL	15974 ± 9547	4790 ± 2566	1099 ± 968
TSH	23033 ± 22877*	8513 ± 5919*	2598 ± 2463 *
ADENOSINE	30515 ± 8470*	14894 ± 4375* **	707 ± 310 **
ADENOSINE AND TSH	33135 ±11987*	15909 ± 3724* **	1064 ± 261**

After incubation the inositol phospholipids were extracted and separated by thin-layer chromatography on silica gel plates. Standards of inositol phospholipids: phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol- 4,5-bisphosphate (PIP_2) were included as **carriers** with each sample to aid localization. PIPs were a sum of PIP and PIP_2. The results are presented in cpm/100 mg of protein as means ± SD.
* $p < 0.05$ in comparison to control group.
** $p < 0.05$ in comparison to TSH.

together with TSH were the most potent activator for PI and total activity present in the slices. The contents of PIPs were at the same level as in the control incubation.

Tab 3. presents the results of estimations of contents of some phospholipids in the thyroid tissue. TSH alone as well as with adenosine significantly increased the contents of phosphatidylinositol in the thyroid tissue. Adenosine alone did not change its contents in the slices. Phosphatidylcholine significantly decreased in the presence of adenosine. The changes after TSH were not significant. TSH and adenosine increase phosphatidilcholine, non-significantly with control, significantly with values after adenosine as a single stimulator. The phosphatidic acid decreased significantly after adenosine. The changes in probes with TSH added were minimal.

DISCUSSION

Activation of both, adenylate cyclase as well as inositol phospholipids hydrolysis and inositol phosphates by TSH were proven in a number of animal experiment models (5-7), and later, in human thyroid (8). The experiments were also performed using healthy tissue or a continous line of functional epithelial cells of rat thyroid.

In Graves' disease with accompanying thyrotoxicosis a stimulatory effect to the thyrocytes is intercepted by Thyroid Stimulating Antibodies (TSAb) attaching to the TSH membrane receptor. Signal transduction stimulates the cAMP cumulation pathway through

Tab 3. Effect of TSH and adenosine on some phospholipids contents of human thyroid in Graves' Disease (n = 10).

	PHOSPHATIDYL-INOSITOL	PHOSPHATIDYL-CHOLINE	PHOSPHATIDIC ACID
CONTROL	42.1 ± 13.0	545.8 ± 176.7	21.2 ± 10.0
TSH	55.1 ± 15.5 *	510.1 ± 141.7	21.9 ± 10.5
ADENOSINE	44.7 ± 3.8 **	476.5 ± 31.8 *	16.1 ± 9.9
ADENOSINE AND TSH	65.0 ± 7.6 ** ***	597.2 ± 58.2 ***	23.6 ± 10.6 ***

The phospholipids were estimated using thin layer two demensional chromatography.
Results are presented in µg of phosphorus /100 mg protein as means ± SD.
* $p < 0.05$ in comparison to control.
** $p < 0.05$ in comprison to TSH.
*** $p < 0.05$ in comparison to adenosine.

adenylate cyclase action (1). Laurent et al. (2) showed that TSAb increased cAMP intracellular level but were unable to activate the phosphatidylinositol-Ca^{2+} cascade, which may confirme the hypothesis about the significance of adenylate cyclase-cAMP pathway in the pathogenesis of Graves' disease.

In this situation thyrotropin is suppressed and not secreted by the pituitary gland. This is caused through the feedback relationship between thyroid hypersecretion of trijodothyronine, hypothalamus and hypophysis. However, Kimura et al (14) recognized human thyrocytes in Graves' disease as normal and also indicated the cumulation of cAMP after TSH stimulation in these cells.

The question remained to be answered, if in a situation of so large an imbalance in signal transduction paths with superiority of adenylate cyclase system in Graves' disease, the phosphatidylinositol path is active in this illness. It is interesting in the context of investigations by Sho et al (15) which indicated the gradual sensitization of TSH by a long period of TSH depletion in a culture medium supplemented with insulin growth factor I and hydrocortisone. After 3 weeks the Ca^{2+} response was induced by a slightly higher concentration of TSH than in serum. This would suggest that the main effects of a certain enviromental situation or factor might cause differential changes in a response mechanism. Our data indicated the considerable activity of the metabolic way in which the inositol phospholipids and inositol phosphates (particullary $InsP_3$) generate.

However the question is about reciprocal influences between both main signal transduction ways in Graves' disease. Adenosine is the key substance connected with many different effects. It is a potent physiological transducer in the cells. ATP released as a cotransmitter is rapidly hydrolysed to adenosine by nucleotidases on cell-surface

membranes (16). Another plausible source of adenosine are thyroid cells themselves. The present study suggests that adenosine participates in the regulation of metabolism of PI as well as InsP and GInsP in responses to TSH. Therefore the adenosine signal may survive longer than ATP and cooperate with TSH as a modulator of the TSH signal, probably even when the neural signal fades. (17). The separate addition of adenosine influences the metabolism of PIPs and $InsP_3$. Our results in human thyroid confirmed observations obtained in experiments with FRTL-5 concerning the reciprocal modulation of agonists of the TSH and PI receptors.

Both, TSH and adenosine together as well as adenosine alone stimulated the metabolism of phosphatidylcholine and phosphatidic acid. This would suggest an action on phospholipid metabolism of these two agonists. It was shown that TSH also stimulates phospholipid methylation in animal models. (18). The enhancement of the actions of different agonists on phosphatidylcholine metabolism suggest a broad field of possibilities (for review see 19).

Our results indicated the sensitivity of phospholipid metabolism in the thyroid in Graves' disease on thyrotropin and adenosine. Adenosine modifies the action of TSH which suggests the reciprocal influences between both main signal transduction systems.

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