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Induction of adrenomedullin 2/intermedin expression by thyroid stimulating hormone in thyroid

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A B S T R A C T

TSH is the important regulator of thyroid function but detailed molecular mechanisms have not been clarified. We first generated the iodine deficient (ID) rat in which goiter is induced by accelerated endogenous TSH secretion. The result of microarray analysis demonstrated markedly increased levels of adrenomedullin 2/intermedin (AM2/IMD) expression in the ID rat thyroid. AM2/IMD is a potent vasodilator. AM2/IMD mRNA expression was induced by TSH in a rat thyroid follicular cell line FRTL-5. Immunohistochemical analysis in human normal and Graves' disease thyroid revealed that AM2/IMD immunoreactivity was detected in follicular cells and more pronounced in Graves' disease. These results indicated that TSH induced AM2/IMD expression in the rat thyroid gland and it could locally work as a potent vasodilator, resulting in the expansion of thyroid inter-follicular capillaries. AM2/IMD could also contribute to facilitate thyroid hormone synthesis possibly via vasodilation effects and/or cAMP stimulating effects in the human thyroid gland.

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1. Introduction

Thyroid-stimulating hormone (TSH) is the most important regulator of thyroid function (Szkudlinski et al., 2002). TSH regulates thyroid activities through its interaction with the TSH receptor (TSHR) via activating the PKA pathway, which subsequently induced expression of various thyroidal proteins including thyroglobulin (Tg) (Van Heuverswyn et al., 1984), sodium iodide symporter (NIS) (Kogai et al., 1997) and thyroid peroxidase (TPO) (Nagayama et al., 1989) resulting in increased synthesis of thyroid hormone and hypertrophy/hyperplasia of thyroid follicles. TSH is also well-known to induce the thyroidal expression of angiogenic factor, VEGF (Klein et al., 2006; Yamada et al., 2006), to secure sufficient blood flow toward efficient hormone production and organ development.

An excess of TSH or TSH-like stimulus results from several pathologies such as iodine deficiency (Zimmermann, 2009) and Graves' disease (Menconi et al., 2014) resulting in follicular cell hyperplasia at histological level and goiter formation at macroscopic level. The characteristics of intracellular TSH signaling has been well studied in the physiology and pathophysiology of thyroid glands but it is still considered important to examine the underlining molecular events activated by TSH stimulus to obtain a better understanding of the pathogenesis of thyroid disorders.

Therefore, in this study, we first generated the rat model fed with iodide deficient diet continuously for 14 weeks to accelerate endogenous TSH secretion as a result of marked shortage of thyroid hormone secretion. These rats all demonstrated goiter formation. We studied these thyroid glands as well as normal control using microarray analysis for comprehensive understanding of TSH effects on thyroid and firstly demonstrated that adrenomedullin 2/intermedin (AM2/IMD) mRNA expression was markedly increased 58.7-fold in the ID rat thyroid than that of control. AM2/IMD is a member of calcitonin gene-related peptide (CGRP)/calcitonin (Roh et al., 2004; Takei et al., 2004) family and has been reported to have various biological effects including vasodilation (Roh et al., 2004; Takei et al., 2004) and angiogenesis (Smith et al., 2009). However, AM2/IMD expression has not been studied in thyroid. We therefore studied AM2/IMD expression and its changes by TSH in rat and human thyroid glands.

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2. Materials and methods

2.1. Animals and treatments

Eight weeks male Sprague-Dawley rats (Charles River Laboratories Japan Inc., Kanagawa, Japan) were tentatively classified into two groups (n = 8 each), control and iodine deficiency (ID) treated ones following a 1-week acclimatization period. Control and ID group rats were given a commercial diet (AIN-93G; Oriental Yeast Co., Ltd., Chiba, Japan) and a low iodine diet (modified AIN-93G; Oriental Yeast) for 14 weeks, respectively. At the end of the feeding period, blood samples were collected for hormone analyses, and rats were then euthanized with exsanguination and their thyroid glands were carefully removed for later analyses. One lobe of each thyroid gland was fixed with 10% phosphate-buffered formalin for histological evaluations for 36 hr at room temperature and the other lobe was immediately immersed in RNA stabilization reagent (RNAlater, QIAGEN GmbH, Hilden, Germany) and stored for gene expression analysis. Research protocols for all animal experimental procedures were approved by the Animal Research Committee of ASKA Pharmaceutical Co., Ltd.

2.2. Measurement of hormones, AM2/IMD and urinary iodine concentration

Plasma thyroxin (T4), triiodothyronine (T3) and TSH concentrations were measured by ELISA using commercially available kits (for T4 and T3: DRG International, Inc., Springfield, NJ, for rat TSH: Endocrine Technologies, Inc., Newark, CA). The AM2/IMD peptide concentration in FRTL-5 culture medium was measured by ELISA using commercially available kit (Phoenix Pharmaceuticals, Inc., Mountain View, CA). The antibody used in this kit recognized all three AM2/IMD subtypes, AM2/IMD$_{8–47}$, AM2/IMD$_{1–47}$ and AM2/IMD-53 (Takahashi et al., 2011). All the procedure was conducted according to the instruction manual of the manufacture. Urinary iodine concentration was analyzed with the method based on the Sandell–Kolthoff reaction (Ohashi et al., 2000).

2.3. Gene expression analysis

In order to obtain comprehensive gene expression profiles of the ID rat thyroid, total RNA was extracted from the thyroid tissue using mirVana miRNA isolation kit (Life Technologies, Carlsbad, CA), and then examined in SurePrint G3 Rat Gene Expression 8 × 60K Microarray (Agilent Technologies, Santa Clara, CA). We then focused on genes expressed at high levels in ID status based upon the results of microarray analysis and subsequently selected the genes of which known functions are related to the abnormal pathophysiology or histopathology of TSH excess however remotely. We then focused on the AM2/IMD whose expression was 58.7-fold higher in the ID rat thyroid than that of control. The increased expression of AM2/IMD was also validated at mRNA levels using qRT-PCR analysis. Total RNA was extracted using RNeasy mini kit (QIAGEN). cDNA was synthesized using QuantiTect reverse transcription kit (QIAGEN) and real-time PCR was performed using the LightCycler System and FastStart DNA Master Plus SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). The PCR primer sequence of AM2/IMD, NIS, and β-actin used in this study was summarized as follows: AM2/IMD (NM_201426); forward 5′-CCTGGGTAAGGGACTGAGAC-3′ and reverse 5′-TCCAGACTACAGGCCGAAG-3′, NIS (NM_052983); forward 5′-GGCTTGGCTGTATCCCT-3′ and reverse 5′-CCGTGTCCATTCCAGAAC-3′, GAPDH (NM_017008); forward 5′- TGACAACTTTGGCATCGT-3′ and reverse 5′-ATGCAGGGATGATGTTGT-3′, β-actin (NM_031144); forward 5′-ATGAGCTGCCTGACGGT-3′ and reverse 5′-GTGTTGGCATAGAG GTCTTTA-3′. TSH stimulation alters the stability of GAPDH mRNA in FRTL-5 cell (Wadsworth et al., 1990), therefore, β-actin was used as housekeeping gene for the RT-PCR analysis of FRTL-5 cell and GAPDH was used for the thyroid tissue. cDNAs of known concentrations for target genes, and the housekeeping gene were used to generate standard curves for real-time quantitative PCR in order to determine the quantity of target transcripts to be studied. The mRNA level in each case was represented as a ratio of housekeeping gene and was evaluated as a fold induction compared with that of each control in all the studies.

2.4. Cell culture

Fisher rat thyroid cell line FRTL-5 cells were cultured in Ham's F-12K medium (Life Technologies) supplemented with 5% fetal bovine serum, 0.045 mg/mL ascorbic acid, 0.01 μg/mL somatostatin, 5 μg/mL transferrin, 10 ng/mL glycyl-L-histidyl-L-lysine acetate, 10 nM hydrocortisone, 10 mg/mL insulin and 10 mIU/mL bovine TSH at 37 °C under 5% CO_2 atmosphere. As a TSH starvation treatment, FRTL-5 cells were cultured in the medium without TSH (starvation medium) for one week.

For the analyses of TSH-dependent AM2/IMD expression and secretion, FRTL-5 cells were seeded on a 35 mm dish with starvation medium and cultured for 1 week, and then treated with TSH in each condition. In the subsequent TSH dose dependency analysis, FRTL-5 cells were cultured with TSH at 0, 0.1, 0.5, 1, 5 and 10 mIU/mL for 24 hr. In TSH time dependency analysis, these cells were cultured at 10 mIU/mL for 0, 3, 6, 12 and 24 hr. In the analysis of TSH signal-dependent AM2/IMD expression, FRTL-5 cells were treated with 10 mIU/mL TSH, 10 mM adenylate cyclase agonist forskolin or cell permeable cAMP analog 8-Br-cAMP for 24 hr after 1 week TSH starvation treatment.

2.5. Histological and immunohistochemical analysis

In histological (hematoxylin and eosin stained slides) and/or immunohistochemical analysis, stained specimens were evaluated by M.F. (DVM) for rat and H.S. (MD) for the human thyroid gland. The Histofine Kit (Nichirei biosciences Inc., Tokyo, Japan) was used for the immunohistochemistry of AM2/IMD. A rabbit polyclonal antibody against AM2/IMD (Bioss Inc., Woburn, MA) was used with a dilution of 1:1000 in immunohistochemistry of rat thyroid gland. The antiserum against human AM2/IMD (Peptide Institute, Inc., Minoh, Japan) raised in a rabbit was used at 1:4000 dilution in immunohistochemical analysis of human thyroid specimens (Hirose et al., 2008; Morimoto et al., 2008). Antibodies used in this study recognized all three AM2/IMD subtypes, AM2/IMD$_{8–47}$, AM2/IMD$_{1–47}$ and AM2/IMD-53 (Takahashi et al., 2011). The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution and counterstained with hematoxylin. As a negative control, normal rabbit IgG was used instead of the primary antibodies. In the absorption test, AM2/IMD antibody solution was incubated with excess amount of synthetic rat or human AM2/IMD peptide (Peptide Institute, Inc.) solution with gentle agitation. After the incubation, the antibody–peptide mixture was centrifuged at 10,000 × g for 1 hr and the supernatant then subjected to further immunohistochemical procedure.

When evaluating immunoreactivity in human thyroid cases, the following system of evaluation was used: negative, –; weak immunoreactivity in follicular cells or marked immunoreactivity in follicular cells with <5%, +; marked immunoreactivity in follicular cells with 5–25%, ++; marked immunoreactivity in follicular cells with more than 25%, +++.

2.6. Human thyroid specimens

15 cases of thyroid specimens were retrieved from the pathology files of Department of Pathology, Tohoku University Graduate

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