The International Journal of Biochemistry & Cell Biology 44 (2012) 290–301

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Comparative proteomic analysis to dissect differences in signal transduction in activating TSH receptor mutations in the thyroid

Kerstin Krause[a,1], Alexandra Boisnard[a,1], Christian Ihling[b], Marian Ludgate[c], Markus Eszlinger[a], Knut Krohn[a], Andrea Sinz[b], Dagmar Fuhrer[d,*]

[a] Clinic for Endocrinology and Nephrology, University of Leipzig, Liebigstr. 20, D-04413 Leipzig, Germany
[b] Department of Pharmaceutical Chemistry & Bioanalytics, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, D-06120 Halle (Saale), Germany
[c] Centre for Endocrine and Diabetes Sciences, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK
[d] Department of Endocrinology and Metabolism, Division of Laboratory Research, University Hospital Essen, Hufelandstr. 55, 45147 Essen, Germany

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form 9 October 2011
Accepted 27 October 2011
Available online 3 November 2011

Keywords:
Thyroid hormone stimulating receptor (TSHR)
cAMP signaling
Toxic thyroid nodules
2D-DIGE proteomics
Mass spectrometry

ABSTRACT

In the thyroid, cAMP controls both thyroid growth and function. Gain-of-function mutations in the thyroid-stimulating hormone receptor (TSHR) lead to constitutive cAMP formation and are a major cause of autonomous thyroid adenomas. The impact of activating TSHR mutations on the signal transduction network of the thyrocyte is not fully understood.

To gain more insights into constitutive TSHR signaling, rat thyrocytes (FRTL-5 cells) with stable expression of three activating TSHR mutants (mutTSHR: A623I, L629F and Del613-621), which differ in their functional characteristics in vitro, were analyzed by a quantitative proteomic approach and compared to the wild-type TSHR (WT-TSHR). This study revealed (1) differences in the expression of Rab proteins suggesting an increased TSHR internalization in mutTSHR but not in the WT-TSHR; (2) differential stimulation of PI3K/Akt signaling in mutTSHR vs. WT-TSHR cells, (3) activation of Epac, impairing short-time Akt phosphorylation in both, mutTSHR and WT-TSHR cells.

Based on the analysis of global changes in protein expression patterns, our findings underline the complexity of gain-of-function TSHR signaling in thyrocytes, which extends beyond pure cAMP and/or IP formation. Moreover, evidence for augmented endocytosis in the mutTSHR, adds to a new concept of TSHR signaling in thyroid autonomy. Further studies are required to clarify whether the observed differences in Rab, PI3K and Epac signaling may contribute to differences in the phenotypic presentation, i.e. stimulation of function and growth of thyroid autonomy in vivo.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

The TSH receptor (TSHR) couples to multiple G proteins (Laugwitz et al., 1996) but most of the effects of TSH are mediated through Gsα and cAMP (Kimura et al., 2001; Medina and Santisteban, 2000). cAMP activates multiple downstream targets, including PKA and guanine nucleotide exchange factors for Rap (de Rooij et al., 1998; Kawasaki et al., 1998a) and Ras (Pak et al., 2002; Pham et al., 2000). Besides the control of differentiated functions, cAMP inhibits or stimulates cell proliferation depending on the cell type. In the thyroid, chronic cAMP stimulation leads to toxic thyroid hyperplasia in transgenic mice (e.g. due to adenosine A2 receptor overexpression) as well as hyperthyroidism and goiter in individuals with germline gain-of-function TSHR mutations (Vassart and Dumont, 1992; Paschke and Ludgate, 1997; Corvilain et al., 2001). In addition, presence of TSHR autoantibodies also results in the chronic activation of the TSHR (Morshed et al., 2009). Somatic activating TSHR mutations can be found in 60% of patients with toxic thyroid nodules. Functional characterization of TSHR mutants is usually performed in COS-7 cells, a non-thyroid cell system, and shows that the mutants differ in their potency to activate Gα and Gq signaling. The in vitro findings however do not correlate with the biological behaviour of the respective mutants in vivo. We have previously shown that the biological effects of TSHR mutations must be investigated in the correct cellular context and that biological net effects in terms of stimulation of thyroid growth and function cannot be deduced purely on measurement of second messengers (Fuhrer et al., 2003). Thus it is likely that still unknown signaling

Abbreviations: PKA, protein kinase A; GEF(s), guanine nucleotide exchange factor(s); EPAC, exchange proteins activated by cAMP; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; 8-CPT, 8-CPT-2-O-Me-cAMP.

* Corresponding author. Tel.: +49 201 723 2821.
E-mail address: Dagmar.fuehrer@uk-essen.de (D. Fuhrer).

[1] These authors contributed equally to this study.

1357-2725/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2011.10.024

Table 1
Summary of biological properties of the used mTSHR and the TSHR-WT.

cAMP:	623 > 629 > Del > WT > Neo
IP:	Del > 623 > 629 > WT/Neo
hTSHR copy number:	WT > Del > 629 > 623 > Neo
DNA synthesis:	629 > Del > WT > 623 > Neo
Growth:	623 > 629 > Del > WT/Neo

Adapted from Fuhrer et al. (2003).

mechanisms, e.g. exerting a counter-regulatory effect could contribute to the biological impact. In addition, TSHR signaling to cascades alternative to cAMP-PKA and IP-PKC must be considered.

In this paper we aimed to decipher constitutive TSHR signaling in the thyroid using a proteomic approach, since this allows a comprehensive analysis of global changes in protein expression which might reflect the biological situation more adequately than mRNA expression analysis. As a model we chose previously characterized FRTL-5 cell clones with stable expression of three different gain-of-function TSHR mutations (Fuhrer et al., 2003). In addition, this model has been recently used to demonstrate a modulatory effect of TSH on calcium signaling (Lorenz et al., 2010). Besides the wild-type TSHR (WT-TSHR) we used the A623I (623), L629F (629) and Del613-621 (Del) TSHR mutants, which are all located within the exon 10 encoded transmembrane domain of the THSR and which differ in their degree of constitutive activation of cAMP and inositol phosphate formation (Wonerow et al., 2000; Fuhrer et al., 2003). To decipher the differences in signaling triggered by the different activating mutations, we used two dimensional differential in-gel electrophoresis (2D-DIGE) analysis with targeted Western blot analysis for confirmation, followed by in vitro validation of the detected differential stimulation of PI3K/Akt and MAPK pathways. These data provide first evidences for differences between the mutants in receptor endocytosis and the stimulation of PI3K/Akt and MAPK pathways by PKA-dependent and -independent signaling cascades.

2. Materials and methods

2.1. Plasmids and retroviral gene transfer

We have studied the SB5 sub-clone of FRTL-5 cells stably expressing the wild-type (WT) TSHR or 3 different gain-of-function TSHR mutants, A623I, L629F and Del613-621, introduced by retroviral infection as previously described (Fuhrer et al., 2003). The empty plasmid served as control (Neo). Briefly, the cDNAs of the human TSHR mutants A623I, L629F, Del613-621 and the TSHR-WT were subcloned into the pLNSX vector. For retroviral transduction the 'Phoenix' transient retroviral producer packaging cell line was used (Danos and Mulligan, 1988). Retroviral supernatants were harvested at 48 h after transfection and assessed for (1) retroviral titers in human epithelial cell line A431; (2) TSHR expression by specific [125-I]TSH binding of G418 selected A431 colonies; and (3) cell surface expression in the human thyroid cell line hORI3 by flow cytometry using the polyclonal TSHR Ab p60 as described before (Fuhrer et al., 2003). Twenty-four hours after retroviral infection, FRTL-5 cells were passaged and selected under two conditions: (1) G418 resistance and (2) G418 resistance plus TSH withdrawal (4H medium). In each of four separate infection experiments, all FRTL-5 clones/retroviral construct were pooled (Fuhrer et al., 2003). Table 1 summarizes the biological properties of the mutTSHR and the TSHR-WT and the empty vector control (Neo).

2.2. Cell culture and stimulation

Cells were split into 10 cm dishes with 1×10^6 cells/dish (in triplicates) and maintained in 5H medium consisting of 2:1:1 mixture of DMEM:Ham's F12:MCDB104 (all from Life Technologies, Carlsbad, CA, USA) supplemented with 5% calf serum (Life Technologies), 10 μg/ml insulin, 0.4 μg/ml hydrocortisone (Merck Biosciences, Darmstadt, Germany), 45 μg/ml ascorbic acid, 5 μg/ml transferring and 5 mU/ml bovine TSH (all from Sigma–Aldrich, Munich, Germany).

After 24 h of growing, cells were starved for 48 h in 5H medium containing 0.2% calf serum without TSH. Afterwards they were stimulated with 250 μM isobutylmethylxanthine (IBMX; Sigma–Aldrich) for 4 h. Cells were then washed with PBS and shock-frozen in liquid nitrogen.

For analysis of the PI3K/Akt or MAPK pathway, cells were grown in 6-well plates (3×10^5/well). For activation of adenyl cyclase, after 3 days cells were stimulated with 10 μM forskolin for 5 min. For inhibition of PKA, PI3K or mTOR, cells were incubated with 10 μM H89, 10 μM LY294002 or 10 μM Rapamycin 60 min before stimulation with 10 μM H89 for 5 min. Forskolin and H89 were from Sigma–Aldrich (Munich, Germany), LY294002 and Rapamycin were obtained from Merck Biosciences (Darmstadt, Germany) and 8-pCPT-2′OMe-cAMP was from BIOLOG Life Science Institute (Bremen, Germany).

2.3. Cell lysis and protein precipitation

Cells were lysed in buffer containing 10 mM Tris, 400 mM NaCl, 1 mM EDTA, 0.1% NP-40 (all from Sigma–Aldrich). Proteins were precipitated using chloroform–methanol–precipitation. Briefly, protein samples (500 μl) were diluted with 2000 μl chloroform and 500 μl of methanol (all from Sigma–Aldrich). After vortexing, 1000 μl H_2O was added. Samples were vortexed and centrifuged at 14,000 × *g* for 15 min at 4 °C. The aqueous top layer was removed and 400 μl methanol were added. After vortexing, samples were spun at 14,000 × *g* for 1 min at 4 °C. The supernatant was removed and the samples were air-dried at room temperature and stored at −80 °C.

2.4. 2D-DIGE and image analysis

Precipitated protein pellets were solubilized in IPG-buffer consisting of 8 M urea, 2 M thiourea, 4% chaps, 0.5% IPG-buffer pH 3–10 and 1.2% DeStreak (all from GE Healthcare, Freiburg, Germany). The protein content was determined using the 2D Quant Kit (GE Healthcare, Freiburg, Germany) according to the manufacturer's description. For DIGE analysis the CyDye minimal labelling kit (GE Healthcare, Freiburg, Germany) was used according to the manufacture's description. Isoelectric focussing was performed in 24 cm long non-linear pH 3-10 IPG strips (GE Healthcare, Freiburg, Germany) followed by SDS-PAGE in 8–16% gradient gels (Krause et al., 2006). All samples were run in triplicates.

Afterwards, the gels were scanned using a TyphoonTM imager (GE Healthcare). Gel analysis was performed using DeCyder 2-D Differential Analysis Software v7.0 (GE Healthcare) to co-detect, normalize and quantify the protein spots in the images. The DeCyder biological variation (BVA) module was used for comparison between the TSHR mutants and the TSHR-WT to the mixed internal standard and for the calculation of normalized spot volumes/protein abundance. The resulting spot maps corresponding to the individual gels were used to determine average abundance changes and paired Student's *t*-test *p*-values for each protein across the different gels. Protein spots which were consistently expressed across the replicate gels with a significant (Student's *t*-test, $p < 0.05$) change in abundance and at least ≥1.5-fold compared to the Neo and a least ≥1.3-fold change in spot quantity between the respective samples were selected for further characterization by mass spectrometry. Mass spectrometry was performed on spots which

Download English Version:

https://daneshyari.com/en/article/1984093

Download Persian Version:

https://daneshyari.com/article/1984093

Daneshyari.com