TSH induces metallothionein 1 in thyrocytes via $G_{q/11}$ - and PKC-dependent signaling

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Abstract

Metallothioneins (MTs) are cytoprotective proteins acting as scavengers of toxic metal ions or reactive oxygen species. MTs are upregulated in follicular thyroid carcinoma and are regarded as a marker of thyroid stress in Graves' disease. However, the mechanism of MT regulation in thyrocytes is still elusive. In other cellular systems, cAMP-, calcium-, or protein kinase C (PKC)-dependent signaling cascades have been shown to induce MT expression. Of note, all of these three pathways are activated following the stimulation of the TSH receptor (TSHR). Thus, we hypothesized that TSH represents a key regulator of MT expression in thyrocytes. In fact, TSHR stimulation induced expression of MT isoform 1X (MT1X) in human follicular carcinoma cells. In these cells, induction of MT1X expression critically relied on intact $G_{q/11}$ signaling of the TSHR and was blocked by chelation of intracellular calcium and inhibition of PKC. TSHR-independent stimulation of cAMP formation by treating cells with forskolin also led to an upregulation of MT1X, which was completely dependent on PKA. However, inhibition of PKA did not affect the regulation of MT1X by TSH. As in follicular thyroid carcinoma cells, TSH also induced MT1 protein in primary human thyrocytes, which was PKC dependent as well. In summary, these findings indicate that TSH stimulation induces MT1X expression via $G_{q/11}$ and PKC, whereas cAMP–PKA signaling does not play a predominant role. To date, little has been known regarding cAMP-independent effects of TSHR signaling. Our findings extend the knowledge about the PKC-mediated functions of the TSHR.

Key Words

- ▶ TSH receptor
- ▶ metallothionein
- ▶ signal transduction
- ▶ $G_{q/11}$
- ▶ Calcium
- ▶ protein kinase C

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Introduction

Metallothioneins (MTs) are small, cysteine-rich proteins that act as scavengers of reactive oxygen species and are involved in the homeostasis of zinc and the detoxification of bivalent heavy metals (Park *et al.* 2001). The inducible MT isoforms, MT1 and MT2 (MT2A), are upregulated in

inflammatory processes via cytokines or increased oxidative stress and are important cytoprotective proteins (Viarengo *et al.* 2000). In humans, several MT1 variants (e.g. MT1G, MT1H, and MT1X) exist, which appear to exert redundant biological functions.

A role of MTs in the thyroid gland has recently been put forward in Graves' disease (GD). A transcriptomic analysis of the thyroid samples of GD patients revealed an upregulation of MTs (Ruiz-Riol *et al.* 2011), which was inversely correlated with aberrant HLA class II expression (Ruiz-Riol *et al.* 2012). Thus, MTs were proposed to form part of the danger-associated molecular pattern in autoimmune thyroid disease representing an adaptive cytoprotective and anti-inflammatory response. Furthermore, a role of MTs in thyroid cancer has been suggested since MT expression is dysregulated in follicular and papillary thyroid carcinomas (Huang *et al.* 2003, Ferrario *et al.* 2008, Królicka *et al.* 2010). Although these findings support the hypothesis that MTs exhibit relevant biological functions in the thyroid gland, the mechanisms of MT regulation in thyroid cells are only incompletely understood.

The main homeostatic stimulus regulating the functional integrity of the thyroid gland is the hypothyseal glycoprotein hormone TSH, which controls the growth and survival of thyroid cells as well as the synthesis and secretion of thyroid hormones (for review, see Vassart & Dumont (1992) and De Felice *et al.* (2004)). TSH acts on the G-protein-coupled TSH receptor (TSHR) activating G-proteins of all four families, i.e. G_s , $G_{i/o}$, $G_{12/13}$, and $G_{q/11}$ (Laugwitz *et al.* 1996).

Most molecular TSHR targets are regulated in a G_s -cAMP-dependent manner (for review, see De Felice *et al.* (2004)). Recently, we have identified a G_{13} -dependent signaling pathway of the TSHR leading to the activation of p44/42 MAPK (Büch *et al.* 2008). Another TSHR-promoted signaling pathway in thyrocytes is the $G_{q/11}$ -dependent activation of phospholipase C (PLC; Van Sande *et al.* 2006) with a subsequent increase in inositol trisphosphate (IP_3) and diacylglycerol levels, a rise in the intracellular calcium concentration ($[Ca^{2+}]_i$), and an activation of protein kinase C (PKC). Remarkably, more distal effectors of the $G_{q/11}$ -dependent TSHR signaling pathway are still largely unknown. The dearth of mechanistic insights notwithstanding, the general importance of $G_{q/11}$ -regulated signaling in thyroid cells is highlighted by the observation that conditional deletion of G_{α_q} proteins in murine thyrocytes results in hypoplastic thyroid glands and severe hypothyroidism (Kero *et al.* 2007). Furthermore, a human TSHR mutation has been identified impairing the activation of the $G_{q/11}$ -PLC cascade without affecting G_s -cAMP signaling (Grasberger *et al.* 2007). This TSHR mutation results in partial resistance of the receptor with elevated TSH levels and increased iodine uptake

but reduced thyroid hormone synthesis (Grasberger *et al.* 2007). Collectively, these findings indicate that $G_{q/11}$ -dependent pathways of the TSHR regulate relevant biological functions in thyroid cells.

Herein, we demonstrate that the MT1X is a TSHR-regulated target in a human thyroid carcinoma cell line stably expressing the recombinant TSHR as well as in primary human thyrocytes (PHTs) endogenously expressing the TSHR. The upregulation of MT1X by TSHR stimulation was cAMP independent and promoted by a $G_{q/11}$ - and PKC-dependent pathway. These findings define a novel $G_{q/11}$ -promoted function of the TSHR and, thus, extend our knowledge about the G_s -cAMP-independent functions of this receptor.

Materials and methods

Materials

Bovine TSH, forskolin (FSK), ionomycin, phorbol 12-myristate 13-acetate (PMA), ATP, bradykinin (BK), bisindolylmaleimide II and X, BAPTA/AM, cadmium chloride, insulin, transferrin, somatostatin (Sst), liver cell growth factor (Gly-His-Lys), and fura-2/AM were obtained from Sigma-Aldrich. Rp-cAMPS was procured from Enzo Life Science GmbH (Lörrach, Germany). Protran nitrocellulose transfer membranes were purchased from Whatman (Dassel, Germany). The ECL systems (SuperSignal West Femto Maximum Sensitivity Substrate and SuperSignal West Pico Chemiluminescent Substrate) were obtained from Thermo-Scientific (Bonn, Germany). Rotiblock, milk powder, and all other chemicals that were used were obtained from Carl Roth (Karlsruhe, Germany) unless otherwise indicated.

Thyroid cells

FTC-133 cell lines stably expressing wild-type TSHR (FTC-133 wtTSHR) or a mutated TSHR variant with disrupted $G_{q/11}$ coupling (FTC-133 Y601H) have been established and functionally characterized as described previously (Büch *et al.* 2008). Additionally, three PHT cell lines (SD-171, SD-191, and SD-197) were used in this study. The PHT cell lines were established from three thyroidectomized patients with GD and Struma nodosa. For this purpose, thyroid tissue samples were dissected with scissors and digested with 1 mg/ml collagenase (Roche Applied Science) in Iscove's modified Dulbecco's medium (IMDM) with L-glutamine (PAA, Pasching, Austria) for 2 h at 37 °C. Thereafter, the cells were passed

through a sieve and washed in IMDM containing 10% FCS, 100 IU/ml penicillin, and 50 µg/ml streptomycin. Isolated cells were seeded in 25 cm² Falcon primary tissue culture flasks (BD Primaria, Heidelberg, Germany) and passaged twice before being used for the experiments. Both FTC-133 and PHT cells were grown in IMDM, additionally containing 10% (v/v) FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 10 ng/ml liver cell growth factor (Gly-His-Lys), and 10 ng/ml Sst.

Determination of the [Ca²⁺]_i

To load the cells with fura-2/AM, they were detached by chelation of extracellular calcium with EDTA (2 mM), and a cell suspension (5 ml with 1×10⁶ cells in the culture medium) was supplemented with 1.25 µM fura-2/AM for 15 min at 37 °C. The cells were then centrifuged and resuspended in a 5 ml assay buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 10 mM HEPES at pH 7.4, supplemented with 2 mM CaCl₂, 5.5 mM glucose, and 0.1% (w/v) BSA. The cells were seeded in clear-bottom 96-well plates at a density of 2×10⁴ cells/well. Fluorescence was recorded at 37 °C in a PolarSTAR plate reader obtained from BMG (Offenburg, Germany) according to the manufacturer's protocol. Agonists were automatically injected after 10 s. Fluorescence signals were monitored for at least 70 s after the addition of agonists. The free calcium levels were monitored from the changes in the fluorescence ratio produced by excitation at 340 and 380 nm.

Quantitative RT-PCR

Total RNA from the cells was isolated using the TRI reagent (Sigma-Aldrich). First-strand synthesis was carried out with oligo dt₍₁₈₎ primers, using the REVERTAID reverse transcriptase (MBI-Fermentas, Sankt Leon-Roth, Germany). Products were amplified using specific intron-spanning primer pairs for MT1X (MT1X for and MT1X rev) and β-actin primers as controls (ACT for and ACT rev). Primer sequences were as follows: MT1X for, 5'-AGG CAC AGG AGC CAA CAG-3'; MT1X rev, 5'-CTT CTC CTT GCC TCG AAA TG-3'; ACT for, 5'-GAG TAC TTG CGC TCA GGA GG-3'; and ACT rev, 5'-GGC TAC AGC TTC ACC ACC AC-3'. The primers were purchased from Metabion (Martinsried, Germany). Quantitative RT-PCR was carried out using the 2× master mix from the SYBR Green I Master PCR Kit (Roche Applied Science) containing a HotStar Taq polymerase, a buffer, nucleotides, 2.5 mM MgCl₂, and SYBR Green. Subsequently, 10 pmol of each primer pair and 8 µl

of the pre-diluted (1:100) first-strand synthesis sample were added to the reaction mixture, and PCR was carried out in a lightcycler apparatus (Roche Applied Science) using the following conditions: 15 min of initial activation and 45 cycles of 10 s at 95 °C, 10 s at 55 °C, and 10 s at 72 °C, each. Fluorescence intensities were recorded after the extension step at 72 °C after each cycle. Samples containing primer dimers were excluded by a melting curve analysis and identification of the products was done by agarose gel electrophoresis. Crossing points were determined by a software program. The relative gene expression was quantified using the following formula:

$$2^{(\text{crossing point of } \beta\text{-actin} - \text{crossing point X})} \times 100 = \text{percentage of reference gene expression.}$$

Western blot

For western blot analysis, the cells were grown in six-well plates (3×10⁵ cells/well) for 24 h. The cells were growth-arrested for 18 h by incubation in a serum-free medium, stimulated as indicated, washed with ice-cold PBS, and solubilized in 200 µl lysis buffer (125 mM Tris-Cl, 2% (w/v) SDS, 10% (v/v) glycerol, 4% (v/v) β-mercaptoethanol, and 50 µg/ml bromophenol blue). Proteins were separated by SDS-PAGE (9% gel) and electroblotted onto Hybond-C Extra membranes. Blots were incubated in Rotiblock for 1 h to saturate nonspecific binding sites, washed in PBS, and incubated with a murine monoclonal anti-MT1 antibody (UC1MT, Abcam, Cambridge, UK) diluted to 1:1000 in PBS with 5% (w/v) nonfat dried milk. Reblots were prepared as loading controls using a murine monoclonal anti-actin antibody (Santa Cruz Biotechnology) diluted to 1:2000 in PBS with 5% (w/v) nonfat dried milk. As the secondary antibody, a peroxidase-coupled goat anti-mouse antibody (Sigma) diluted to 1:2000 in PBS with 5% (w/v) nonfat dried milk was used.

Immunocytochemistry

Thyroid cells were cultivated on glass cover slips for 18 h in serum-free IMDM. After stimulation (as indicated), the cells were washed with PBS and fixed with pre-warmed (37 °C) 4% paraformaldehyde (diluted in PBS) for 15 min. After additional washing with PBS (three times), the cells were permeabilized with 0.1% Triton X-100 and subsequently blocked in 10% goat serum (in PBS; Goat Serum Donor Herd; Sigma) for 1 h at room temperature. Next, anti-MT1 antibody (UC1MT, Abcam; 1:100, in 5% goat serum) was added and the mixture was incubated for 2 h at room temperature. After additionally washing three times

with PBS, the cells were incubated with the secondary antibody (anti-mouse coupled to FITC, Santa Cruz Biotechnology; 1:1000 in 5% goat serum) for 1 h at room temperature in the dark. Finally, the cells were fixed with a mounting medium (DakoCytomation Fluorescent Mounting Medium; Dako, Hamburg, Germany) on glass slides. Samples were evaluated using an Axiovert 200M fluorescence microscope (Zeiss, Jena, Germany). Fluorescence was determined at 490 nm excitation.

Statistical analysis

Bars represent the means of at least three independent experiments $\pm$ S.E.M. Statistical significance of differences was assessed by one-way ANOVA with the Tukey–Kramer *post hoc* test.

Results

TSH induces *MT1X* mRNA and promotes an increase in intracellular calcium levels in FTC-133 wtTSHR cells

To test the hypothesis that TSHR signaling is involved in the regulation of MTs, we first used follicular thyroid carcinoma cells recombinantly expressing the human TSHR (FTC-133 wtTSHR). These cells have been extensively characterized previously (Büch *et al.* 2008). As has been shown, FTC-133 wtTSHR cells specifically bind to TSH and demonstrate a robust increase in the cAMP levels upon TSH stimulation (Büch *et al.* 2008) indicative of the functional expression of the TSHR. Thus, we performed a quantitative RT-PCR analysis with primers detecting the *MT1X* in FTC-133 wtTSHR cells. The stimulation of the cells with TSH for 12 h led to an induction of *MT1X* mRNA in a concentration-dependent manner (Fig. 1A).

As has been reported previously, an upregulation of MTs in anaplastic thyroid carcinoma cells after treatment with cadmium is mediated via an elevation of the intracellular calcium levels (Liu *et al.* 2007). Therefore, we tested whether treatment of FTC-133 wtTSHR cells with TSH also promotes a calcium increase. In fact, TSH stimulation led to a pronounced elevation of calcium levels, which returned to near-baseline levels within 200 s (Fig. 1B).

Other calcium-mobilizing stimuli such as ATP and carbachol do not induce *MT1X* in FTC-133 wtTSHR cells

To test the specificity of *MT1X* induction in FTC-133 wtTSHR cells, we next evaluated the responses toward a

Figure 1

Quantitative analysis of *MT1X* mRNA levels by RT-PCR of total RNA from FTC-133 wtTSHR cells following a 12-h stimulation with TSH in the indicated concentrations (A). Total RNA was reverse-transcribed, and the products of the first-strand synthesis were analyzed for the presence of amplification products obtained with primer pairs against *MT1X* or β -actin (as the reference gene). The ratio of *MT1X* to β -actin expression was determined and values for unstimulated cells (basal) were set at 1. Furthermore, calcium measurements were performed using the ratiometric calcium dye fura-2/AM (B). Cells labeled with fura-2/AM were stimulated with 50 mU/ml TSH or HBS buffer, and the change in fluorescence ratio was recorded using an excitation wavelength of 340 or 380 nm.

number of TSHR-independent stimuli. Remarkably, treatment with ATP, BK, carbachol (CCh), or Sst failed to significantly affect *MT1X* expression (Fig. 2A). This finding was surprising since at least BK, CCh, and ATP elicited an increase in the $[Ca^{2+}]_i$ in FTC-133 wtTSHR cells (Fig. 2B, C and D). Of note, the peak calcium response after CCh treatment was in the same order of magnitude as that in the case of TSH treatment (Fig. 2D), whereas ATP produced an even higher calcium peak (Fig. 2B).

In principle, different kinetics of the calcium signal could represent one possible explanation for the fact that the calcium-mobilizing stimuli differed with regard to their capacity to induce *MT1X*. Thus, we performed longer measurements comparing the responses upon TSH and

Figure 2

Quantitative analysis of *MT1X* mRNA levels by RT-PCR of total RNA from FTC-133 wtTSHR cells following a 12-h stimulation with 2 mM ATP, 1 μM bradykinin (BK), 100 μM carbachol (CCh), or 30 nM somatostatin (Sst) (A). Total RNA was reverse-transcribed, and the products of the first-strand synthesis were analyzed for the presence of amplification products obtained with primer pairs against *MT1X* or β -actin (as the reference gene). The ratio of *MT1X* to β -actin expression was determined and values for unstimulated cells (basal) were set at 1. Additionally, calcium

measurements were performed using the ratiometric calcium dye fura-2/AM (B, C, D, E and F). The cells were stimulated with 2 mM ATP (B), 1 μM BK (C), 100 μM CCh (D), or 30 nM Sst (E), and the change in fluorescence ratio was recorded using an excitation wavelength of 340 or 380 nm. Moreover, comparative calcium measurements for a longer period of time were performed after stimulation with 50 mU/ml TSH, 100 μM CCh, or HBS buffer (F).

CCh treatment. However, both stimuli showed very similar kinetics (Fig. 2F). Calcium responses after TSH or CCh treatment returned to the levels of buffer injection after > 250 s. Since differences in amplitude or time course of the calcium signals could not explain why TSH led to an upregulation of *MT1X* and other stimuli did not, a rise in intracellular calcium levels alone does not appear to be sufficient for the induction of *MT1X* expression.

Induction of *MT1X* mRNA in FTC-133 cells is dependent on intact $G_{q/11}$ signaling of the TSHR

To further elucidate the mechanism of *MT1X* induction by TSH, we compared FTC-133 wtTSHR cells with FTC-133 Y601H cells. As has been described previously (Büch *et al.* 2008), the latter cell line stably expresses a mutated TSHR not capable of coupling to $G_{q/11}$ proteins. However,

FTC-133 wtTSHR and FTC-133 Y601H cells display comparable levels of TSHR expression and TSHR-dependent cAMP formation indicative of unaltered G_s coupling (Büch *et al.* 2008). In contrast, TSHR stimulation in FTC-133 Y601H cells does not mediate an increase in $[Ca^{2+}]_i$ due to disrupted $G_{q/11}$ coupling of the mutated receptor (Büch *et al.* 2008), whereas FTC-133 wtTSHR cells show a pronounced calcium response (Fig. 1B).

Using these cells, we performed quantitative RT-PCR analyses with *MT1X*-specific primers and compared the time-dependent regulation of *MT1X* upon TSH stimulation. In FTC-133 wtTSHR cells, 25 mU/ml TSH induced *MT1X* expression by a factor of 2.4 when compared with untreated cells after a 3-h stimulation and by a factor of 3.8 after an incubation of 12 h (Fig. 3A, left). In contrast, no induction of *MT1X* mRNA expression was observed in FTC-133 Y601H cells (Fig. 3A, right). In response to

Figure 3

Quantitative analysis of *MT1X* mRNA levels by RT-PCR of total RNA from FTC-133 wtTSHR and FTC-133 Y601H cells following stimulation with 25 mU/ml TSH (A) and 100 mU/ml TSH (B) for the indicated time. Total RNA was reverse-transcribed, and the products of the first-strand synthesis were analyzed for the presence of amplification products obtained with primer pairs against *MT1X* or β -actin (as the reference gene). The ratio of *MT1X* to β -actin expression was determined and values for unstimulated cells (basal) were set at 1.

higher TSH concentrations (100 mU/ml), *MT1X* mRNA expression was stimulated by a factor of 6.2 after 12 h in FTC-133 wtTSHR cells (Fig. 3B, left), while FTC-133 Y601H cells were unaffected (Fig. 3B, right). Thus, an intact $G_{q/11}$ coupling of the TSHR is a prerequisite for the TSH-promoted induction of *MT1X* expression.

TSH-promoted *MT1X* mRNA induction in FTC-133 wtTSHR cells depends on calcium and PKC

To characterize the mechanism of *MT1X* induction by TSH in detail, we tested the role of calcium and PKC,

two classical downstream effectors of $G_{q/11}$ signaling. The stimulation of FTC-133 wtTSHR cells with the PKC-activating phorbol ester PMA mimicked the effects of TSH (Fig. 4A) and led to a >3.8-fold induction of *MT1X* mRNA levels, suggesting that PKC activation may be involved in *MT1X* regulation in thyroid cells. A comparable effect was observed in FTC-133 Y601H cells (Fig. 4B), suggesting that these cells, in principle, are able to induce *MT1X* in a TSH-independent mechanism. Furthermore, stimulation with the calcium ionophore ionomycin only modestly induced *MT1X* in FTC-133 wtTSHR cells as well as in FTC-133 Y601H cells (Fig. 4A and B). The small and nonsignificant increase in *MT1X* expression after ionomycin stimulation is in line with the above-mentioned finding that other calcium-mobilizing stimuli such as ATP and CCh (Fig. 2B and D) failed to induce *MT1X* in FTC-133 wtTSHR cells (Fig. 1A). Of note, the effect of the unspecific calcium ionophore ionomycin on intracellular calcium levels is much more

Figure 4

Quantitative analysis of *MT1X* mRNA levels by RT-PCR of total RNA from FTC-133 wtTSHR or FTC-133 Y601H cells following stimulation with 10 μ M PMA (A and B), 0.4 μ M ionomycin (A and B), or 100 mU/ml TSH (C and D) for 12 h. Where indicated, the cells were additionally pretreated with 10 μ M BIM II (C) or 1 μ M BAPTA/AM (D) 30 min before TSH treatment and throughout the stimulation with TSH. Total cellular RNA was reverse-transcribed, and the products of the first-strand synthesis were analyzed for the presence of amplification products obtained with primer pairs against *MT1X* or β -actin (as the reference gene). The ratio of *MT1X* to β -actin expression was determined and values for unstimulated cells (basal) were set at 1.

pronounced than the increases evoked by the stimulation of specific receptors.

In line with the assumption that $G_{q/11}$ effectors were involved in the regulation of MT1X, pretreatment with the PKC inhibitor BIM II inhibited TSH-promoted induction of MT1X expression (Fig. 4C), as did the calcium chelator BAPTA/AM (Fig. 4D). Thus, an activation of PKC and an increase in intracellular calcium levels are at least necessary for TSH-promoted induction of MT1X expression. However, as has been stated above, the

calcium increase alone is most likely not sufficient for MT1X upregulation by TSH.

cAMP–PKA signaling is not required for MT1X mRNA induction by TSH in FTC-133 wtTSHR cells

Previous studies in different cells using stimuli other than TSH have reported that elevations of cAMP might mediate an induction of MT expression (Cousins *et al.* 1986). A contribution of cAMP signaling to the TSH-promoted regulation of MT1X would be plausible since a G_s -dependent increase in cAMP levels is the best established cellular response after TSH stimulation. Thus, we tested whether treatment with the direct adenylyl cyclase activator FSK would induce MT1X. In fact, incubation of cells with FSK increased MT1X mRNA levels (Fig. 5A). This finding appeared to be at odds with the lack of TSH-dependent MT1X upregulation in FTC-133 Y601H cells (Fig. 3A and B), which display a robust cAMP accumulation upon TSH stimulation (Büch *et al.* 2008). To shed light on this conundrum, we investigated the role of PKA in MT1X regulation. Of note, the PKA inhibitor Rp-cAMPS precluded the effect of FSK on MT1X upregulation in FTC-133 wtTSHR cells but not the effect of TSH (Fig. 5B). Thus, cAMP–PKA signaling is not an obligatory requirement for TSHR-dependent MT1X upregulation in FTC-133 wtTSHR cells (Fig. 5B).

TSH stimulation induces MT1 protein in FTC-133 wtTSHR cells but not in FTC-133 Y601H cells

Next, we studied the effects of TSH on MT1 protein expression in FTC-133 wtTSHR and FTC-133 Y601H cells. In immunoblots of FTC-133 wtTSHR cells, anti-MT1 antibodies recognized an 8 kDa protein, which was induced upon treatment of cells with TSH (100 mU/ml, 48 h) or – as a positive control – cadmium chloride (CdCl_2 , 200 μM for 48 h) (Fig. 6A). Further immunocytochemical analysis confirmed that stimulation with 100 mU/ml TSH for 48 h strongly induced MT1 in about 30% of FTC-133 wtTSHR cells (Fig. 6B, upper panel). However, TSH was without any effect in FTC-133 Y601H cells characterized by defective $G_{q/11}$ coupling (Fig. 6B, lower panel). To rule out a more general cellular dysfunction of FTC-133 Y601H cells, CdCl_2 (200 μM , 48 h) was added, which resulted in a clearly discernible induction of MT1 protein in these cells (Fig. 6C). Of note, the proportion of CdCl_2 -responsive FTC-133 Y601H cells (Fig. 6C) was similar to that of TSH-responsive FTC-133 wtTSHR cells (Fig. 6B, upper panel).

Figure 5

Quantitative analysis of MT1X mRNA levels by RT-PCR of total RNA from FTC-133 wtTSHR cells following stimulation with 10 μM forskolin (FSK; A and B), 0.4 μM ionomycin (Iono; A), or 100 mU/ml TSH (A and B) for 12 h. Where indicated, the cells were additionally pretreated with 10 μM Rp-cAMPS 30 min before TSH or FSK treatment and throughout the stimulation (B). Total cellular RNA was reverse-transcribed, and the products of the first-strand synthesis were analyzed for the presence of amplification products obtained with primer pairs against MT1X or β -actin (as the reference gene). The ratio of MT1X to β -actin expression was determined and values for unstimulated cells (basal) were set at 1.

Figure 6

Western blot analysis of MT1 expression in FTC-133 wtTSHR cells (A, upper panel) subjected to treatment with either 100 mU/ml TSH or 200 μ M CdCl₂. The expression of actin was used as a loading control (A, lower panel). MT1 immunostaining in FTC-133 wtTSHR cells (B) and FTC-133 Y601H cells (C). Where indicated, the cells were stimulated with 100 mU/ml TSH or 200 μ M CdCl₂ for 48 h. For immunostaining, the cells were fixed with para-formaldehyde and permeabilized with Triton X-100. The bars indicate 50 μ M.

Induction of MT1 protein by TSH is dependent on PKC in the primary thyroid cell line SD-191

Since FTC-133 wtTSHR cells express the recombinant TSHR, the responsiveness of MT1X to TSH challenge might represent an unphysiological response. Therefore, we additionally examined the effects of TSH stimulation on several PHT cell lines (SD-191, SD-171, and SD-197) endogenously expressing the TSHR.

Owing to the very low proliferation rate of these PHT cells, we could not establish MT1 expression data on mRNA and protein levels for all the cell lines used. In SD-171 and SD-197 cells, we could only perform

quantitative PCR analyses showing a tendentious although not significant induction of *MT1X* mRNA in SD-197 cells (Supplementary Figure 1A, see section on supplementary data given at the end of this article) and a significant induction in SD-171 cells (Supplementary Figure 1B). Furthermore, the TSH-promoted induction of MT1X expression in SD-171 cells could not be mimicked by treatment with FSK (Supplementary Figure 2A, see section on supplementary data given at the end of this article) and was dependent on PKC (Supplementary Figure 2B).

Using the cell line SD-191, we could monitor the protein expression of MT1 upon stimulation with TSH. In these cells, immunoblots revealed an induction of MT1 protein in response to 48-h treatment with TSH (100 mU/ml), which was counteracted by pretreatment with the PKC inhibitor BIM (Fig. 7A). In addition, immunocytochemical analysis confirmed that both TSH (100 mU/ml for 48 h) and CdCl₂ (200 μ M for 48 h) robustly induced MT1 in these cells (Fig. 7B, C and D), further supporting the notion that MT1 is a physiological downstream TSH effector in thyroid cells.

Discussion

In this study, we identified MT1 as a novel TSH-regulated target in thyroid cells that is induced by engaging a cAMP-independent and PKC-dependent signaling pathway (Fig. 8). To study the effects of TSHR stimulation on induction of MT1 expression, we used follicular carcinoma cells recombinantly expressing either the wild-type TSHR (FTC-133 wtTSHR) or a receptor with disrupted G_{q/11} coupling (FTC-133 Y601H). Furthermore, we made use of primary thyroid cells endogenously expressing the TSHR to exclude artifacts resulting from recombinant expression. A general difficulty in studying thyroid functions *in vitro* is based on the fact that the immortal thyroid cell lines available are dedifferentiated in many aspects (van Staveren *et al.* 2007, Saiselet *et al.* 2012) and show a greater similarity to anaplastic thyroid carcinoma than to differentiated thyroid carcinoma, let alone to a normal thyroid gland. Even primary cells, which are usually obtained from patients with goiter or GD, exhibit some genetic alterations or modified protein expression patterns when compared with normal thyrocytes (Xu *et al.* 2007, Davies *et al.* 2010, Park *et al.* 2005). However, these cell culture models still have some advantages to study the principal mechanisms of TSHR signaling when compared with *in vivo* models as discussed previously (van Staveren *et al.* 2006). Particularly, the relative homogeneity of conditions in cell culture and the possibility to exactly

Figure 7

Western blot analysis of MT1 expression in the PHT cell line SD-191 (A, upper panel) subjected to treatment with 100 mU/ml TSH. Where indicated, the cells were additionally pretreated with 10 μM of the PKC inhibitor BIM 30 min before TSH stimulation. The expression of actin was

used as a loading control (A, lower panel). MT1 immunostaining in PHTs (B, C and D). The cells were stimulated with 100 mU/ml TSH (C) or 200 μM CdCl₂ (D) for 48 h. For immunostaining, the cells were fixed with para-formaldehyde and permeabilized with Triton X-100. The bars indicate 10 μm.

control the dosage of and time of exposure to TSH and to refer to untreated controls are considerable advantages of cell culture when compared with *in vivo* models (van Staveren *et al.* 2006). Of note, the TSHR is a prime example of a receptor possessing a very complex signaling by coupling to G_s, G_{q/11}, G_{i/o}, and G_{12/13} proteins (Laugwitz *et al.* 1996). In this case, highly controlled *in vitro* conditions are a major advantage to allow basic investigations on fundamental signaling events.

In this connection, it is worth mentioning that even in this *in vitro* model a remarkable heterogeneity of cellular responses occurs as is illustrated by the fact that MT1 upregulation in FTC-133 cells after TSH stimulation as well as after treatment with the unspecific inducer CdCl₂ occurred in only about 30% of the cells. The reason for this divergent responsiveness of cells with regard to MT1 upregulation is not clear. Since a positive correlation between proliferative markers such as Ki-67 and MCM-2 and the expression levels of MT1 in tumor cells has been described (Werynska *et al.* 2011), one could speculate that the upregulation of MT1 in FTC-133 cells might be dependent on the cell-cycle phase.

Although the TSHR is capable of coupling to all four classes of G-proteins, most cellular effects of TSHR activation described to date rely on the G_s-cAMP-PKA pathways. Recently, we have observed that the activation of G₁₃ proteins by the TSHR entails the transactivation of the EGF receptor and stimulation of p44/42 MAPK

(Büch *et al.* 2008). However, the molecular identity of cAMP-independent and G_{q/11}- and PKC-regulated effectors of the TSHR is largely unknown.

Figure 8

Synopsis of the signaling molecules involved in upregulation of MT1 in thyroid cells by treatment with TSH or with the direct adenyl cyclase (AC) activator forskolin (FSK). Both TSH and FSK lead to an increase in cAMP levels and subsequent activation of cAMP-dependent protein kinase A (PKA). The latter is involved in the upregulation of MT1 in the case of FSK but not in the case of TSH. Regarding TSH-promoted induction of MT1 expression, an increase in intracellular calcium levels is necessary, although not sufficient. Additionally, the activation of PKC is involved.

A well-established mechanism of TSHR-induced gene regulation comprises G_s - and cAMP-dependent activation of PKA and subsequent cAMP-responsive element-binding protein (CREB) binding to cAMP-responsive promoter elements. Of note, cAMP-dependent gene regulation has also been described for MTs in nonthyroid cell systems (Cousins *et al.* 1986). By comparing the effect of the unspecific adenylyl cyclase activator FSK and that of TSH in FTC-133 wtTSHR cells on MT1X regulation, we found that FSK induced MT1X in a strictly PKA-dependent mechanism, whereas TSH-promoted induction of MT1X expression did not require any contribution of PKA. Furthermore, TSH completely failed to induce MT1 in FTC-133 Y601H cells expressing a TSHR variant with disrupted $G_{q/11}$ coupling but intact G_s -cAMP signaling (Büch *et al.* 2008).

The difference between the effects of FSK and those of TSH in FTC-133 wtTSHR cells may be explained by the fact that the cellular compartmentalization of the TSHR as well as the complement of specific adenylyl cyclase subtypes impinges on TSHR-dependent G_s signaling (Calebiro *et al.* 2009), whereas FSK as an unspecific activator of membrane-associated adenylyl cyclases does not discriminate between cellular compartments. Remarkably, in primary thyrocytes, FSK completely failed to induce MT1X. Thus, we conclude that FSK-dependent cAMP signaling is qualitatively different from TSH-dependent signaling (Fig. 8) and that the cAMP-PKA cascade is not required and not substantially involved in upregulation of MT1X by TSH.

The activation of $G_{q/11}$ proteins by TSH leads to an increase in cytosolic calcium concentration and an activation of PKC. In this study, disruption of $G_{q/11}$ signaling, chelation of intracellular calcium, and inhibition of PKC abrogated the induction of MT1X expression by TSH in FTC-133 wtTSHR cells. However, calcium-mobilizing stimuli such as ATP and CCh did not evoke an induction of MT1X expression. Furthermore, treatment with ionomycin leading to a massive influx of calcium only modestly, if at all, induced MT1X in FTC-133 wtTSHR cells and had no statistically significant effect in PHT cells. Thus, an increase in calcium levels appears to be necessary but not sufficient for a receptor-mediated upregulation of MT1X in thyroid cells. Of note, previous investigations have demonstrated that treatment of thyroid carcinoma cells with cadmium (Cd^{2+}) led to an increase in intracellular calcium levels as well as an activation of p44/42 MAPK (Liu *et al.* 2007), which were both necessary for MT induction (Liu *et al.* 2009). In contrast, we found that inhibition of p44/42 MAPK did not

impact MT1X induction by TSH (Büech & Stohr, 2012, unpublished data), giving evidence that calcium signaling via the TSHR differs from the Cd^{2+} -promoted activation of calcium and p44/42 MAPK pathways.

Classically, bivalent cations such as Zn^{2+} and Cd^{2+} induce MTs by interacting with the transcription factor *MTF1* (metal-responsive transcription factor; Heuchel *et al.* 1994). After nuclear translocation, *MTF1* binds to metal-responsive DNA elements and induces the transcription of MT mRNA (Brugnera *et al.* 1994). Apart from this signaling pathway, PKC can also directly phosphorylate and activate *MTF1* (LaRochelle *et al.* 2001), thus providing an explanation for the stimulatory effects of the PKC-activating phorbol ester PMA on *MT1X* gene transcription as well as for the involvement of PKC in TSH-elicited induction of MT1X expression.

MTs are expressed in numerous tissues including normal and neoplastic thyroid cells (Nartey *et al.* 1987) and have been shown to protect cells from potentially harmful heavy metal ions such as Cd^{2+} (Park *et al.* 2001). Interestingly, MT-deficient mice display a conspicuous impairment of thyroid function upon perinatal exposure to low doses of Cd^{2+} , emphasizing a physiologically relevant protective role of MTs in the thyroid gland (Mori *et al.* 2006). Furthermore, MTs exhibit cellular resistance to reactive oxygen species, e.g. H_2O_2 and other peroxides (Li *et al.* 2004, Baird *et al.* 2006). The latter function is likely to be of special importance in thyroid cells, because high levels of peroxides are required in these cells to oxidize iodide ions, allowing for tyrosine iodination during the multistep synthesis of thyroid hormone (Schweizer *et al.* 2008). Since $G_{q/11}$ -dependent signaling in thyrocytes is involved in the stimulation of H_2O_2 generation (Kimura *et al.* 1995), a simultaneous TSHR- and $G_{q/11}$ -promoted upregulation of intracellular peroxide scavengers is mechanistically reasonable. In this regard, it is worth mentioning that co-regulation of the expression of thyroperoxidase, a key enzyme regulating peroxide generation, with MT expression has been described in thyroid cells (Eszlinger *et al.* 2006).

Moreover, a functional role of MTs in a wide array of cancer types including differentiated thyroid cancer has been postulated (for review, see Cherian *et al.* (2003) and Pedersen *et al.* (2009)). In both follicular and papillary thyroid tumors, MT expression has been described (Nartey *et al.* 1987). Interestingly, several MT1 isoforms have been found to be downregulated in papillary thyroid carcinoma when compared with a normal thyroid gland (Huang *et al.* 2003, Ferrario *et al.* 2008), probably via epigenetic silencing (Huang *et al.* 2003). Accordingly, a role of MTs

as oncosuppressors in this tumor entity has been proposed (Ferrario *et al.* 2008). Of note, enhanced oxidative stress has been associated with the formation of goiter and dysregulation of thyroid cell growth (Poncin *et al.* 2010) and has been put forward as an important factor for the pathogenesis of thyroid carcinoma (Wang *et al.* 2011, Xing 2012). Thus, the antioxidant capacity of MTs may contribute to the proposed oncosuppressive effect in papillary carcinoma. However, in contrast to these findings, in follicular carcinoma, an upregulation of MTs when compared with the normal thyroid tissue and follicular adenoma has been described (Króllicka *et al.* 2010). In fact, especially the MT1X appears to be differentially regulated in follicular and papillary thyroid carcinomas (Ferrario *et al.* 2008). The biological consequences of the divergent MT expression in these two types of differentiated thyroid carcinomas are, however, not elucidated.

In the context of these findings, the observed $G_{q/11}$ -dependent regulation of MT1 expression may represent an important mechanism responsible for the protection of normal thyroid cells from oxidative stress and/or malignant transformation. In this regard, it should be enlightening to investigate the functional consequences of manipulating MT1 expression in the thyroid gland *in vivo*.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-12-0200>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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