

RESEARCH ARTICLE

Ghrelin-mediated inhibition of the TSH-stimulated function of differentiated human thyrocytes *ex vivo*

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Abstract

Ghrelin is a peptide hormone produced mainly in the gastrointestinal tract known to regulate several physiological functions including gut motility, adipose tissue accumulation and hunger sensation leading to increased bodyweight. Studies have found a correlation between the plasma levels of thyroid hormones and ghrelin, but an effect of ghrelin on the human thyroid has never been investigated even though ghrelin receptors are present in the thyroid. The present study shows a ghrelin-induced decrease in the thyroid-stimulating hormone (TSH)-induced production of thyroglobulin and mRNA expression of thyroperoxidase in a primary culture of human thyroid cells obtained from paranodular tissue. Accordingly, a trend was noted for an inhibition of TSH-stimulated expression of the sodium-iodine symporter and the TSH-receptor. Thus, this study suggests an effect of ghrelin on human thyrocytes and thereby emphasizes the relevance of examining whether ghrelin also influences the metabolic homeostasis through altered thyroid hormone production.

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Introduction

Ghrelin is a 28 amino acid peptide hormone released mainly from the gastric oxyntic glands that acts through a G-protein coupled receptor called the ghrelin receptor (GhrR) [1]. The receptor is expressed in many regions of the brain including the hypothalamus [2], the anterior pituitary gland [2] and the hippocampus [3]. Moreover, though to a much lesser extent than in the pituitary, the receptor is also expressed in several peripheral tissues [4]. The most well-studied area of ghrelin is its influence on metabolic homeostasis in an orexigenic and adipogenic direction due to its effect on the energy expenditure, appetite, adipocyte metabolism [5–9] and gastrointestinal motility [10, 11]. All these effects of ghrelin, and maybe more, are likely to play a role in body mass homeostasis. Thus the plasma ghrelin concentration correlates negatively with the body mass [12, 13], weight loss correlates with an increase in plasma ghrelin [14] and weight gain with a reduction [15]. The production of ghrelin is influenced by the caloric load, ingested macronutrients [16] and external food cues [17].

The thyroid gland plays a central role in regulation of metabolism, why it is important to understand any interaction between ghrelin and thyroid function. Most studies have most consistently found an inverse correlation in untreated hyper- and hypothyroid patients and in the euthyroid state [18–29]. However, other studies found opposite correlations, especially among patients with Hashimoto's thyroiditis [28, 30–32] and subclinical hypothyroidism [33]. These association studies, however, do not reveal any causality between ghrelin and thyroid hormones but suggest a possible interaction of ghrelin with the hypothalamus-pituitary-thyroid (HPT) axis. Several intervention studies have been performed [5, 34–40]. *In vivo* studies in rats show that ghrelin injection causes a decline in thyrotropin releasing hormone (TRH) and thyroid-stimulating hormone (TSH) [5, 34, 35] as well as a decline of triiodothyronine (T₃) and thyroxine (T₄) [39, 40]. Similar studies in humans confirm the inhibiting impact of ghrelin on the plasma concentration of TSH [36, 38]; one [36], in contrast to the aforementioned studies [39, 40], also showing an elevated T₄ plasma concentration. However, other studies in humans showed no effect [37]. A few studies have been performed to look further into the effect of ghrelin on the HPT axis *in vitro*. These studies will be described below.

GhrR is present in the hypothalamus [2], where ghrelin stimulates the activity of neuropeptide Y (NPY) and agouti-related protein (AGRP)-synthesizing neurons [41]. Activation of these two types of neurons has been shown to inhibit the activity of TRH neurons [42–44], but direct inhibition of the activity of TRH neurons by ghrelin has not been examined. Furthermore, the opposite functioning hormone leptin reduced fasting-induced increases in NPY and AGRP mRNA and prevented fasting-induced reduction in pro-TRH mRNA levels in the hypothalamus leading to a decrease in circulating thyroid hormone levels [45]. A similar study has not yet been performed for ghrelin. Less is known about the relationship between ghrelin and the thyrotrophs of the pituitary. Ghrelin stimulates the somatotrophs to synthesize GH [46] and studies also show the presence of GhrR in the thyrotrophs [47]. The percentage of thyrotrophs expressing GhrR in the pituitary seems to increase when mice are calorie restricted [47]. If this translates to humans, it might indicate that the pituitary could be causally and directly involved in the correlation between ghrelin and thyroid hormones in plasma. The potential function of ghrelin on the human thyroid isolated from the rest of the HPT axis is unknown, whereas two studies have been performed in rat thyroid cell lines [48, 49]. These studies showed an enhanced proliferation of the thyrocytes [48] and a potentiation of the TSH-induced expression of thyroglobulin (Tg), thyroperoxidase (TPO) and the sodium iodide symporter (NIS) by ghrelin [49].

The hypothesis of this study was that some of the adipogenic effect of ghrelin could be due to an impact on the thyroid-influenced metabolic rate. Thus, the present study is the first to investigate a direct effect of ghrelin on the TSH-induced human thyroid cell function *ex vivo*.

Material and methods

Primary thyroid cell cultures

Tissue samples from 9 patients were obtained from thyroidectomies due to non-toxic thyroid adenomas performed at the Department of ENT-Head and Neck surgery, Rigshospitalet, University of Copenhagen. The study was performed with the participants' written informed consent and approval by the Danish Committees on Health Research Ethics, Capital region (Protocol number: H-1-2012-110) which also represents the institutional review board in Denmark. Prior to the operation the patients had not received any drugs known to influence the function of the thyroid. The paraadenomatous thyroid tissue was washed in a calcium and magnesium free PBS (Gibco, Invitrogen Thermo Fischer Scientific, Waltham, MA, USA). The tissue samples were sliced before incubation with collagenase I (1 mg/mL) (Sigma-Aldrich,

St. Louis, MO) and dispase II (2.4 mU/L) (Roche, Basel, Switzerland) at 37°C for 75 min. The suspension was filtered through a 100 µm pore strainer (Falcon, BD bioscience, NJ) and cultured in HAM's F-12 medium supplemented with 1% L-glutamin (Panum Institute, Copenhagen University, Denmark), 1% non-essential amino acids (Gibco, Invitrogen, Carlsbad, CA, USA), 5% fetal bovine serum (FBS) (Biological Industries, Beit HaEmek, Israel), 1% penicillin and streptomycin (Invitrogen) which will be referred to as the *medium mixture* for the remainder of this article. It was then centrifuged at 1200 x G for 5 minutes and re-suspended in the above mentioned medium mixture after addition of six nutritional factors: TSH (1 IU/L) (Sigma-Aldrich, St. Louis, MO), insulin (10 mg/L) (Eli Lilly, Herlev, Denmark), transferrin (6 mg/L) and glycyl-histidyl-lysine acetat (10 µg/L) (Sigma-Aldrich, St. Louis, MO), somatostatin (10 µg/L) (Calbiochem, EMD Millipore, Billerica, MA) and hydrocortisone (10⁻⁸ M) (Calbiochem, EMD Millipore, Billerica, MA). The cells were cultured under similar conditions to the cell line of epithelium cells from rats (FRTL-5) [50]. The cells were cultured as monolayers in a humidified atmosphere (5% CO₂) at 37°C until a confluent monolayer was visualized in the wells for a maximum of 10 days. Afterwards, cells were starved from TSH for 72 hours and the following measurements were carried out in the presence of ghrelin (10⁻⁷ M) (PolyPeptide, Limhamn, Sweden), the above mentioned 6 nutritional factors including varying concentrations of TSH (0.1; 0.5; or 1 IU/L) and in absence of FBS. In optimization experiments, ghrelin at 6 different concentrations from 10⁻¹¹ to 10⁻⁶ M gave almost similar responses and 10⁻⁷ M was chosen arbitrarily in the remaining experiments. Cell cultures were exposed for 72 hours after which supernatants and cells were harvested. Relevant controls without TSH and/or ghrelin were included. Cell supernatants were temporarily stored at -20°C until used for cAMP and Tg measurements described in section 2.1.2 and 2.1.3, respectively. For real-time quantitative polymerase chain reaction (RT-qPCR) analysis, cell remnants from the cultures incubated with 0.1 IU/L TSH were harvested using incubation with lysis buffer (Qiagen, Hilden, Germany) followed by addition of 70% ethanol and the preparation was stored at -80°C until analysis. Tissue samples from two more patients were amplified by RT-qPCR before culture and after 1, 5, 12 days and 6, 9, 10, 13 days, respectively, to examine the stability of the expression of the GhrR-1a (henceforth referred to as GhrR) in the thyroid cells compared to human brain GhrR (Table 1). The amplification products were aligned with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene (Table 1).

cAMP. 3-Isobutyl-1-methylxanthine (IBMX) diluted in alcohol (final ethanol concentration 1%) was used for cAMP assessment and added to the cell cultures concurrently with ghrelin. The negative controls were added 1.1% ethanol. Cells were harvested as described above, and the cAMP concentration was measured by a competitive protein binding method as

Table 1. Primer sequences for RT-qPCR.

| Gene | Forward primer (5'-3' Sequence) | Reverse primer (5'-3' Sequence) |
|---------|---------------------------------|---------------------------------|
| Tg | GGGCGGGCAGTCAGCAGAGAGTG | ACCATAGTGGGCAGCCTCGGGTGAG |
| TSH-R | GAATGCTTTTCAGGGACTATGCAAT | ACAGCAGTGGCTTGGGTAAGAA |
| TPO | GGAGAGTGTGGGATGGAAG | GGATTTGCCTGTGTTGGAA |
| NIS | CCTTAGCTGACAGCTTCTATGCCA | CCCCAAGAAAAACAGACGATCC |
| IL-6 | AGAGTAACATGTGTGAAAGCAGCAA | CCTCAAAGTCCAAAAGACCAAGTGA |
| GhrR-1a | ACCAGAACCACAAGCAAACC | CAGGCTCAAAGGATTTGGAA |
| GAPDH | CATGAGAAGTATGACAACAGCCT | AGTCCTTCCACGATACCAAGT |

Tg, Thyroglobulin; TSH-R, thyroid-stimulating hormone receptor; TPO, thyroperoxidase; NIS, sodium iodide symporter; IL-6, interleukin 6; GhrR-1a, growth hormone secretagogue receptor 1a; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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described elsewhere [51] in which paper controls stimulating cells with forskolin were performed. The detection limit of the assay was 0.004 μM [52]. The calibration range was 0.05 to 2.0 μM . The intra-assay variation at the concentration of 0.4 μM was 4.7% and 7.2% at the concentration of 1.4 μM ($n = 8$ duplicates for each control level). For the low control, the inter-assay variation was 13.5% (range 0.29–0.45 μM) and 9.7% for the high control (range 1.10–1.71 μM) ($n = 5$ samples in duplicates for each control) [52].

Thyroglobulin. The Tg levels were assessed in supernatants by enzyme-linked immunosorbent assay (ELISA). Wells of polystyrene microtiter plates were coated with mouse anti-human Tg-antibody (Tg-Ab) (TF33, 3.1 g/L, AbD Serotec, Oxford, UK) and blocked with 200 μL TBS/0.5% bovine serum albumin (BSA) for 20–24 hours at 4°C. The plates were washed and incubated with supernatants for 60 minutes at 37°C and with rabbit anti-human Tg-Ab (K14, diluted 1:2 x 10⁵) for another 60 minutes at 37°C. After washing, peroxidase-conjugated polyclonal porcine anti-rabbit immunoglobulin (P399, Dako, Glostrup, Denmark, diluted 1:2 x 10³) and murine serum (Dako) were added and incubated for 60 minutes. Plates were washed again and a chromogenic substrate was added (TMB One, KEM EN TEC diagnostics, Taastrup, Denmark). Sulphuric acid (0.18 M) was added to stop the reaction and the results were measured by an ELISA reader (BioTek Synergy 2) at 450 nm. The calibration range was 10 to 500 $\mu\text{g/L}$ [52]. When ELISA was performed, the samples were diluted until Tg levels measured were in compliance with the detection range. Afterwards, the results were adjusted in accordance with the dilution series. The intra-assay variation at the concentration of 52 $\mu\text{g/L}$ was 9.5% and 8% at 101 $\mu\text{g/L}$ ($n = 7$ and 6 duplicates for the low and high control level, respectively). For the low control, the inter-assay variation was 22.3% (range 32–65 $\mu\text{g/L}$) and 17.5% for the high control (65–121 $\mu\text{g/L}$) ($n = 5$ samples in duplicates for each control) [52].

RT-qPCR. RT-qPCR was used for measuring the mRNA encoding Tg, NIS, TPO, TSH receptor (TSH-R) and interleukin-6 (IL-6), the latter used for controlling the specificity of the ghrelin-induced effect. Total RNA was extracted from cultured, primary human thyroid cells from 7 patients with Qiagen Rneasy mini kit according to the manufacturer's protocol. Nano-Drop spectrophotometer was used for quantification of isolated RNA. For each sample, cDNA was synthesized (Superscript VILO synthesis kit, Invitrogen) by mixing 4 μL of the VILO reaction mix, 2 μL of the Superscript enzyme mix, the RNA (same amount from each sample) and RNAase free water to a total volume of 20 μL . Samples were incubated for 10 minutes at 25°C, 60 minutes at 50°C and 5 minutes at 85°C, whereupon 80 μL of 0.5X Tris-EDTA-buffer (Sigma-Aldrich) were added. The RT-qPCR analysis was performed with SYBR® Green JumpStart Taq Ready Mix (Sigma-Aldrich). A pool of undiluted cDNA was used for standards. 4 μL of SYBR Green JumpStart Taq ReadyMix, 10 μL of H₂O and 1 μL of primers (1 μM final concentration of each primer) were added to each reaction. RT-qPCR was performed on Lightcycler 480 II (Roche, Basel, Switzerland) with an initial denaturation at 94°C for 2 minutes, 45 cycles consisting of 30 seconds at 94°C, 45 seconds at 59°C, 1.30 minutes at 72°C. The analysis was followed by a melting curve analysis. The cycle threshold (Ct) values obtained from the RT-qPCR were normalized to the reference gene beta-2-microglobulin (Table 1).

Statistics

Results were analyzed in GraphPad Prism 7 (2016 GraphPad Software, Inc.) and represented as means + or \pm SEM. P-values lower than 0.05 were considered statistically significant. All experiments were carried out in triplicate. When cell cultures with and without ghrelin with the same concentration of added TSH were compared, the paired, the non-parametric Wilcoxon signed-rank test was used (i.e. in all statistics performed) [53, 54]. In the Tg and cAMP

assays, the basal levels, i.e. the values in the absence of TSH, were subtracted, before the groups were compared. Absolute values for the basal levels of Tg and cAMP are shown in the *Supporting information* section.

Results

Ghrelin receptors are expressed in thyroid cells

To ensure that the culturing procedure of the thyroid tissue did not affect the expression level of GhrR we tested the expression of the receptor in two human thyroid tissue samples before culture and after 12–13 days of culture. We found that the concentration of GhrR in the thyroid was about the same as in human brain tissue before and after 12–13 days (Fig 1).

Role of ghrelin as an inhibitor of TSH-induced thyroglobulin production

To test whether ghrelin affected the TSH-induced Tg production, we treated cells with TSH alone and in combination with ghrelin, respectively. TSH stimulated an equal increase in Tg production at all three concentrations used, in accordance with a former study [53] in which the TSH effect on Tg production was the same at 0.1 to 10 IU/L TSH. After addition of ghrelin, a significant decrease in the 0.1 IU/L TSH-induced production of Tg was observed ($n = 8$, $p = 0.039$) (Fig 2A). Although not significant, the same tendency was seen for concentrations of TSH of 0.5 and 1 IU/L in which a decrease was found ($n = 6$, $p = 0.16$ for both). Importantly, ghrelin only decreased the TSH-induced Tg production, whereas the production of Tg without TSH stimulation was unaffected by ghrelin treatment (S2A Fig).

Ghrelin did not influence the TSH-stimulated cAMP generation

To see which part of the TSH receptor signaling pathway that ghrelin inhibits, we examined if ghrelin treatment affected TSH-induced cAMP expression. No effect of ghrelin on the TSH-

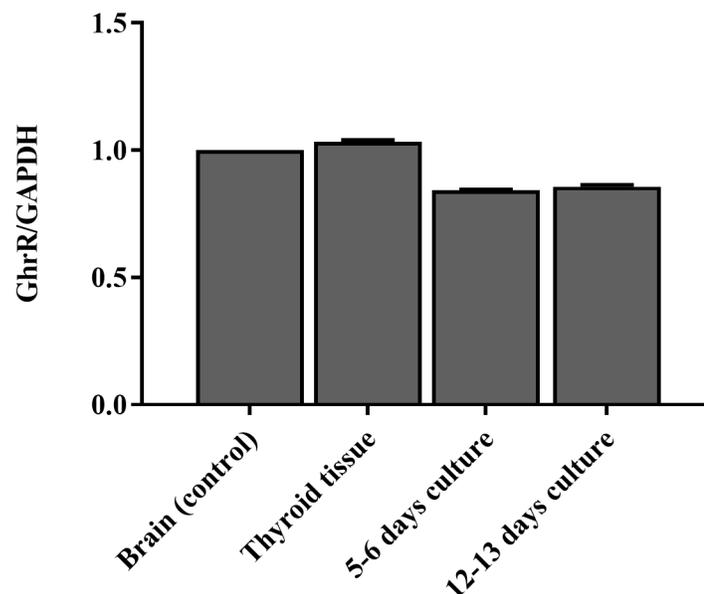


Fig 1. Ghrelin receptor (GhrR) mRNA expression level. GhrR mRNA expression level in relation to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression level in human brain, thyroid tissue and cell cultures measured by real-time quantitative polymerase chain reaction (RT-qPCR). $n = 2$.

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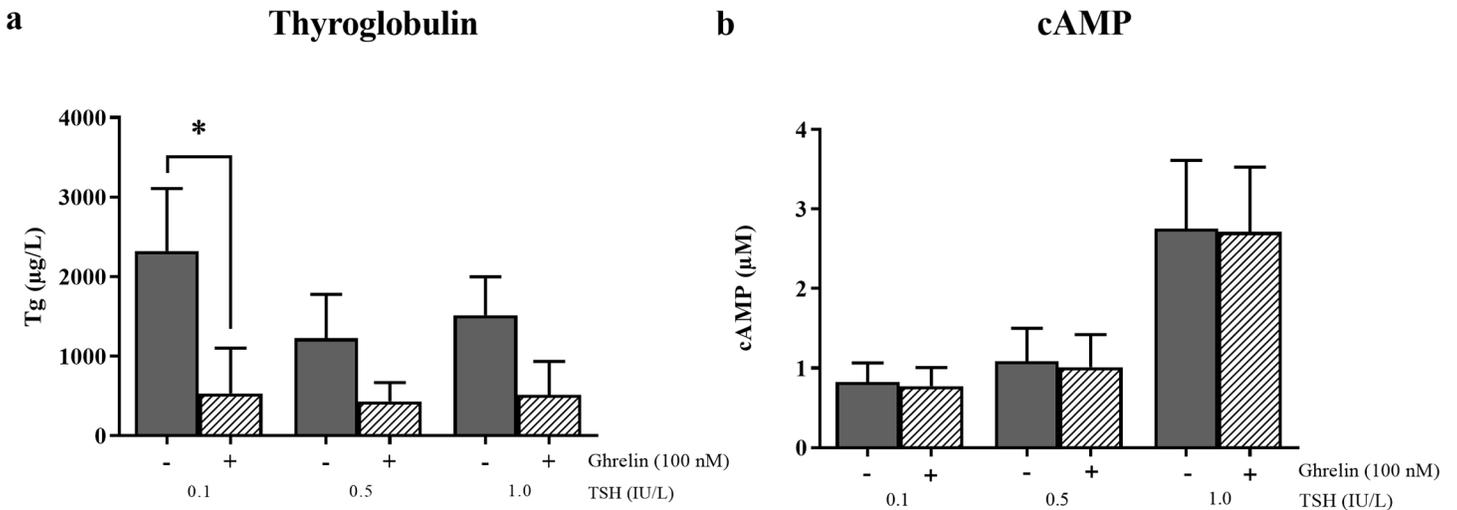


Fig 2. The influence of ghrelin on the thyroid-stimulating hormone (TSH)-induced increase in thyroglobulin (Tg) and cAMP production. The influence of ghrelin on the TSH-induced increase in Tg and cAMP production at three different concentrations of TSH (0.1 IU/L, 0.5 IU/L and 1 IU/L). The basal levels, i.e. the values in the absence of TSH, were subtracted, before the groups were compared. Grey = vehicle, pattern = ghrelin (100 nM). Means (+SEM). *P < 0.05 compared to the control (vehicle). **A)** Ghrelin inhibited the TSH-induced increase in Tg production measured by enzyme-linked immunosorbent assay (ELISA) in primary cultures of human thyroid cells for the TSH concentration of 0.1 IU/L. n = 8 (0.1 IU/L) and n = 6 (0.5 and 1 IU/L) in triplets. Two patient samples were excluded due to lack of basal TSH-induced Tg production. **B)** No influence of ghrelin on the TSH-induced increase in cAMP production at three different concentrations of TSH (0.1 IU/L, 0.5 IU/L and 1 IU/L) measured by a competitive protein binding method in primary cultures of human thyroid cells. n = 8 (0.1 IU/L and 1 IU/L) and n = 6 (0.5 IU/L) in triplets.

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induced production of cAMP was observed which indicates that the inhibiting effect on the ghrelin-induced Tg secretion is at least not involving the steps upstream of the adenylate cyclase in the $G_{\alpha s}$ coupled pathway of TSH (Fig 2B). It should be noted that some of the cAMP values measured are below calibration range, though above the detection limit. However, this does not change the conclusion that no effect of ghrelin on the TSH-induced production of cAMP was found.

Ghrelin decreased the TSH-induced expression of TPO

To analyze changes in key thyroid components upon the addition of ghrelin, we performed RT-qPCR analysis of the expression levels of Tg, NIS, TPO and TSH-R. All cell cultures responded to the addition of TSH (0.1 IU/L) by multi-fold increases above the basal levels (Fig 3). Combined addition of ghrelin and TSH inhibited TPO upregulation significantly (p = 0.031). Tg, NIS and TSH-R decreased as well, though not obtaining statistical significance. Importantly, the reference gene IL-6 was unaffected by TSH and the combination with ghrelin.

Discussion

The present study is the first to investigate whether ghrelin acts directly on human thyrocytes, that have previously been described to express the GhrR [4, 55, 56], and accordingly the ability to modulate the secretion of several key components that are important to the production of thyroid hormones.

We confirmed the presence of GhrR on the thyroid tissue as well as on the cultured thyrocytes. The expression levels were lower in the cultured thyrocytes compared to the tissue samples but the expression levels were stable from day 5–6 to day 10–13 of culture (Fig 1). We showed that ghrelin decreased the TSH-induced protein level of Tg significantly for the lowest

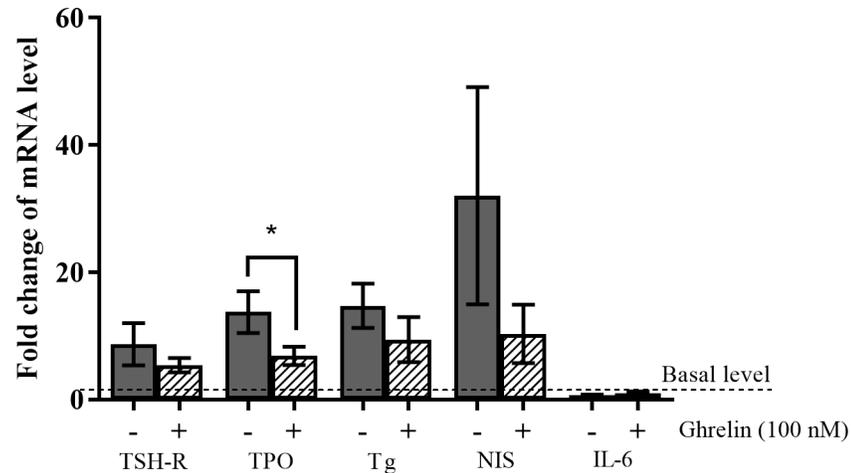


Fig 3. The influence of ghrelin on the thyroid-stimulating hormone (TSH)-induced (0.1 IU/L) mRNA expression of four thyroid components. The expression of the TSH receptor (TSH-R), thyroperoxidase (TPO), thyroglobulin (Tg) and sodium iodide symporter (NIS) measured by real-time quantitative polymerase chain reaction (RT-qPCR) in a primary culture of human thyroid cells in presence and absence of ghrelin. Indicated as fold change of mRNA expression compared to basal level (dashed line). IL-6 was used as a negative control. Grey = vehicle, pattern = ghrelin (100 nM). Means (\pm SEM), $n = 6$. * $P < 0.05$ compared to the control (vehicle). Two patients were excluded due to unknown sample material.

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concentration of TSH and only a trend was observed for the two higher concentrations. The non-significance for these two concentrations might have been due to a large inter-culture variation of 19.9% [53] combined with the lower number of cultures at these two concentrations. Additionally, the mRNA expression of TPO was decreased and a tendency of inhibition of NIS, Tg and TSH-R was observed in thyrocytes from paranodular thyroid tissue, indicating a suppressive role of ghrelin on the thyrocytes (Figs 2 and 3). The production of Tg is influenced by the amount of TPO and NIS, hence ghrelin may mediate its inhibitory effect on Tg through NIS and/or TPO. Importantly, ghrelin did not affect the basal level of Tg and cAMP (S2 Fig), NIS, TPO or TSH-R in the absence of TSH. To explore where in the TSH signaling pathway ghrelin could cause its influence, we measured the concentration of cAMP in the cultures with or without ghrelin. This particular component is relevant because the TSH receptor is $G_{\alpha s}$ coupled [57] and therefore induces cAMP expression when activated in contrast to the GhrR which is $G_{\alpha q}$ coupled [58, 59]. We found no change in cAMP production when ghrelin was added along with TSH, suggesting that ghrelin influences the TSH pathway downstream of the adenylate cyclase (Fig 2B).

Therefore, our results indicate that there could be an antagonizing function of ghrelin on the TSH-induced function of human thyrocytes; although a direct translation from our *ex vivo* experiments to the real situation should be made with caution. Importantly, a limit of our study is that it does not take into account the feed-back mechanisms which occur in a whole organism.

Similar studies in rats

An effect of ghrelin downstream of the adenylate cyclase is apparently supported by an *in vitro* study of a rat cell line (FRTL-5) which found evidence of crosstalk occurring downstream of cAMP through ghrelin-induced intracellular calcium signaling which changed the TSH-induced proliferation of the thyrocytes, possibly mediated by the p66Shc pathway [48]. This led to an enhanced proliferation of the thyrocytes, whereas the function of the cells remained

unexamined. Another study in rat tumor thyroid cells (PC-CI3) found a potentiation of the TSH-induced expression of Tg, TPO and NIS by ghrelin [49]. However, cell lines are known to lose properties by passaging and therefore are not necessarily good markers of human physiology [60, 61]. Furthermore, studies have shown that thyroid cell lines, as a result of dedifferentiation during passaging, become highly proliferative, but often lack their primary function e.g. producing Tg, wherefore proliferation rate and function do not consistently correlate positively [61]. Our study is the only one investigating the effect of ghrelin on *human* thyroid tissue and moreover in *primary* cell cultures.

New contributions to understand the role of ghrelin on the hypothalamus-pituitary-thyroid-axis

Thus, our findings are new contributions to understanding the complex effects of ghrelin on the HPT axis and thereby on the levels of thyroid hormones in health and disease. It may therefore contribute to our understanding of the correlation between the plasma levels of ghrelin and thyroid hormones. The inhibiting effect of ghrelin on the thyroid components found in this study could be due to an energy saving strategy in which the orexigenic effect of ghrelin together with the decreased metabolism leads to a less catabolic state. This is in accordance with the inverse relationship between thyroid hormones and ghrelin found in patients with especially hyperthyroidism [18–29] as well as the inhibiting effect of ghrelin on the HPT axis shown in several *in vivo* studies [5, 34–40]. The adipogenic effect of ghrelin has until now mostly been attributed to increased food intake and increased fat accumulation but with this study we propose a new mechanism, though, more studies need to be done to clarify mechanisms.

Conclusions

This study demonstrates for the first time a direct effect of ghrelin on human thyrocytes *ex vivo* and thereby suggests a new possible role for ghrelin in regulating the production of thyroid hormones in humans. The shown suppressive impact of ghrelin on the thyrocytes is one more minor step towards understanding the role of ghrelin in human energy homeostasis and may in the long run contribute to the development of new therapeutic strategies in thyroid and metabolic disorders.

Supporting information

S1 Fig. The influence of ghrelin on the thyroid-stimulating hormone (TSH)-induced increase in thyroglobulin (Tg) and cAMP production for each patient. The influence of ghrelin on the TSH-induced increase in thyroglobulin Tg and cAMP production at three different concentrations of TSH (0.1 IU/L, 0.5 IU/L and 1 IU/L). The basal levels, i.e. the values in the absence of TSH, were subtracted, before the groups were compared. Grey = vehicle, pattern = ghrelin (100 nM). Means (+SEM). *P < 0.05 compared to the control (vehicle). **A)** Ghrelin inhibited the TSH-induced increase in Tg production measured by enzyme-linked immunosorbent assay (ELISA) in primary cultures of human thyroid cells for the TSH concentration of 0.1 IU/L. n = 8 (0.1 IU/L) and n = 6 (0.5 and 1 IU/L) in triplets. Two patient samples were excluded due to lack of basal TSH-induced Tg production. **B)** No influence of ghrelin on the TSH-induced increase in cAMP production at three different concentrations of TSH (0.1 IU/L, 0.5 IU/L and 1 IU/L) measured by a competitive protein binding method in primary cultures of human thyroid cells. n = 8 (0.1 IU/L and 1 IU/L) and n = 6 (0.5 IU/L) in triplets. (TIF)

S2 Fig. The influence of ghrelin on the level of thyroglobulin (Tg) and cAMP in the absence of TSH. Grey = vehicle (without ghrelin), pattern = ghrelin (100 nM). Means (+SEM).

*P < 0.05 compared to the control (vehicle). n = 8 in triplets. **A)** Ghrelin did not influence the basal level of Tg ($\mu\text{g/L}$) in the absence of TSH measured by enzyme-linked immunosorbent assay (ELISA) in primary cultures of human thyroid cells. **B)** No influence of ghrelin on the basal level of cAMP ($\mu\text{mol/L}$) was observed in the absence of TSH, when measured using a competitive protein binding method in primary cultures of human thyroid cells. (TIF)

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Writing – review & editing: Maria Barington, Marianne Møller Brorson, Jacob Hofman-Bang, Åse Krogh Rasmussen, Birgitte Holst, Ulla Feldt-Rasmussen.

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