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Thyroid-stimulating hormone stimulates lipolysis in adipocytes in culture and raises serum free fatty acid levels in vivo

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Abstract

Thyroid-stimulating hormone (TSH) stimulates adipocyte lipolysis, but signal transduction pathways activated by TSH for this response have not been directly studied. Using differentiated 3T3-L1 adipocytes as well as primary human adipocytes, we characterized the lipolytic action of TSH with dose-response and time-course studies, and compared it with isoproterenol. Thyroid-stimulating hormone stimulated phosphorylation of perilipin and hormone-sensitive lipase (HSL). Inhibition of protein kinase A with H89 blocked TSH-stimulated lipolysis as well as phosphorylation of perilipin and HSL. Thyroid-stimulating hormone stimulated lipolysis in vivo, as indicated by an elevation in serum free fatty acid (FFA) levels after recombinant human TSH administration to thyroidectomized patients (42% increase, n = 19, $P < .05$). For patients with a body mass index less than 30 kg/m^2, the TSH-induced increase in serum FFA levels was 53% (n = 11, $P < .05$), whereas levels in patients with a body mass index of at least 30 kg/m^2 (n = 8) did not change after TSH treatment. In summary, TSH stimulates lipolysis and phosphorylation of perilipin and HSL in a protein kinase A–dependent manner in differentiated adipocytes in culture and raises serum FFA levels in vivo.
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1. Introduction

Lipolysis is a critical and highly regulated adipocyte function for energy homeostasis. Insulin suppresses lipolysis through activation of phosphodiesterase 3B, reducing cyclic adenosine monophosphate (cAMP) levels and thereby the activity of cAMP-activated protein kinase (protein kinase A [PKA]). Protein kinase A stimulates lipolysis by phosphorylating and activating hormone-sensitive lipase (HSL). Protein kinase A also phosphorylates the lipid droplet coat protein, perilipin, which facilitates access of HSL to the stored triglyceride to mediate free fatty acid (FFA) and glycerol release. Catecholamines, classic activators of lipolysis, act on G protein–coupled β-adrenergic receptors linked to adenylate cyclase to stimulate PKA. Inappropriately elevated FFA levels are associated with insulin resistance and cardiovascular disease [1,2].

Thyroid-stimulating hormone (TSH) has been implicated as an activator of lipolysis for several decades. Early work examining G protein–coupled receptor function and its link to adenylate cyclase and lipolysis included studies with TSH [3-5]. Several studies have demonstrated that adipocytes express functional TSH receptors in rodents as well as humans [6-10]. Activation of the TSH receptor can raise cAMP levels in adipocytes in culture, but the requirement for this second messenger with respect to TSH-induced lipolysis was not directly determined by inhibitor studies [11]. The impact of TSH on HSL and perilipin has also not been reported.

TSH levels are persistently elevated in subclinical hypothyroidism as a compensatory mechanism to restore thyroid hormone production to normal in mild thyroid gland failure [12]. This population appears to be at higher risk for cardiovascular disease based on cross-sectional and

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longitudinal studies [13,14]. Traditional risk factors such as hypertension or hypercholesterolemia do not explain the association. Elevated serum FFA levels have been reported in patients with subclinical hypothyroidism [15], consistent with the possibility that TSH may exert extrathyroidal effects in vivo, up-regulating FFA release from adipocytes. A similar role for TSH as a trigger for lipolysis during the neonatal period has also been reported, a time when serum TSH levels surge dramatically [16].

We have now examined the effect of TSH on the lipolytic response of 3T3-L1 and human differentiated adipocytes, and delineated upstream signaling events that involve PKA, perilipin, and HSL. Finally, serum levels of FFAs were measured in thyroidectomized patients treated with recombinant human (rh) TSH as a model to assess the action of TSH on human adipocytes in vivo.

2. Materials and methods

2.1. Murine 3T3-L1 cell culture and differentiation

3T3-L1 preadipocytes (ATCC, Manassas, VA) were grown to confluence in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum, 0.1 mg/mL streptomycin, and 100 U/mL penicillin (henceforth referred to as *antibiotics*). Differentiation was initiated 2 days postconfluence by placing the cells in DMEM supplemented with 10% fetal bovine serum and antibiotics and with 0.5 mmol/L isobutylmethylxanthine and 0.25 μmol/L dexamethasone for the first 2 days, and 1 μmol/L insulin for the first 4 days. Medium was replaced every 2 days. On day 8, adipocytes were placed in DMEM supplemented with 1% calf serum and antibiotics overnight before stimulation.

2.2. Isolation, culture, and differentiation of human subcutaneous abdominal preadipocytes

Human abdominal subcutaneous adipose tissues were obtained during elective abdominal surgery from a total of 18 patients (14 women, 4 men), with a mean (±SD) age 49 ± 10 years and a mean (±SD) body mass index (BMI) of 26 ± 4 kg/m^2. The study was approved by the Ottawa Hospital Research Ethics Board (1995023-01H). The stromal preadipocytes were isolated as previously described [17]. Briefly, tissues were dissected to remove blood vessels and fibrous tissue, then digested with collagenase (CLS type I, 600 U/g of tissue). Stromal preadipocytes were obtained after sequential centrifugation, size filtration, and treatment with erythrocyte lysis buffer. Preadipocytes were grown in DMEM supplemented with 10% fetal bovine serum, antibiotics, and 50 U/mL nystatin (growth medium). Cells were either seeded for a maximum of 3 passages or cryopreserved until required. For differentiation, cells were seeded at a density of 3×10^4 cells per square centimeter in growth medium. Differentiation was induced the following day by the addition of growth medium supplemented with 0.85 μmol/L insulin, 100 μmol/L indomethacin, 0.5 μmol/L dexamethasone, and 0.25 mmol/L isobutylmethylxanthine for 14 days; and this resulted in about 50% to 60% of the cells differentiating into adipocytes [18]. On day 14, the medium was removed; cells were washed once with growth medium and then placed in growth medium for 2 days before stimulation.

2.3. Measurement of glycerol and FFA release

Stimulations were performed in assay buffer (LIP-1-NC-lipolysis kit; Zen-Bio, Research Triangle Park, NC) supplemented with 1% calf serum and antibiotics. Differentiated adipocytes were stimulated with 0 to 50 mU/mL TSH, 1 μmol/L isoproterenol, or vehicle control for the specified times. When indicated, adipocytes were pretreated with 20 μmol/L H89 (Calbiochem, San Diego, CA) or vehicle (0.1% dimethyl sulfoxide [DMSO]) for 1 hour before stimulation. The cell culture supernatants were collected and centrifuged at 500*g* to remove cell debris. Triacylglycerol accumulation in each cell culture was determined as previously described [18] and was similar between treatments for each experiment. Glycerol release was quantified using the LIP-1-NC lipolysis kit (Zen-Bio) according to the manufacturer's instructions. Free fatty acid release was quantified with a commercial kit (NEFA-C; Wako Chemicals, Richmond, VA).

2.4. Immunoblot analysis

Human differentiated adipocytes were stimulated for 15 to 90 minutes with 50 mU/mL TSH, 1 μmol/L isoproterenol, or vehicle. When indicated, adipocytes were pretreated with 20 μmol/L H89 (Calbiochem) or vehicle (0.1% DMSO) for 1 hour before stimulation with 50 mU/mL TSH. Adipocytes were lysed in Laemmli buffer [19] supplemented with 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 5 mmol/L EGTA, and 1 mmol/L Na_3VO_4. Equal amounts of solubilized protein (25 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose membrane. Nonspecific immunogenic sites were blocked; and membrane was probed with perilipin (1:2000; Progen, Heidelberg, Germany), phospho–hormone-sensitive lipase (pHSL) Ser563 (1:1000; Cell Signaling), or HSL (1:2000, Cell Signaling, Danvers, MA), followed by incubation with the appropriate horseradish peroxidase–conjugated secondary antibodies. Immunoreactivity was detected by enhanced chemiluminescence. Relative densitometry was measured with AlphaEaseFC software (Alpha Innotech, San Leandro, CA), and data are expressed as integrated optical density units.

2.5. Measurement of FFA in human serum

Participants (approved by the Ottawa Hospital Research Ethics Board, 2006558) were 15 women and 4 men, with a mean (±SD) age of 50 ± 11 years, who had previously undergone thyroidectomy and radioablative iodine therapy for thyroid cancer. None had evidence of metastatic disease,

and they were otherwise healthy. In the context of routine surveillance for thyroid cancer recurrence, the patients received 2 separate intramuscular doses of rhTSH (0.9 mg), administered 24 hours apart on days 1 and 2, without discontinuation of ongoing L-thyroxine therapy. Fasting blood samples were drawn in the morning of days 1 (before the first dose of rhTSH), 3, and 5. Serum FFA levels were determined using a commercial kit (SFA-1, Zen-Bio).

2.6. Statistical analysis

Data were analyzed by analysis of variance, followed by post hoc Newman-Keul tests for multiple comparisons, with $P < .05$ taken as significant.

3. Results

3.1. TSH stimulates lipolysis in mouse 3T3-L1 adipocyte studies

We compared the effect of 5 mU/mL TSH with 1 μmol/L isoproterenol on glycerol release from 3T3-L1 adipocytes over 4 hours. The basal level of glycerol was 82 ± 5 μmol/L. Thyroid-stimulating hormone elicited a 2.3-fold increase vs a 5.2-fold increase due to isoproterenol (Fig. 1A). A dose-response curve showed a gradual enhancement of glycerol release as concentrations rose to 50 mU/mL TSH (Fig. 1B). The lowest dose that yielded an increase in glycerol release was 0.5 mU/mL. Most of the effect on glycerol release by TSH occurred at 4 hours (Fig. 1C). Basal glycerol release rose over the period tested.

To evaluate whether PKA signaling was required for the lipolytic effect of TSH, 3T3-L1 adipocytes were pretreated with 20 μmol/L H89 before the addition of TSH. In the presence vs absence of H89, the TSH-stimulated rise in glycerol release over basal was reduced by 68% (Fig. 1D).

3.2. TSH stimulates lipolysis in human abdominal subcutaneous differentiated adipocytes

The effect of 5 mU/mL TSH compared with 1 μmol/L isoproterenol was examined in primary cultures of differentiated human adipocytes. The glycerol level under basal conditions was 30 ± 12 μmol/L. The stimulation of glycerol release over 4 hours for either agonist was significant compared with basal, rising 3.0-fold and 4.0-fold in response to TSH and isoproterenol, respectively (Fig. 2A). Lipolysis was also assessed by measuring FFA release. The stimulation of FFA release was also significant compared with basal values, with values rising to 140 ± 52 μmol/L for TSH and

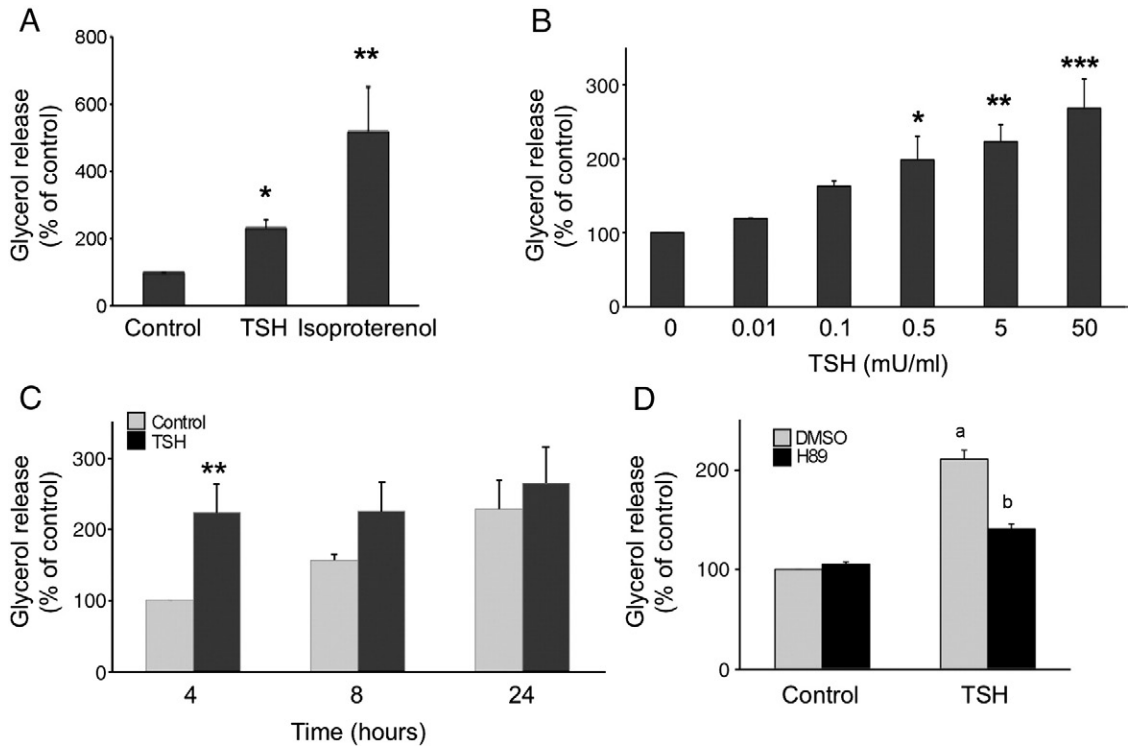

Fig. 1. Thyroid-stimulating hormone stimulates lipolysis in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate for 8 days. On day 8, cells were placed in serum-reduced medium overnight before stimulation. A, Cells were stimulated with 5 mU/mL TSH, 1 μmol/L isoproterenol, or vehicle control for 4 hours. B, Cells were stimulated with indicated concentrations of TSH for 4 hours. C, Cells were stimulated for 4, 8, or 24 hours with 5 mU/mL TSH or vehicle control. D, Cells were pretreated with 20 μmol/L H89 or DMSO (control) for 1 hour before stimulation with 5 mU/mL TSH for 4 hours. Cell culture supernatants were collected and assayed for glycerol release. Results represent the mean ± SE of 3 (B and C) or 4 (A and D) independent experiments, each performed in duplicate. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ compared with vehicle control. $^{a}P < .001$ compared with control, and $^{b}P < .001$ compared with TSH condition.

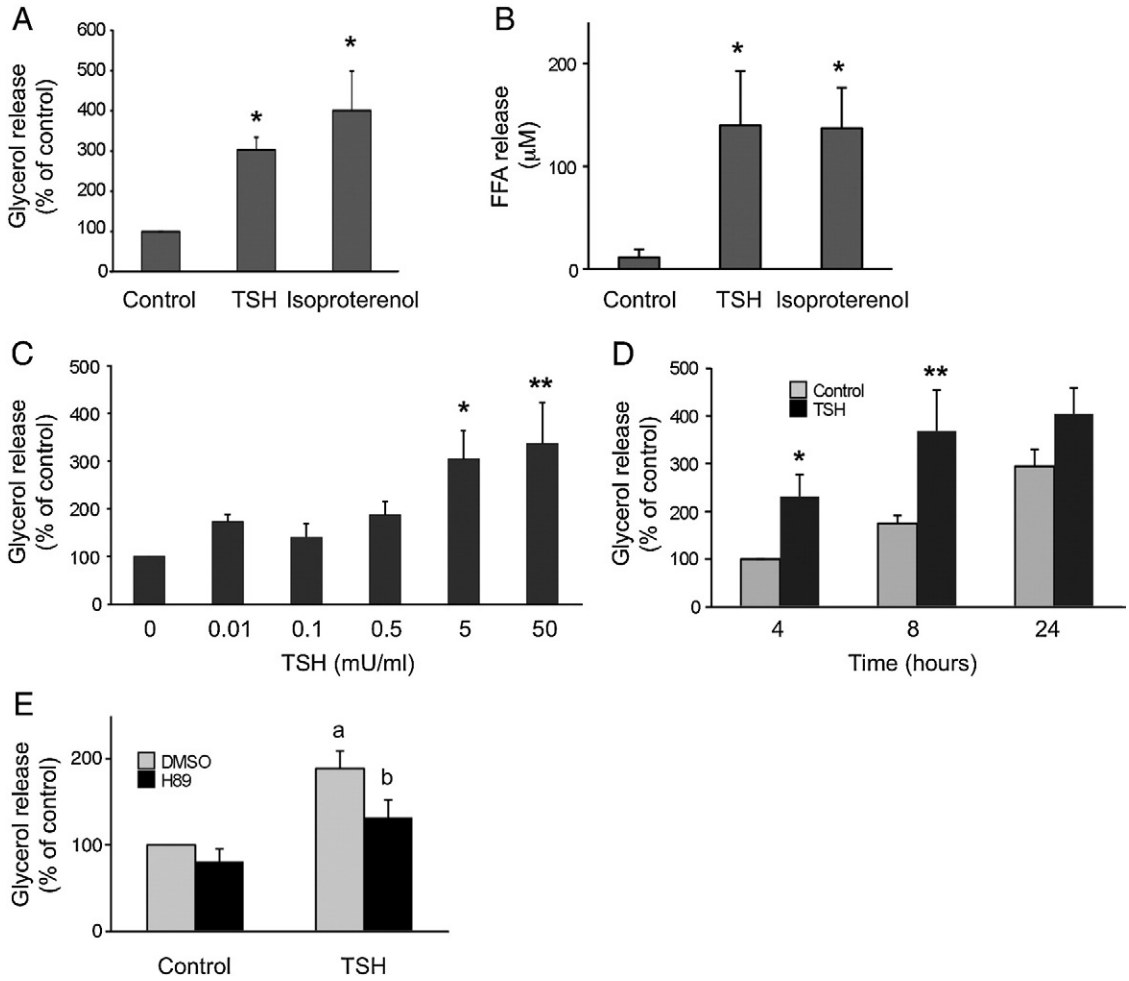

Fig. 2. Thyroid-stimulating hormone stimulates lipolysis in human abdominal subcutaneous adipocytes. Human preadipocytes were induced to differentiate for 14 days. A and B, Cells were stimulated with 5 mU/mL TSH, 1 μmol/L isoproterenol, or vehicle control for 4 hours. C, Cells were stimulated with indicated concentrations of TSH for 4 hours. D, Cells were stimulated for 4, 8, or 24 hours with 5 mU/mL TSH or vehicle control. E, Cells were pretreated with 20 μmol/L H89 or DMSO (control) for 1 hour before stimulation with 5 mU/mL TSH for 4 hours. Cell culture supernatants were collected and assayed for glycerol (A, C-E) or FFA (B) release. Results represent the mean ± SE of 3 (B), 4 (A, C, E), or 8 (D) separate patient samples, each determined in duplicate. $^{*}P < .05$ and $^{**}P < .01$ compared with vehicle control. $^{a}P < .01$ compared with control, and $^{b}P < .05$ compared with TSH condition.

151 ± 49 μmol/L for isoproterenol (Fig. 2B). Overall, the TSH vs the isoproterenol responses of the human differentiated adipocytes were not significantly different (Fig. 2, $P > .05$). Dose-response experiments revealed that the lowest effective dose of TSH that stimulated glycerol release was 5 mU/mL and that there was no further enhancement at 50 mU/mL (Fig. 2C). The lipolytic action of TSH persisted up to 8 hours (Fig. 2D). Basal glycerol rose slowly over the measured period. Inhibition of PKA via H89 pretreatment significantly inhibited the TSH-stimulated glycerol release over basal from the human differentiated adipocytes by 43% compared with conditions without H89 (Fig. 2E).

3.3. TSH stimulates phosphorylation of perilipin and HSL in human abdominal subcutaneous differentiated adipocytes

Lipolytic agonists acting through their cognate G protein-couple receptor (GPCR) are known to stimulate the PKA-mediated phosphorylation of perilipin and HSL. We treated human differentiated adipocytes with either TSH or isoproterenol for 1 hour and assessed perilipin phosphorylation by following its characteristic mobility shift on SDS-PAGE (Fig. 3A). A clear shift to the upper band, that is, reduction in mobility, occurred in response to either agonist. Thyroid-stimulating hormone treatment also increased the levels of pHSL by 2.5-fold; phosphorylation at serine 563 by PKA is indicative of activation (Fig. 3B). H89 pretreatment, which inhibited lipolysis in human differentiated adipocytes (Fig. 2E), also inhibited HSL as well as perilipin phosphorylation in these cells (Fig. 3B).

3.4. Elevation of serum FFA levels in response to TSH administration to thyroidectomized patients

To determine if TSH was able to induce lipolysis in vivo and thereby raise serum FFA levels, we studied 19 patients

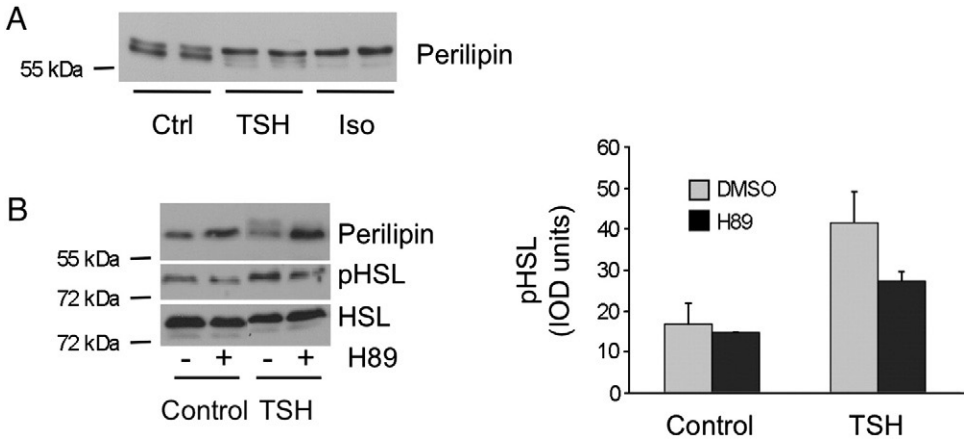

Fig. 3. Thyroid-stimulating hormone stimulated perilipin and HSL phosphorylation in human abdominal subcutaneous adipocytes. Human preadipocytes were induced to differentiate for 14 days. A, Cells were stimulated with 5 mU/mL TSH, 1 μmol/L isoproterenol, or vehicle for 1 hour. Representative immunoblots from 3 patient samples are shown. B, Cells were pretreated with 20 μmol/L H89 or DMSO (control) for 1 hour before stimulation with 50 mU/mL TSH for 1 hour. Solubilized proteins were separated by SDS-PAGE and immunoblotted for perilipin, pHSL, or HSL, as indicated. Representative immunoblots from 2 patient samples are shown. Densitometric data for pHSL from 2 independent patient samples are expressed as mean ± range. Iso indicates isoproterenol; Ctrl, control.

(15 female, 4 male) who had previously undergone a surgical thyroidectomy followed by radioactive ablation of any residual thyroid tissue. As part of their routine surveillance for thyroid cancer involving measurement of serum thyroglobulin and radioactive iodine body scanning, rhTSH (0.9 mg) was administered on days 1 and 2. Blood samples were drawn on days 1 (before rhTSH injection), 3, and 5. Thyroid-stimulating hormone was 0.4 ± 0.9 mU/L at baseline, increased to greater than 100 mU/L on day 3, and was 15 ± 8 mU/L on day 5.

The fasting concentration of FFA in the serum was 298 ± 44 μmol/L at baseline on day 1 before rhTSH administration. No significant change in fasting FFA serum levels was seen on day 3; but on day 5 of the rhTSH protocol, they increased significantly by 42% (Fig. 4A). Analyzing the FFA response according to whether patients were obese (BMI ≥30 kg/m^2, n = 8) or nonobese (BMI <30 kg/m^2, n = 11) revealed that the nonobese group was responsive to rhTSH with an increase of 53%. There was no increase after rhTSH administration in the obese group (Fig. 4B), possibly because this group already had higher FFA concentrations than the nonobese group at baseline. The baseline FFA serum level was 217 ± 29 μmol/L for those with a BMI less than 30 kg/m^2 vs 406 ± 80 μmol/L for those with a BMI of at least 30 kg/m^2 ($P < .05$). It is also possible that there are differences in adipocyte TSH receptor expression in these obese vs nonobese subjects.

4. Discussion

Our data provide a detailed characterization of TSH-stimulated lipolysis in human differentiated adipocytes in culture, including an analysis of the phosphorylation of perilipin and HSL. A requirement for PKA signaling for these events was demonstrated by their inhibition in response to H89. Evidence for this response in vivo was shown by the ability of rhTSH administration to raise FFA serum levels in patients.

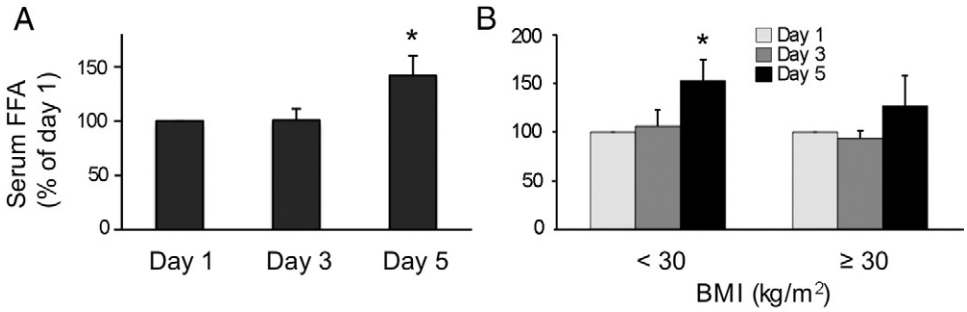

Fig. 4. Thyroid-stimulating hormone elevates serum FFAs in thyroidectomized patients. Patients received 2 injections of rhTSH, 24 hours apart, on day 1 and day 2. Serum samples were collected on days 1 (basal level before rhTSH administration), 3, and 5. A, Serum FFA levels; all patients (n = 19). B, Serum FFA levels; patients separated based on whether or not they were obese as defined by BMI (BMI <30 kg/m^2, n = 11; BMI ≥30 kg/m^2, n = 8). *$P < .05$ compared with day 1.

Extrathyroidal expression of functional TSH receptor occurs in adipocytes [6-10]. Considering this in conjunction with the elevated risk of cardiovascular disease in patients with subclinical hypothyroidism [13,14], we speculate that adipocytes may be "innocent bystanders" that are inappropriately activated by the elevated serum levels of TSH in these patients. We have previously reported that TSH-stimulated adipocytes increase production of the proinflammatory, proatherogenic cytokine interleukin-6 (IL-6) [17,20-22]. Elevation of serum IL-6 levels in response to rhTSH in thyroidectomized patients has also been noted [22,23].

We have extended our studies here to examine TSH-stimulated lipolysis because inappropriate elevation of FFA has also been associated with cardiovascular disease risk [1,2]. The notion that TSH can act on adipocytes to regulate lipolysis has been explored for several decades [3-5]. It has been suggested that this physiologic regulation occurs in the neonatal period associated with a surge in serum TSH levels [16]. A recent report found that persistently elevated serum TSH levels occurring in adults with subclinical hypothyroidism were also associated with higher serum FFA levels compared with euthyroid controls [15].

The concentration of TSH required to elicit the release of glycerol from adipocytes in our in vitro studies was in the same dose range as those used for thyrocytes in culture [24]. Our comparison studies indicated that the TSH-stimulated adipocyte responses were in the same range as those to 1 μmol/L isoproterenol. This suggests that TSH may act as a physiologic regulator of lipolysis.

Thyroid-stimulating hormone, as well as TSH receptor stimulating antibodies, has been reported to raise cAMP and stimulate lipolysis in human adipocytes; and it was assumed that cAMP was the responsible second messenger for the lipolytic response [11]. However, because inhibitor studies were not performed by those investigators, the evidence for their assumption was only correlative. In our studies, we used H89 to selectively inhibit the cAMP-responsive kinase PKA. Our results demonstrate that PKA is required for TSH-stimulated lipolysis for differentiated 3T3-L1 and human adipocytes.

The proximal signaling events leading to lipolysis induced by TSH have not been delineated previously. We observed that TSH clearly stimulated the phosphorylation of perilipin and HSL. Perilipin is a lipid droplet-associated coat protein whose phosphorylation on 6 serines (81, 222, 276, 433, 492, and 517) by PKA permits HSL to access the stored triacyglycerol [25,26]. The phosphorylation of HSL on serines 563 and 565, as well as serines 659 and 660, leads to its translocation from cytosol to the droplet [26,27]. Consistent with what we observed for lipolytic responses, H89 prevented the TSH-stimulated phosphorylation of perilipin and HSL. The inhibition was not complete, suggesting the possibility of roles for other kinases, such as extacellular signal-regulated kinase (ERK)1/2, which has been reported for β3-adrenergic receptor regulation of lipolysis [28]. We have also recently identified activation of another kinase, inhibitor of kappaB kinase β (IKKβ), as a required event for TSH-stimulated human differentiated adipocyte IL-6 production [17].

To determine if TSH could induce lipolysis in vivo, we obtained fasting serum FFA level measurements from patients, previously treated for thyroid cancer with surgical thyroidectomy and radioactive iodine ablation, after rhTSH administration. Thyroid-stimulating hormone raised serum FFA levels in the group overall, with those having a BMI less than 30 kg/m^2 accounting for the response. This observation is consistent with the report of elevated FFA levels in patients with subclinical hypothyroidism [15]. Their serum level of TSH was much lower than that achieved after rhTSH administration, suggesting that long-term exposure of adipocytes to low-dose TSH may be sufficient to induce lipolysis in vivo.

Our study has some limitations. Because inhibition of PKA was pharmacologic, complete specificity cannot be ensured; however, H89 continues to be used by leading adipocyte research groups as a useful tool to probe the role of PKA in adipocyte responses [28-30]. Our data from adipocytes in culture were derived from preadipocytes induced to differentiate in vitro. It will be useful to confirm our data on freshly isolated adipocytes, although the fact that rhTSH treatment in vivo raised fasting FFA levels is reassuring with respect to adipocyte responsiveness. Assessing cellular TSH receptor expression in the differentiated adipocytes and in isolated adipocytes would add to our understanding of TSH signaling in these cell models. Finally, with respect to our in vivo study, it is unclear why the rhTSH-induced rise in fasting FFA serum levels was only evident at day 5 and not at day 3. It is possible that our sampling times missed an earlier response between days 1 and 3. Thyroid-stimulating hormone receptor down-regulation has been reported to occur in adipocytes [31]. If this occurred in our subjects, the expected lower number of receptors could have delayed the FFA response until day 5, under the influence of the declining but persisting rhTSH in circulation at that time.

In summary, our data draw attention to TSH as a potentially important regulator of lipolysis. That, together with its actions to increase the production of proinflammatory, proatherogenic adipokines, should prompt more research into extrathyroidal TSH signaling as a potential mechanism underlying the association of subclinical hypothyroidism and cardiovascular disease.

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