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TSH signaling pathways that regulate MCP-1 in human differentiated adipocytes

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ABSTRACT

Objective. Adipose tissue is an extra-thyroidal thyroid-stimulating hormone (TSH) target. Increases in lipolysis and in expression and release of interleukin-6 (IL-6) occur in TSH-stimulated adipocytes, and levels of circulating free fatty acids and IL-6 rise following TSH administration to patients with previous thyroidectomy and radioablation for thyroid cancer. Our first objective was to compare how TSH stimulates protein kinase A (PKA) and inhibitor of κB (IκB) kinase (IKK)-β. Our second objective was to investigate whether TSH induces other cytokines besides IL-6.

Methods. TSH stimulation of either CHO cells expressing human TSH receptor or human abdominal subcutaneous differentiated adipocytes.

Results. Signaling studies showed TSH increased NADPH oxidase activity, and either diphenyleneiodonium (oxidase inhibitor) or *N*-acetyl cysteine (scavenger of reactive oxygen species) reduced IKKβ phosphorylation. Phosphorylation of protein kinase C-δ, an upstream regulator of NADPH oxidase, was increased by TSH, and rottlerin (PKCδ inhibitor) reduced TSH-stimulated IKKβ phosphorylation. TSH upregulated monocyte chemoattractant protein-1 (MCP-1) mRNA expression and the release of MCP-1 protein in human abdominal differentiated adipocytes. H89 (PKA inhibitor) and sc-514 (IKKβ inhibitor) each blocked TSH-stimulated MCP-1 mRNA expression and protein release, suggesting PKA and IKKβ participate in this pathway.

Conclusions. These data provide new information about TSH signaling in human differentiated adipocytes, and add to the evidence that TSH is a pro-inflammatory stimulus of adipocytes.

Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovarian; CREB, cAMP response element-binding protein; DMEM, Modified Eagle's Medium; DPI, diphenyleneiodonium; FBS, fetal bovine serum; FFA, free fatty acids; GPCR, G protein-coupled receptor; IκB, inhibitor of κB; IKK, inhibitor of IκB kinase; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; PKA, protein kinase A; PKC, protein kinase C; RBP4, retinol binding protein 4; rh, recombinant human; RT, reverse transcriptase; TSH, thyroid-stimulating hormone; TSHR, TSH receptor.

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1. Introduction

In thyrocytes, thyroid-stimulating hormone (TSH) binds to its receptor (TSHR), a G protein-coupled receptor (GPCR) that can communicate with several G proteins, including G_s and G_q. A variety of downstream signaling pathways are consequently activated, regulating thyrocyte growth and hormone production [1–4]. Extra-thyroidal expression of TSHR in adipocytes has been documented [5–8], but little is known about how TSH influences adipocyte signaling and cellular responses.

In vivo, TSH stimulates the release of pro-inflammatory molecules and free fatty acids (FFA) when administered acutely to patients previously treated for thyroid cancer by total thyroidectomy and radioablation [9–11]. Furthermore, serum levels of interleukin-6 (IL-6), tumor necrosis factor-α, C-reactive protein, and FFA are elevated in patients with subclinical hypothyroidism, a condition whereby chronically elevated TSH serum levels compensate for mild thyroid failure to maintain normal thyroid hormone levels [12–15]. These inflammatory and metabolic disturbances might explain the epidemiological association of accelerated cardiovascular disease with subclinical hypothyroidism [16,17].

Studies on adipocyte cell cultures reveal similar TSH-stimulated responses to the ones described above for *in vivo* studies. TSH stimulates release of FFA in human differentiated adipocytes and neonatal adipocytes in culture, and requires protein kinase A (PKA) as well as protein kinase C (PKC) [11,18,19]. TSH also dose-dependently stimulates IL-6 production and release in human abdominal differentiated adipocytes, and this depends on the inhibitor of κB kinase (IKK) β/nuclear factor (NF) κB pathway [9,20]. This pathway is also activated by TSH in orbital adipose cells and other cell types [21–24]. The downstream activation of IKKβ by other GPCR may occur via the activation of NADPH oxidase [25]. The generation of reactive oxygen species by NADPH oxidase results in the activation of IKKβ [26].

Our objectives were to investigate how TSHR activates the IKKβ pathway, and to evaluate whether adipokines other than IL-6 are regulated by TSH. We examined if there is a role for NADPH oxidase and PKC-δ upstream of TSH-stimulated IKKβ, and also whether monocyte chemoattractant protein-1 (MCP-1) is a TSH-induced adipokine.

2. Material and methods

2.1. Chinese hamster ovarian (CHO) cell culture

Parental CHO cells (CHO-JP02) and human TSHR overexpressing CHO cells (CHO-JP2626, or CHO-hTSHR) were kindly provided by J. E. Dumont, Erasme University Hospital, Free University of Brussels, Brussels, Belgium [27]. Cells were plated and grown in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin), and 400 μg/ml G418 (all from Life Technologies, Burlington, ON, Canada). Medium was changed every 2 days until confluence.

2.2. Isolation, culture, and differentiation of human abdominal subcutaneous stromal cells

Human abdominal subcutaneous adipose tissue was obtained from 22 patients (19 female, 3 male), with mean age 49 ± 9 years (±S.D.) and mean body mass index 27 ± 6 (±S.D.), undergoing elective abdominal surgery (approved by the Ottawa Hospital Research Ethics Board, protocol 1995023-01H). They were weight-stable, did not have diabetes, and were not on steroid therapy. The stromal preadipocytes were isolated as previously described [28]. Briefly, blood vessels and fibrous tissue were carefully removed, followed by collagenase digestion (CLS type I; 600 U/g of tissue; Worthington Biochemical Corporation, Lakewood, NJ, USA), and sequential centrifugation, size filtration, and treatment with erythrocyte lysis buffer. Preadipocytes were cultured in growth medium which was Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies) supplemented with 10% FBS and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 U/ml nystatin; EMD Millipore, Billerica, MA, USA). Upon reaching 80%–90% confluence, cells were either re-plated for a maximum of three passages or cryopreserved until needed. The presence of macrophages (CD14) or endothelial cells (CD31) is <2% in our culture conditions. For differentiation, preadipocytes were seeded at a density of 3×10^4 cells/cm^2 and allowed to adhere overnight. Adipogenesis was induced with growth medium supplemented with 0.85 μmol/L insulin, 0.25 mmol/L isobutylmethylxanthine, 100 μmol/L indomethacin, and 0.5 μmol/L dexamethasone for 14 days. Cells were then placed in growth medium for 2 days prior to stimulation studies.

2.3. Acute stimulation

CHO cells, CHO-hTSHR cells, or human differentiated adipocytes, in DMEM supplemented with 4% fatty acid-free bovine serum albumin (BSA) or 1% calf serum (Life Technologies), were stimulated with 50 mU/ml Calbiochem bovine TSH (C-bTSH; EMD Millipore), Sigma bovine TSH (S-bTSH; Sigma-Aldrich, Oakville, ON, Canada), an analog of recombinant human (rh) TSH (TR1401, a kind gift from MW Szkudlinski,Trophogen, Rockville, MD), or vehicle (H_2O) for 0 to 4 h, as shown. Where indicated, cells were pre-treated for 30 min with 10 μmol/L diphenyleneiodonium (DPI; Sigma-Aldrich), 10 μmol/L rottlerin (EMD Millipore), or vehicle (0.1% DMSO); for 1 h with 100 μmol/L sc-514, 20 μmol/L H-89 (both from EMD Millipore), or vehicle (0.1% DMSO); or for 2 h with 10 mmol/L N-acetylcysteine (NAC; Sigma-Aldrich) or vehicle (H_2O), prior to stimulation with TSH. Cells were then lysed and processed for immunoblot analysis, NADPH oxidase activity, or RNA isolation and qPCR. Conditioned medium was collected and assessed for cytokine content by ELISA.

2.4. Immunoblot analysis

Cells were lysed in Laemmli buffer [29] containing 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophostate, 5 mmol/L EGTA, and 1 mmol/L sodium orthovanadate. Protein was quantified by the modified Lowry assay (BioRad; Bio-Rad, Hercules, CA, USA), with BSA as standard. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose. Non-specific antigenic sites were blocked and