Interleukin-6 release from human abdominal adipose cells is regulated by thyroid-stimulating hormone: effect of adipocyte differentiation and anatomic depot

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Submitted 25 October 2005; accepted in final form 5 January 2006

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Interleukin-6 (IL-6) is a proinflammatory adipokine that is associated with an elevated risk of cardiovascular disease (CVD) in longitudinal prospective studies (6, 30, 32, 38). It may act directly at the vessel wall or indirectly by stimulating hepatic production of C-reactive protein (CRP), a novel CVD risk marker (25, 31). Because adipose tissue makes a significant contribution to the level of IL-6 in the circulation (27), it is important to identify the factors that influence IL-6 secretion from this tissue. We have demonstrated that TSH stimulates IL-6 release from 3T3-L1, 3T3-F442A, and human differentiated adipocytes in culture (5).

Elevated TSH secretion in subclinical hypothyroidism (mild thyroid gland failure) serves to maintain normal thyroid hormone levels, and, interestingly, this thyroid disorder has been associated with CVD (8, 13, 15, 19, 22, 33, 39). Traditional CVD risk factors do not clearly account for the association, but recently CRP was found to be higher in patients with subclinical hypothyroidism (7, 36). This clinical finding is consistent with our cellular studies linking TSH to IL-6 production in adipocytes, given that IL-6 is a major regulator of CRP production (25).

To broaden our knowledge of TSH action on IL-6 release from human adipose cells, we have investigated the effects of stage of adipocyte differentiation and anatomical site of adipose tissue depot on this process. Stromal preadipocytes and differentiated adipocytes derived from paired samples of abdominal subcutaneous and omental fat depots were studied. In addition, serum levels of IL-6 were measured in thyroidectomized patients treated with recombinant human (rh) TSH as a measure of extrathyroidal action of TSH in vivo.

MATERIALS AND METHODS

Isolation, culture, and differentiation of human stromal preadipocytes. Paired samples of abdominal subcutaneous and omental adipose tissues (~1–2 g wet wt) were obtained from five patients (4 female, 1 male) undergoing elective abdominal surgery (3 total abdominal hysterectomies, 1 bowel resection, and 1 gastrojejunostomy) with the approval of the Ottawa Hospital Research Ethics Committee (no. 1995023-01H). Their age was 51.4 (SD 7.3) yr, and body mass index (BMI) was 26.6 (SD 3.7) kg/m². The hospital record noted they were not acutely ill, were weight stable, and did not have diabetes, renal, or infectious disease. Two had well-controlled hypertension, one of whom was also known to have stable coronary artery disease, and were treated with candesartan, hydrochlorothiazide, losartan, verapamil, and simvastatin. Of the four women, two were postmeno-
pausal, one of whom was treated with estrogen (transdermal) and medroxyprogesterone. Stromal preadipocytes were isolated from each depot, and processed in parallel, as previously described (2). Blood vessels and fibrous tissue were carefully removed from the adipose tissue, followed by collagenase (CLS type I; 200 U/g of tissue) digestion. After centrifugation, size filtration, and treatment with erythrocyte lysis buffer, equal numbers of stromal preadipocytes from each depot for each patient were subsequently seeded (the number varied between patients, averaging $7 \times 10^5$ cells/cm$^2$) and grown in DMEM supplemented with 20% FBS and antibiotics. The following day, cells were either induced to differentiate in DMEM supplemented with 10% FBS, 0.85 mol/l insulin, 100 μmol/l indomethacin, 0.5 μmol/l dexamethasone, 0.25 mmol/l isobutyl methylxanthine, and antibiotics or maintained in control medium (DMEM with 10% FBS and antibiotics). After the 14-day differentiation period, both stromal preadipocytes and differentiated adipocytes from each of the two depots were placed in DMEM supplemented with 1% calf serum and treated with 1 μmol/l TSH (Sigma) or vehicle (water) for 4 h. At the end of each experiment, medium was collected and centrifuged (500 g for 5 min at 4°C) to eliminate cell debris. The supernatant was either kept at 4°C and analyzed in the following day or frozen at $-80°C$ until assayed for IL-6 protein.

Measurement of IL-6 protein in conditioned medium. IL-6 protein was measured by enzyme immunometric assay (Assay Designs, Ann Arbor, MI), according to the manufacturer’s instructions. After the collection of the medium, the adherent cells were lysed in Laemmli buffer (without bromphenol blue dye; see Ref. 24), and protein concentration was determined by modified Lowry method (Bio-Rad; Mississauga, ON, Canada), using BSA as a standard.

Measurement of IL-6 levels in human serum. The study subjects (approved by the Institutional Review Board of University of Pisa) included five men aged 43 (SD 2) yr who had undergone thyroidectomy and radioablative iodine therapy for thyroid cancer. They have been described previously (23). None had evidence of metastatic disease and were otherwise healthy. As part of ongoing routine follow-up care, and without discontinuation of thyroid hormone therapy, they received two separate doses of rhTSH (0.9 mg im) administered 24 h apart on days 0 and 1. Serum samples were obtained on days 0 (before the 1st dose), 1, 2, 3, and 4 and were then frozen. IL-6 and TSH were measured in thawed samples by an ELISA kit (Alpco Diagnostics, Windham, NH) and by chemiluminescence (Elecsys 2010; Roche Diagnostics, Indianapolis, IN), respectively.

Statistical analysis. Data were analyzed by ANOVA and the Newman-Keul’s test for multiple comparisons ($P < 0.05$ considered significant).

RESULTS

Basal IL-6 release was measured in medium obtained after 4 h of incubation with the cell cultures. To examine the effect of stage of differentiation and adipose tissue depot, we compared basal IL-6 secretion from stromal preadipocytes and differentiated adipocytes derived from either the abdominal subcutaneous or omental adipose tissue depots. As shown in Fig. 1, mean basal IL-6 release was 30-fold ($P < 0.01$) and 8-fold ($P < 0.001$) higher from stromal preadipocytes vs. differentiated adipocytes from the subcutaneous and omental depots, respectively. In the case of stromal preadipocytes, those from the omental depot released 1.6-fold more IL-6 ($P < 0.05$). Omental differentiated adipocytes also released more IL-6 than subcutaneous differentiated adipocytes, but this difference did not reach significance.

The response to treatment with 1 μmol/l TSH was assessed by comparing each condition with its respective control, given the very different basal IL-6 values (Fig. 1). TSH increased IL-6 release by differentiated subcutaneous adipocytes by 90% over basal (Fig. 2). This approximately twofold stimulation is consistent with our previous study on subcutaneous differentiated adipocytes (5). The responses of the preadipocytes from either depot, as well as the differentiated omental adipocytes, were ~35–40% and did not reach significance. The omental preadipocytes did not become more TSH responsive upon differentiation, in contrast to the differentiation of subcutaneous preadipocytes.

To address the question of whether TSH could act on extrathyroidal tissue targets in vivo and elicit an increase in serum levels of IL-6, we obtained serial blood samples for measurement of TSH and IL-6 from five male patients who had previously undergone surgical thyroidectomy followed by radioactive iodine ablation of any residual thyroid tissue. They received rhTSH injections in preparation for routine radioactive iodine body scans for thyroid cancer surveillance. rhTSH (0.9 mg) was administered on days 0 and 1; values for serum...
TSH and IL-6 levels on days 0–4 are shown (Fig. 3). The mean baseline TSH was 0.63 ± 0.05 (SE) mU/l (n = 5), and rose as expected in response to rhTSH administration. The mean baseline IL-6 value was 1.7 ± 0.9 (SE) pg/ml, with a range from undetectable to 4.6 pg/ml. IL-6 levels began to increase by day 1, and by day 4 the mean value reached 4.7 ± 0.8 pg/ml. This approximately threefold response of IL-6 over the 4 days just failed to reach significance (P < 0.05).

Samples from one of the five patients, although also showing a clear response to TSH, started from a much higher baseline IL-6 concentration for unknown reasons. Reanalysis of the data without this patient results in a baseline IL-6 of 0.9 ± 0.7 pg/ml, reaching a value of 4.4 ± 0.9 pg/ml on day 4, an approximately fivefold rise (P < 0.01).

To reduce the variation between all five subjects, the data were normalized by setting the IL-6 value of the final time point, day 4, at 100% and comparing the earlier time points as a percentage of the respective day 4 value for each patient. The rise in the serum IL-6 level was approximately threefold on days 3 and 4 (P < 0.05, n = 5). Taken together, the data indicate that rhTSH has raised serum IL-6 in these thyroidec- tomized patients by acting on extrathyroidal tissue(s).

DISCUSSION

We previously demonstrated that mouse (3T3-L1, 3T3-F442A) and human abdominal subcutaneous differentiated adipocytes secrete IL-6 in response to TSH (5). We now report on basal and TSH-stimulated IL-6 release in human adipose cells at different stages of differentiation (preadipocytes and differentiated adipocytes) and from different anatomical locations (subcutaneous abdominal and omental adipose tissue depots).

Our data indicating that basal IL-6 release is higher from stromal preadipocytes vs. differentiated adipocytes is consistent with several previous studies. The contribution of isolated adipocytes to IL-6 secretion was found to be only 10% compared with that of adipose tissue fragments (14). These authors therefore concluded that the stromal-vascular cellular fraction in intact adipose tissue must be an important IL-6 source. A subsequent study concurred, describing higher IL-6 secretion from stromal preadipocytes vs. isolated adipocytes from adipose tissue obtained from lean control or leptin-deficient obese ob/ob mice (17). Differentiation of human subcutaneous prea- dipocytes into adipocytes was also recently reported to mark- edly reduce IL-6 mRNA expression, although there was a return to preadipocyte levels with prolonged culture time (40).
However, the literature on IL-6 production from adipose tissue cellular fractions is somewhat controversial. In a comparison of human isolated adipocytes with adipose stromal cells, an equivalent amount of IL-6 secretion was observed; in addition, a vaguely defined undigested matrix component of adipose tissue was reported to be responsible for a large part of the IL-6 produced by adipose tissue (12). On the other hand, immunohistochemistry analysis of human breast adipose tissue did not detect any IL-6 reactivity in preadipocytes (29). The reason for this is unclear, but technical limitations are a possibility, since earlier work demonstrated IL-6 mRNA expression in stromal cells from breast adipose tissue, making it unlikely that breast preadipocytes are unique compared with other preadipocytes (9). Another study reported that IL-6 secretion increased during differentiation of human subcutaneous preadipocytes into adipocytes. However, the experimental design precluded a true assessment of IL-6 secretion from control preadipocytes, since their first measurement actually occurred after 6 days of differentiation treatment (37).

We found that stromal preadipocytes behaved in a depot-specific fashion, with omental cells releasing more IL-6 under basal conditions than subcutaneous cells. A parallel trend favoring the omental depot for the differentiated adipocytes was not significant. These responses correspond to those of isolated adipocytes and adipose tissue fragments, which also show this depot-related characteristic of IL-6 secretion (14). Another study also detected the same depot-specific effect of IL-6 secretion from adipose tissue samples; for isolated adipocytes, the trend was in the same direction, but not significant, perhaps because of differences in collection times (12). Furthermore, we provide novel data that stromal preadipocytes also exhibit this same depot-dependent response. The molecular basis for this depot-dependent difference in IL-6 production is not known. The previous two studies cited above used freshly isolated adipocytes, leaving open the possibility that in vivo influences before tissue removal might still have been operating during the relatively brief culture period for those cells. Because our data were derived from cells that were maintained for 2 wk in control or differentiation medium beyond the time for the initial cell passages, it would seem that the depot-related difference of IL-6 release is based on inherent attributes of the adipose cells themselves.

Adipogenesis resulted in the acquisition of IL-6 responsiveness to TSH for subcutaneous, but not omental, differentiated adipocytes. In previous work, we did not detect any consistent changes in TSH receptor (TSHR) protein expression upon human adipocyte differentiation or between depots that would account for this result; larger studies to more rigorously examine TSHR expression are warranted (4). However, we have observed that, in the 3T3-L1 preadipocyte cell line model, adipogenesis is associated with the ability of TSH to elevate intracellular cAMP levels (3). Future work will need to address whether downstream differences in TSH signaling molecules may be operative between depots and/or during human adipogenesis as well. It should be noted that, although the release of IL-6 by abdominal subcutaneous differentiated adipocytes is TSH responsive, the actual amount released under these culture conditions is smaller than that released basally by stromal preadipocytes. Whether this will be the case for other adipokines implicated in metabolic and cardiovascular dysfunction, such as tumor necrosis factor-α, is an open question. Finally, it should be noted that the number of patients in our study was small and somewhat heterogeneous (e.g., age, sex, medical history). With a larger and more homogeneous sample, more subtle regulatory aspects of IL-6 release may be revealed in the future.

To investigate the possibility of nonthyroidal action of TSH in vivo, we used the paradigm of rhTSH administration to patients who had been previously treated for thyroid cancer with surgical thyroidectomy and ablative doses of radioactive iodine. The elevation of serum IL-6 concentration in response to rhTSH injection complements and strengthens our experiments on cultured adipose cells reported here and previously (5). The design of the study, conducted in vivo, does not allow precise identification of adipose tissue as the site of nonthyroidal IL-6 production. To answer that question, adipose tissue biopsy and measurement of IL-6 expression before and after exposure to elevated TSH levels will be required.

Others have recently used rhTSH administration in thyroidectomized patients to explore extrathyroidal action of TSH. rhTSH treatment reduced serum vascular endothelial growth factor levels in such patients (35). In another study, short-term elevation of serum TSH levels did not elevate serum testosterone (23). These studies, together with our findings, suggest that extrathyroidal action of TSH and its regulation of pro-atherogenic adipokines will require careful investigation, since substantial tissue selectivity of TSH responsiveness appears to exist.

In summary, we show here for the first time that differentiated adipocytes derived from the abdominal subcutaneous, but not the omental, depot are TSH responsive in terms of IL-6 release. We further report that IL-6 release in the circulation occurs in thyroidectomized patients receiving rhTSH, indicating an extrathyroidal tissue(s) response to TSH.

ACKNOWLEDGMENTS

We thank the patients and surgeons of the Ottawa Hospital for valuable participation.

GRANTS

This work was supported, in part, by Grant T5307 from the Heart and Stroke Foundation of Ontario (to A. Sorisky) and National Institutes of Health Grants EY-008976, EY-11708, and DK-063121 (to T. J. Smith).

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