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TSH Induces Insulin Receptors That Mediate Insulin Costimulation of Growth in Normal Human Thyroid Cells

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The mitogenic/goitrogenic effects of thyrotropin (TSH) on human thyrocytes *in vitro* and *in vivo* depend on permissive comitogenic effects of insulin-like growth factors (IGFs), which are mimicked *in vitro* by the low-affinity binding of high supraphysiological concentrations of insulin to IGF-I receptors. Contrary to general assumption, we show here that very low concentrations of insulin, acting through insulin receptors but not IGF-I receptors, can also support the stimulation of DNA synthesis by TSH in primary cultures of normal human thyrocytes. Moreover, TSH through cAMP increases the content of insulin receptors demonstrated by Western blotting and the cells' responsiveness to low insulin concentrations. These observations provide the first *in vitro* evidence in normal human thyroid cells of a functional interaction between TSH and insulin acting through its own receptor.

Key Words: **human thyroid; goitrogenesis; TSH; insulin receptor; diabetes; cell proliferation; cyclic AMP.**

In various thyroid cell culture systems, including primary cultures of canine (1, 2) and human thyrocytes (3) and rat thyroid cell lines (4), the stimulation of proliferation by TSH depends on the supportive comitogenic effects of insulin-like growth factors (IGFs). Presumably the same paradigm applies to the human thyroid *in vivo* and therefore to goitrogenesis (5, 6). In culture, IGF-I effects are mimicked by supraphysiological micromolar concentrations of insulin, which are believed to act through their low-affinity binding to IGF-I receptors (4). Indeed, despite the similarity of their receptors and signal transduction pathways, insulin is mostly regarded as a regulator of glucose, protein and lipid metabolism, whereas IGFs mainly act as mitogenic hormones (7, 8). Nevertheless increased expression of insulin receptors has been described in cancers including breast (9) and thyroid carcinomas (10), and insulin has been found to signal a mitogenic pathway through its own receptors rather than IGF-I receptors in several cancer cell lines (8, 11, 12) and transfected cells that overexpress insulin receptors (13). Whether insulin can stimulate cell proliferation through its own receptor in normal cells containing normal levels of insulin and IGF-I receptors is far less clear. Using primary cultures of dog thyrocytes, we have found that insulin receptors are functionally equivalent to those of IGF-I in their capacity to transmit a comitogenic stimulus that permits the stimulation of proliferation by TSH, and even more intriguingly that the presence of insulin receptors strictly depends on TSH (14). Here we extend these unexpected observations to the physiologically relevant model of normal human thyrocytes in primary culture.

MATERIALS AND METHODS

Thyroid tissue was obtained from 5 patients aged 25–53 years undergoing surgery for a hypofunctioning thyroid nodule, following a protocol approved by the Ethics Committee of the Medical School. Only histologically normal perinodular tissue was used. In one more case, results were confirmed using thyroid tissue from a healthy donor deceased from a cerebral hemorrhage. The thyrocytes were cultured as described (3) from follicles released by enzymatic digestion of minced tissue. Cells seeded at a density of $\sim 2 \times 10^4$ cells/cm^2 were cultured in a mixture of DMEM/Ham's F12/MCDB104 medium (2:1:1 by vol) supplemented with 2.5 μg/ml human transferrin, 40 μg/ml ascorbic acid and antibiotics. One percent fetal calf serum was added for the first 24 h to ensure the spreading of the cells as a monolayer (3). Medium was renewed after 1 day and then every 2 days. Cells were stimulated at day 5 in the presence of 500 μg/ml bovine serum albumin (crystallized, Serva-Boehringer, Heidelberg, Germany).

DNA synthesis. Cells in 3-cm Petri dishes were stimulated for 48 h and bromodeoxyuridine (BrdU) was added for the last 24 h. The incorporation of BrdU was detected by immunofluorescence, and BrdU-labeled nuclei (1000/dish) were counted (2). The results are expressed as the mean and range of measurements on duplicate Petri dishes. The MA-10 blocking insulin receptor monoclonal antibody (12) was a kind gift of Drs. B. Maddux and I. Goldfine (San

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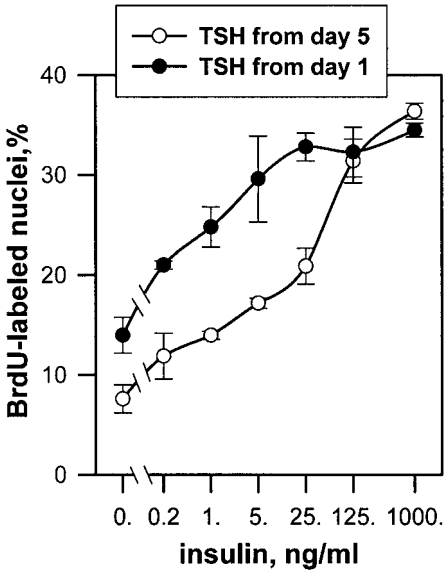

FIG. 1. Concentration–response curves of the comitogenic effect of insulin in the presence of TSH. Thyrocytes from a healthy donor were cultured for 5 days without insulin. They were then stimulated for 48 h with various concentrations of bovine insulin. TSH (0.3 mU/ml) was added at day 5 together with insulin, or was continuously present from day 1.

Francisco, CA), and the αIR3 blocking IGF-I receptor monoclonal antibody (15) was purchased from Calbiochem Corp. (San Diego, CA). Other reagents were as previously described (14).

Western blotting analysis of insulin receptor was performed as described (14) using 25 μg of total cellular proteins separated on SDS–7.5% polyacrylamide gels. Immunodetection was done by chemiluminescence using an anti-human insulin receptor β-subunit polyclonal antibody (Transduction Laboratories, Lexington, KY) and a HRP-conjugated anti-rabbit immunoglobulin.

RESULTS

In the present experimental conditions, human thyrocytes in primary culture express thyroid differentiation, including TSH-dependent iodide uptake (3), and thyroperoxidase and thyroglobulin gene expression (16, 17). In the present experiments, as previously shown (3), important stimulations of DNA synthesis required the synergy of both TSH and insulin or IGF-I. TSH alone had little or no growth promoting effect and insulin alone used at 5 μg/ml (8.3×10^{-7} M) had a low to moderate stimulatory effect which never exceeded half of the maximum response obtained with TSH + insulin.

When quiescent cells were stimulated with TSH and insulin administered together at day 5, the induction of DNA synthesis was mostly observed using high supraphysiological concentrations of insulin, which are assumed to act through low-affinity binding to IGF-I receptors (Fig. 1). Nevertheless a stimulation of DNA synthesis was also detected in the physiological ng/ml range of insulin concentrations. The pretreatment of cells from day 1 with TSH in the absence of insulin markedly increased the DNA synthesis responsiveness to low insulin concentrations added at day 5 in the presence of TSH (Fig. 1). Stimulations were detected with as low as 200 pg/ml (3.2×10^{-11} M) of insulin and maximum responses were reached at 25 ng/ml (4.1×10^{-9} M). Such low insulin concentrations were unlikely to act through IGF-I receptors. These results were representative of the situation observed in 4 primary cultures out of 6. In two other experiments, the comitogenic effects of insulin added together with TSH at day 5 were already close to maximum at low nanomolar concentrations even without the TSH pretreatment (not shown).

Well-characterized blocking antibodies to IGF-I and insulin receptors were added to culture medium in order to directly demonstrate the relative contributions of these receptors in the comitogenic effects of insulin in the presence of TSH (added at day 5) in cells pretreated or not with TSH from day 1 (Fig. 2). In TSH-pretreated cells, the comitogenic effects of a high insulin concentration (5 μg/ml, 8.3×10^{-7} M) were in large part mimicked by IGF-I or a low insulin concentration (10 ng/ml, 1.3×10^{-9} M) (Fig. 2A). The αIR3 IGF-I receptor antibody blocked most of the IGF-I effect but not the effect of the low insulin concentration. αIR3 also slightly lowered the effect of the high insulin concentration until the αIR3-resistant effect of the low insulin concentration (Figs. 2A and 2B). Conversely, the MA-10 insulin receptor blocking antibody completely inhibited the effect of the low insulin concentration, but it weakly affected the effects of IGF-I and the high insulin concentration. By contrast, in cells that were not pretreated with TSH, the effect of the high insulin concentration was resistant to MA-10, but it was in large part inhibited by the IGF-I receptor blocking antibody (Fig. 2B). These results demonstrate that the comitogenic effect of low insulin concentrations, which was mainly observed in TSH-pretreated cells, was mediated by insulin receptors but not by IGF-I receptors. The effect of high insulin concentrations can be mediated by either insulin or IGF-I receptors in TSH-pretreated cells, but mainly by IGF-I receptors in the absence of the TSH-pretreatment.

The presence of insulin receptors was examined by Western blotting in the same experiments in cells pretreated or not with TSH from day 1. Two different experiments are illustrated (Fig. 3A), corresponding either to primary cultures where the responsiveness to low insulin concentrations in the presence of TSH was markedly increased when TSH was administered as a pretreatment at day 1 (experiment 1, also illustrated in Fig. 1), or to the two primary cultures where the already high comitogenic sensitivity to low insulin in the presence of TSH added at day 5 was not further increased by the TSH-pretreatment from day 1 (experiment 2). A low basal presence of insulin receptors was detected (lane 5, exp. 1; lane 3, exp. 2). In all the

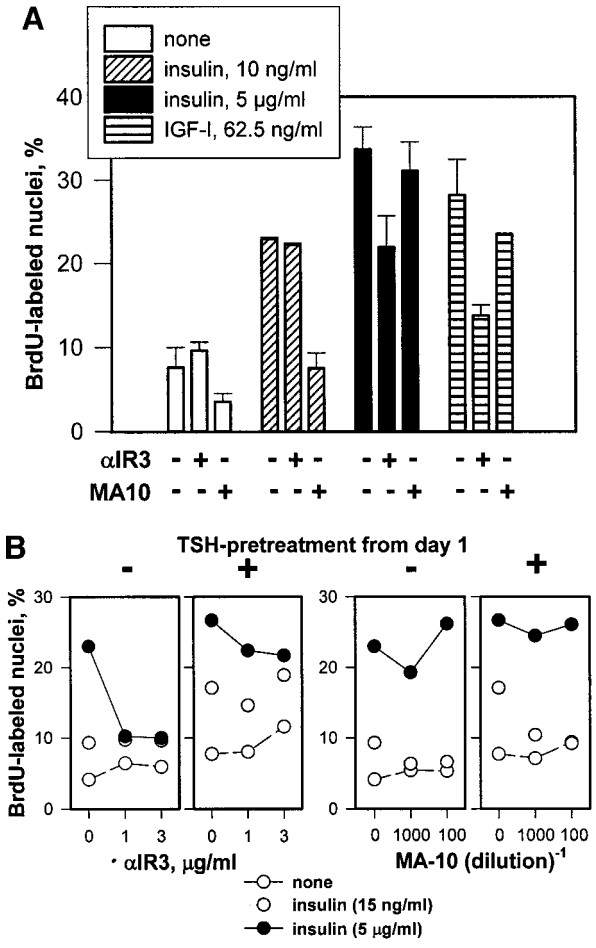

FIG. 2. Inhibition of the comitogenic effects of insulin and IGF-I by specifically blocking IGF-I or insulin receptors. Human thyrocytes were stimulated in the presence of TSH (0.3 mU/ml) at day 5 for 48 h by low or high concentrations of insulin, or IGF-I, as indicated, with or without αIR3 IGF-I receptor blocking antibody (2 µg/ml or as indicated) or MA10 insulin receptor blocking antibody (1/200 or as indicated). In A, cells were pretreated with TSH from day 1. In another experiment (B), DNA synthesis responses were compared in cells pretreated (+) or not (−) with TSH from day 1.

experiments the prolonged TSH treatment from day 1 till day 6 markedly increased the expression of insulin receptors (by a factor of 2.85 ± 1.25.(mean ± SD, n = 5), as analyzed by laser scanning densitometry) (Fig. 3B). In experiment 1, this effect was readily observed after a continuous stimulation by TSH present from day 1 (lane 4 vs lane 5), but hardly after a one-day treatment with TSH added at day 5 (lane 7 vs lane 5), which explained the increased comitogenic responsiveness to low insulin observed in TSH (day 1)-pretreated cells in this culture (Fig. 1). By contrast in experiment 2 (Fig. 3A), a marked induction of insulin receptor accumulation was already observed after the one-day stimulation with TSH added at day 5 (lanes 4 and 5 vs lane 3), which correlated with the observation that in this culture the high comitogenic responsiveness to low insulin in the presence of TSH did not necessitate the pretreatment with this hormone. There was thus a close correlation between the stimulations by TSH of the accumulation of insulin receptors and of the responsiveness to the comitogenic effects of low insulin concentrations. This correlation was reinforced by the comparison of different primary cultures.

The induction by TSH of insulin receptors was mimicked by the adenylyl cyclase activator forskolin (Fig. 3A), and thus mediated by cAMP. Insulin (5 µg/ml) inhibited the accumulation of insulin receptors, either basal (exp. 1, lanes 1 and 6 vs lane 5) or stimulated by TSH (exp. 2, lane 2 vs lane 4) (Fig. 3).

DISCUSSION

Insulin is not generally regarded in classical physiology as an important regulator of thyroid cell function and growth. Insulin-dependent diabetic patients frequently have clinical or subclinical hypothyroidism (18–21). Although often attributed to a multiple autoimmune syndrome, the hypothyroid state was reproduced in experimental diabetic animals, where it was corrected by insulin treatment (21–23). It is generally unclear, however, whether the primary defect involves the hypothalamus, pituitary, the thyroid gland, or peripheral tissues (21). In cultured human thyrocytes, there was some sparse reports of insulin effects in the presence of TSH on thymidine incorporation (24) and iodide uptake (25), but the relatively high concentrations used (10 nM range) did not allow any assertion as for the involvement of receptors of insulin or IGF-I.

In the present study, confirming our previous findings from dog thyroid primary cultures (14), we provide the first *in vitro* evidence in normal human thyroid cells of an important regulatory function of insulin through its own receptor, and of a functional interaction between TSH and insulin. Here we show that the presence of insulin receptor protein in human thyrocytes depends on a sustained stimulation by TSH acting through cAMP. The regulation of insulin receptor gene expression has been found to be especially important for directing insulin to specific tissues. Insulin receptors are expressed ubiquitously, but the classical insulin target tissues contain higher levels of insulin receptors leading to increased insulin sensitivity. Insulin receptors are induced *in vitro* and *in vivo* during muscular, pancreatic and adipose differentiation (26–28). In addition, in various tissues and cell types, insulin receptor expression is positively regulated by glucocorticoids (29) and, as shown here for human thyrocytes, repressed by insulin (28). In other cell types, hormones acting through cAMP have never been reported to stimulate insulin receptor expression or binding capacity (26). The induction of insulin receptors by TSH via cAMP, now observed in two mammalian species, thus appears to be thyroid-specific. As in

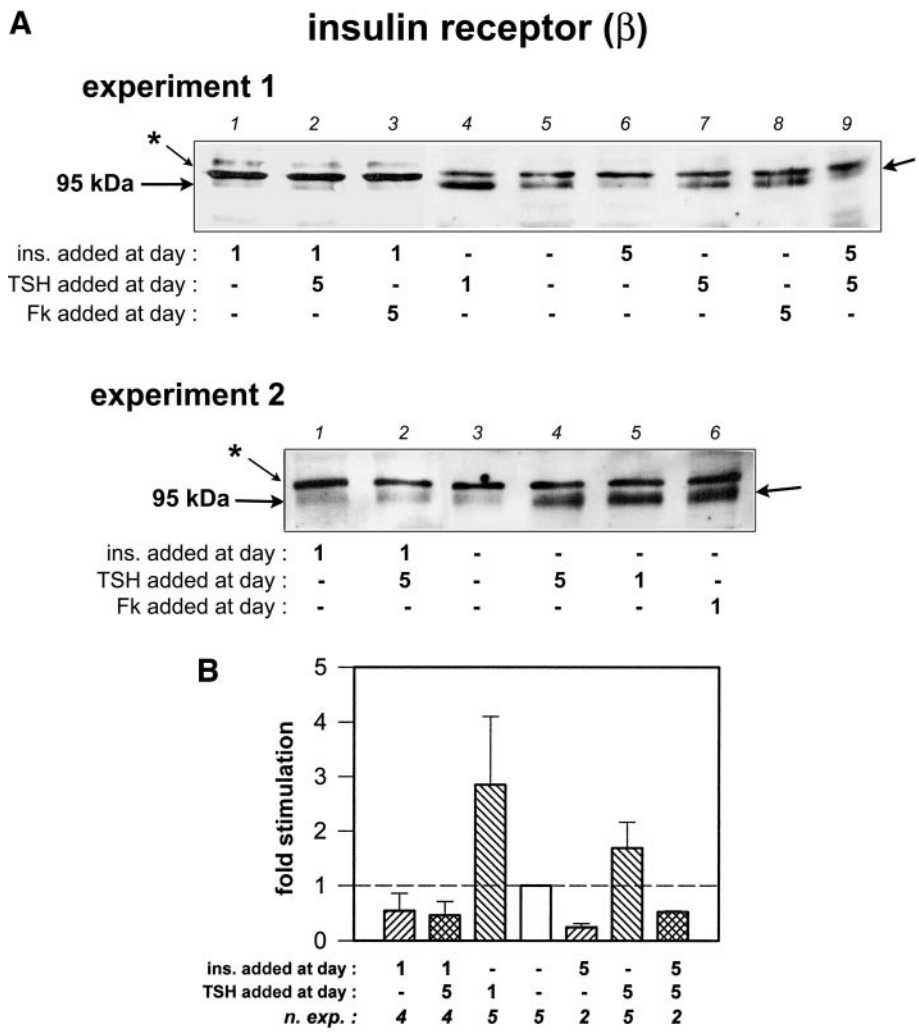

FIG. 3. Western blotting analysis of insulin receptor expression. Human thyrocytes were lysed at day 6 of the culture. TSH (0.3 mU/ml), insulin (5 μg/ml) or forskolin (10^{-5} M) were added at day 1 or 5 as indicated. Arrows indicate the 95-kDa β-subunit of insulin receptor. The asterisk indicates a 110-kDa band nonspecifically revealed by the insulin receptor polyclonal antibody. Two different experiments are shown (A). (B) Summary of TSH and insulin effects on insulin receptor accumulation as measured by laser scanning densitometry of Western blots including those shown in A. Results (mean + SD) are expressed as fold stimulations relative to control cells maintained without insulin and TSH. The number of separate experiments is indicated for each treatment. All the differences versus control cells were significant ($P < 0.05$ according to a one-sample t test with test value equal to 1).

classical insulin target tissues, it is tempting to relate it to the expression of differentiation, which in thyroid is supported by TSH.

The upregulation of insulin receptors by TSH is expected to have an impact on both general and specialized metabolisms of thyrocytes, including some differentiated functions such as thyroglobulin gene transcription which is regulated by both TSH and insulin/IGF-I (30). Here we observed in the presence of TSH a close correlation between the increased presence of insulin receptors and the increased responsiveness to comitogenic effects of very low insulin concentrations (3.2×10^{-11}–4.1×10^{-9} M). As shown by the specific effects of blocking antibodies, this action was mediated solely by insulin receptors but not by IGF-I receptors. This suggests that insulin receptors, once expressed at a sufficient level, can signal a (co)mitogenic cascade as efficiently as IGF-I receptors, as found in human breast carcinoma cells stimulated by progestins (12).

It should be stressed that the increase by TSH of the sensitivity to low concentrations of insulin cannot suffice to explain the growth promoting effects of TSH on human thyrocytes. Indeed it is unlikely to mimic the synergistic triggering of DNA synthesis by TSH and IGF-I or high concentrations of insulin that also act through IGF-I receptors. Nevertheless the present data might be related to the impairment of thyroid growth and function in response to TSH in diabetic rats and mice, which is restored upon insulin administration (22, 23). Our observations thus raise the in-

triguing possibility of TSH/insulin interactions *in vivo*, which could allow circulating insulin, beside locally produced IGF-I (31), to support the growth promoting action of TSH.

The overexpression of insulin receptors may also increase the responsiveness to IGFs. In thyroid cancers the overexpression of insulin receptors leads to an increased formation of insulin receptor/IGF-I receptor hybrids that are activated by IGF-I with high affinity (32). In dog thyrocytes (14) and human thyroid cancers (33), insulin receptors also bind IGF-II with an affinity that does not much differ from the affinity of IGF-II for the IGF-I receptor. They were thus proposed to mediate the autocrine effects of enhanced IGF-II production in thyroid cancers (33) as found in breast carcinoma cells (34). The present demonstration that insulin receptors can deliver a comitogenic stimulus in human thyroid cells *in vitro* supports the hypothesis that the increased presence of insulin receptors reported in thyroid carcinoma cells (10) may indeed give them a selective proliferation advantage.

To conclude, the present study sheds light on the possibility of important roles of insulin and its receptor in thyroid cell physiology and pathology, suggesting that thyroid might well be revealed as a more specific target of insulin than recognized until now.

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