Inhibitors of Specific Aminoacyl-tRNA Synthetases Prevent Thyrotropin-induced Desensitization in Cultured Human Thyroid Cells*

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The effect of specific competitive inhibitors of aminoacyl-tRNA synthetase on thyrotropin (TSH) desensitization in human thyroid cell monolayers was examined. Thyroid cells were preincubated with or without 50 milliunits/ml of TSH for 16 h at 37 °C in histidine-free medium supplemented with either 2 mM histidinol or 2 mM histidine (control medium). All cells were then exposed to TSH for 30 min in fresh, histidine-containing medium, and cellular cAMP levels were then measured. As expected, cells pre-exposed to TSH in control medium had a 55% lower cAMP response than cells preincubated in the same medium without TSH, indicating the development of TSH desensitization. In contrast, cells pre-exposed to TSH in histidinol-containing medium did not develop TSH desensitization. Addition of equimolar (2 mM) histidine to the preincubation medium containing histidinol plus TSH allowed the development of TSH desensitization. TSH stimulation performed in histidinol-containing medium revealed a normal acute cAMP response; however, cAMP levels did not subsequently decline as they did in the presence of histidine. Histidinol was effective in preventing TSH desensitization over a wide range of TSH concentrations (0.1 to 100 milliunits/ml). Once TSH desensitization was induced, histidinol in histidine-free medium was ineffective in reversing this desensitization. The efficacy of histidinol in inhibiting protein synthesis in cultured human thyroid cells was confirmed by determining incorporation into acid-insoluble material of a mixture of 3H-amino-acids. The ability of histidine to reverse protein synthesis inhibition was also demonstrated by the same method. O-Methylthreonine, a competitive inhibitor of isoleucine-tRNA synthetase, similarly prevented TSH desensitization, and this effect was reversed by the concomitant presence of an equimolar concentration of isoleucine. The reversal by histidine of histidinol-induced protein synthesis inhibition was necessary for approximately 90 min before TSH desensitization was produced. These data provide strong evidence for the involvement of protein synthesis in the development of TSH-induced desensitization in cultured human thyroid cells.

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Cells are capable of modulating the extent of their response to specific hormones. Thus, as a general phenomenon, continued hormone stimulation results in the development of partial desensitization. A variety of mechanisms have been implicated in this process (1-3) (also discussed in Ref. 4). These include a decrease in the number of hormone receptors ("down-regulation"), an increase in cyclic nucleotide phosphodiesterase activity, uncoupling of the hormone receptor from the adenylate cyclase complex regulatory protein, and the generation of soluble inhibitory factors.

As with other hormones and their respective target tissues, in vitro exposure of cultured thyroid cells and thyroid slices to TSH1 leads to desensitization to a subsequent TSH challenge (5-9). TSH desensitization in thyroid cells in monolayer culture is prevented by the concurrent presence of cycloheximide (4, 8). These data are consistent with evidence that desensitization to a variety of different hormones is prevented by antimitabolites that block protein synthesis (1), and suggest that the de novo synthesis of an as-yet unidentified protein may be necessary for the development of TSH desensitization.

Confirmation of the existence of an autoregulatory protein that alters hormone responsiveness would have important implications regarding the control of metabolic activity in cells, both benign and malignant. Unfortunately most agents used to inhibit protein synthesis are not specific in that they have known effects on other cellular functions. For example, cycloheximide also blocks sterol synthesis (10) and alters cellular GTP levels (11). Puromycin inhibits cyclic nucleotide phosphodiesterase activity (12). In order to help overcome these difficulties, we have, in the present study, re-examined the role of protein synthesis in TSH desensitization using a system in which protein synthesis in cultured cells is more specifically inhibited at a well defined site. Thus, certain amino acid analogues, in the absence of the natural amino acid, function as competitive inhibitors of aminoacyl-tRNA synthetase. In particular, we utilized L-histidinol (13-16) and L-O-methylthreonine (17) as antagonists for L-histidine and L-isoleucine, respectively. The specificity of this inhibition of protein synthesis is suggested by its reversal with an equimolar concentration of the natural amino acid despite the continued presence of the antagonist. Cellular ATP and GTP levels (14) as well as mRNA production (16) are also unaffected. The present study provides strong evidence that new protein synthesis is indeed necessary for the development of TSH desensitization.

MATERIALS AND METHODS

Cell Cultures—Human thyroid cells were dispersed with collagenase. The abbreviations used are: TSH, thyrotropin, Heps, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.
ase as previously reported (18). The tissue used was either para-

dular normal tissue or from glands affected with Graves’ disease.
The samples were pathological specimens removed surgically for

appropriate clinical reasons. The phenomenon of TSH desensitization

occurs in cells derived from each of these tissues, and the data
described below are representative of all tissues used. Cells were

resuspended after 3 to 6 days of primary culture and stored frozen

in liquid nitrogen as previously described (18). Cells were thawed, re-

plated the day preceding an experiment (approximately 5 x 10^6 cells/35-mm-diameter culture dish) in Dulbecco’s modified Eagle’s medium

containing 10% fetal calf serum, 2 mM glutamine, and nonessential

amino acids.

The experiments were initiated the following day by replacement

of the foregoing medium with fresh medium of similar composition
differing only in the absence of fetal calf serum and with 2 mM

histidinol or O-methylthreonine substituted for histidine and isoleu-

cine, respectively, as indicated in the text. Cells were first rinsed three
times with this medium in order to remove more effectively residual

histidine. When indicated, the medium contained both histidinol and

histidine, or O-methylthreonine and isoleucine. Incubations were

conducted in a water-saturated incubator at 37 °C in 5% CO2 in air.

When a short term (30 min) second incubation followed a long term

first incubation, this second incubation was conducted in L-15 me-
dium, containing 20 mM Hepes, 0.5 mM 3-isobutyl-1-methylxanthine,
pH 7.4, in room air at 37 °C in a water bath, as previously described
(6). L-15 medium was chosen because of the absence of bicarbonate,

thereby allowing incubation in room air. Frequent opening and closing

of the incubator door for short term incubations precludes maintain-

ing stable medium pH.

At the end of the incubation period(s), the medium was rapidly

removed and thyroid cell CAMP content was determined by radioim-

mununoassay (19) as previously described (6).

**Protein Synthesis**—Cells were incubated in medium containing

histidinol, histidine, or a combination of the two, as described above.

This medium was supplemented with a mixture of 3H-amino-acids (2 µCi per dish in 1 ml of medium). Incubations were ended by aspiration

of the medium and the addition of 1 ml of 10% trichloroacetic acid.

After three washes of the still adherent cells with trichloroacetic acid,

the cells were solubilized in 0.6 ml of 1 N NaOH and incorporated

radioactivity was measured by liquid scintillation counting in 10 ml

of Scintiverse (Fisher Scientific Co., Santa Clara, CA).

**Materials**—All culture media, including histidine or isoleucine-free

Dulbecco’s modified Eagle’s medium, were obtained from the Uni-

versity of California, San Francisco Cell Culture Facility. L-Histidinol,

L-O-methylthreonine, Hepes, and 3-isobutyl-1-methylxanthine were

obtained from Sigma. Bovine TSH (Thyrotropin) was purchased from

Armour. 1-amino acid mixture (2H; NET-250) was from New England

Nuclear.

**RESULTS**

In order to determine whether inhibition of protein synthesis

by the substitution in culture medium of histidinol for

histidine would prevent the development of TSH desensit-

ization, thyroid cell monolayers were preincubated for 16 h in

histidine-free medium containing 2 mM histidinol with or

without added TSH (50 milliunits/ml). The extent of the

cAMP response to TSH stimulation in a second, acute (30

min) incubation in full medium (containing histidine without

histidinol) was the same whether or not TSH was present

during the first incubation period (Fig. 1). In contrast, thyroid

cells preincubated in medium containing 2 mM histidine in-

stead of histidinol did develop TSH-induced desensitization

(Fig. 1).

In order to be certain that the substitution of histidinol for

histidine in the first incubation period in the preceding exper-

iment prevented TSH desensitization, it was necessary to

demonstrate that TSH action was unimpeded during this

period. The time course of the cAMP response to TSH stim-

ulation was therefore examined in the presence of either 2 mM

histidine, 2 mM histidinol, or histidinol plus histidine, as described under “Materials and Methods.” The media also contained 200 milliunits/ml of TSH and 0.5 mM 3-isobutyl-1-
methylxanthine. Incubations were ended at the indicated times and

cellular cAMP levels determined as described above. Each point

represents the mean ± S.D. of values obtained in triplicate dishes

of cells. The hatched bars represent cells preincubated in histidinol.

**FIG. 1.** Effect of histidinol inhibition of protein synthesis on

TSH desensitization. Human thyroid cell monolayers were incu-

bated for 16 h in medium containing either histidinol or histidine as

described under “Materials and Methods.” Where indicated, TSH

was also present in this medium. At the end of this first incubation

period, all cells were subjected to a 30-min incubation at 37 °C in L-

15 medium supplemented with TSH and 0.5 mM 3-isobutyl-1-methyl-

xanthine. At the end of this second incubation, cellular cAMP levels

were determined as described under “Materials and Methods.” Each

bar represents the mean ± S.D. of values obtained in triplicate dishes

of cells. The hatched bars represent cells preincubated in histidinol.

**FIG. 2.** Effect of histidinol inhibition of protein synthesis on

the time course of TSH stimulation of thyroid cell cAMP content.

Human thyroid cell monolayers were incubated in histidine-free

medium supplemented either with histidine, histidinol, or histidine

plus histidine, as described under “Materials and Methods.” The

media also contained 200 milliunits/ml of TSH and 0.5 mM 3-isobutyl-

1-methylxanthine. Incubations were ended at the indicated times and

cellular cAMP levels determined as described above. Each point

represents the mean ± S.D. of values obtained in triplicate dishes

of cells.
histidine in this medium prevented the development of TSH-induced desensitization (data not shown).

Once TSH desensitization is induced, removal of TSH is followed by very slow recovery from desensitization. Thus, in dog or human thyroid cell preparations, incubation for between 24 and 72 h in TSH-free medium is necessary before the desensitization process is reversed (4, 21). In addition, although the presence of cycloheximide prevents the development of TSH desensitization, cycloheximide does not reverse desensitization once it is already present (4). It was therefore of interest to examine the effect of histidinol-induced inhibition of protein synthesis on recovery from TSH desensitization. Cultured human thyroid cells were preincubated for 16 h in TSH (first incubation) in order to induce desensitization. The cells were then incubated for an additional 24 h (second incubation) in fresh medium without TSH, containing either histidinol or histidine. All cells were then challenged with TSH for a 30-min period (third incubation) and cellular cAMP levels were then determined. The presence of histidinol during the second (24 h) incubation period did not abolish TSH desensitization (Fig. 3). These data indicate that histidinol is without effect on desensitization once it has been induced.

Although previous data have indicated the efficacy of histidinol in inhibiting protein synthesis in cultured cells, it was important to confirm this effect in the thyroid cells under the conditions of the present study. Incubation of human thyroid cells in amino acid-free culture medium supplemented with a mixture of amino acids showed that new protein synthesis was greatly diminished (by approximately 90%) in the presence of histidinol or histidine. All cells were then challenged with TSH for a 30-min period (third incubation) and cellular cAMP levels were then determined. The presence of histidinol during the second (24 h) incubation period did not abolish TSH desensitization (Fig. 3). These data indicate that histidinol is without effect on desensitization once it has been induced.

In order to determine whether an inhibitor of a different aminoacyl-tRNA synthetase would similarly prevent TSH desensitization, human thyroid cell monolayers were preincubated for 16 h in isoleucine-free medium, supplemented with O-methylthreonine together with 50 milliunits/ml of TSH. Increasing concentrations (up to 40 mM) of O-methyl-threonine progressively decreased the extent of TSH desensitization (Fig. 5). The inclusion of an equimolar concentration of isoleucine to the O-methylthreonine restored the development of TSH desensitization.

One of the advantages of inhibiting protein synthesis with histidinol is that this blockade is rapidly reversible by the

![Fig. 4. Effect of histidinol on thyroid cell protein synthesis.](http://www.jbc.org/content/263/42/13442/F4)

**Fig. 4. Effect of histidinol on thyroid cell protein synthesis.** Human thyroid cell monolayers in 35-mm diameter culture dishes were rinsed in isoleucine-free medium and were then incubated for up to 4 h in the same medium supplemented with a mixture of 3H-amino acids. As indicated, the medium contained either histidinol or histidine. At the indicated time intervals, trichloroacetic acid-precipitable radioactivity was measured, as described under Materials and Methods. Each point represents the mean ± S.D. of values obtained in triplicate dishes of cells.

![Fig. 5. Prevention of TSH-induced desensitization with O-methylthreonine.](http://www.jbc.org/content/263/42/13442/F5)

**Fig. 5. Prevention of TSH-induced desensitization with O-methylthreonine.** Human thyroid cell monolayers were rinsed three times in Dulbecco’s modified Eagle’s medium deficient in isoleucine. The cells were then incubated for 16 h at 37 °C in the same medium supplemented with the indicated concentrations of O-methylthreonine, with or without 50 milliunits/ml of TSH. An additional group of cells was incubated in medium containing 30 mM isoleucine together with 30 mM O-methylthreonine. At the end of this first incubation period, media were removed and all cells were then subjected to a second 30-min incubation in L-15 medium containing 0.5 mM 3-isobutyl-1-methylxanthine and 50 milliunits/ml of TSH. As controls, the medium in two groups of cells was removed immediately after its addition, so that the second incubation was bypassed (second incubation of 0 h). These control groups of cells indicate the degree of TSH desensitization present at the end of the first incubation period. Cellular cAMP levels were then determined as described under Materials and Methods. Each bar represents the mean ± S.D. of values obtained in triplicate dishes of cells.
addition of histidine. It was therefore feasible to determine the period of time required for the development of TSH desensitization after reversal of pre-existing histidinol inhibition of protein synthesis. Human thyroid cell monolayers were rinsed three times with histidine-free Dulbecco’s modified Eagle’s medium as described under “Materials and Methods.” All groups of cells were then preincubated for 1 h in the same medium (1 ml) supplemented with 2 mM histidinol. At zero time in the first incubation, TSH (10 μl of the same medium) was added to the medium to achieve a final concentration of 50 milliunits/ml of TSH. At the same time, histidine was added in each culture dish to attain a final histidine concentration of 8 mM histidine, as well as 2 mM histidinol and 50 milliunits/ml of TSH, was added to the pre-existing 1 ml in each culture dish to attain a final histidine concentration of 4 mM. At the end of 6 h, the media were aspirated and cellular cAMP levels were determined as described under “Materials and Methods.” Each bar represents the mean ± S.D. of values obtained in triplicate dishes of cells. The values are expressed as a percentage of maximum stimulation observed in control dishes of cells subjected to a first incubation under the previously described conditions except for the absence of TSH in the medium. That is 100% of maximum stimulation represents the cAMP response in undesensitized thyroid cells.

**DISCUSSION**

The present data provide the strongest evidence to date that the development of TSH desensitization is related to de novo protein synthesis. The data also indicate the value of applying this system of protein synthesis inhibition to further studies on hormone desensitization in general. Inhibition of protein synthesis with histidinol is more specific (13–16) than with cycloheximide and puromycin (10–12). Further evidence of this specificity is the inability of histidinol to reverse TSH desensitization once it has developed. Thus, it is clear that histidinol is not masking TSH desensitization by a nonspecific stimulatory effect on thyroid cell cAMP generation. Use of a different, but functionally related, system for preventing TSH desensitization, namely O-methylthreonine, and the reversal of this effect with isoleucine, further support the concept that protein synthesis is indeed necessary for the development of TSH desensitization. It should be noted, however, that higher concentrations of O-methylthreonine than 1-histidinol are necessary to prevent TSH desensitization, making the latter agent more desirable in further studies on this phenomenon. The evidence finally suggests that at least 90 min of uninterrupted protein synthesis are necessary for the development of TSH desensitization.

Histidinol-induced inhibition of protein synthesis prevents the development of TSH desensitization over a wide range of TSH concentrations between 100 microunits/ml and 100 milliunits/ml. The former concentration is within the range seen in primary hypothyroidism, and therefore suggests that the phenomenon of TSH desensitization, and the role of de novo protein synthesis therein, is of pathophysiological importance.

Besides the additional evidence for the involvement of new protein synthesis in TSH desensitization shown in the present study, evidence also exists that ADP-ribosylation of as yet unknown proteins is involved in the development of TSH desensitization. Thus, nicotinamide and other inhibitors of ADP-ribosylation reactions prevent the development of TSH desensitization (21), and TSH induces ADP-ribosylation of thyroid cellular proteins independently of cAMP (22). These data raise questions as to the inter-relationship between new protein synthesis and ADP-ribosylation in the induction of TSH desensitization. Because of additivity between cycloheximide and nicotinamide in enhancing thyroid cell cAMP responsiveness to TSH (21), and since nicotinamide does not inhibit protein synthesis (23), it appears that new protein synthesis and ADP-ribosylation are separate, but linked, stages in a cascade of events that ultimately leads to TSH desensitization.

Many possible explanations exist for such an inter-relationship. One such hypothesis takes into account all of the above-mentioned effects, as well as the observation that, once induced, TSH desensitization may persist for days (4, 21), is present in isolated plasma membranes (4, 5, 8), and is only poorly reversible or irreversible by ADP-ribosylation inhibitors (21) and protein synthesis inhibitors (4), respectively. Thus, ADP-ribosylation may activate a labile, rapidly turning protein synthesized in an inactive form. This activated protein may then lead to the covalent modification of unknown components of the adenylate cyclase complex, thereby decreasing TSH stimulation. Inhibition of either ADP-ribosylation or synthesis of this putative protein would prevent accumulation of the active form at a concentration high enough to induce desensitization. The normal responsiveness of a TSH-desensitized thyroid particulate fraction to GTP and fluoride stimulation (4), and the TSH specificity of this desensitization (5, 8) suggests that this modification occurs at the TSH receptor. However, failure to observe an alteration in TSH receptor number in thyroid tissue desensitized to TSH in vitro (4, 5) suggests a possible alteration in the interaction of the TSH receptor and the regulatory protein (N or G/F protein) of the adenylate cyclase complex as shown recently for lutropin (24). Further studies are necessary to investigate these issues.
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REFERENCES
Inhibitors of specific aminoacyl-tRNA synthetases prevent thyrotropin-induced desensitization in cultured human thyroid cells.

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