Heat stress and hydrocortisone are independent stimulators of triiodothyronine-induced growth hormone production in cultured rat somatotrophic tumour cells

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Abstract. We have reported that, in cultured GC cells, the stress of incubation at 41°C enhances thyroid hormone stimulation of growth hormone (GH) in a manner similar to the effects observed in a model of nonthyroidal disease in rats. Since glucocorticoids are potentially involved in stress responses both in vivo and in cell culture, we studied the role of glucocorticoid in the enhancement of (which are rat somatotrophic tumor cells) triiodothyronine (T₃)-induced GH synthesis due to heat stress. Hydrocortisone addition increased T₃-induced GH synthesis and GH mRNA content in cultured GC cells at both 37°C and 41°C. Depletion of glucocorticoid endogenous to serum supplement of the tissue culture medium did not prevent the enhancement of T₃-induced GH synthesis that occurred during incubation at 41°C. The levels and affinity of glucocorticoid cytosolic receptors were not enhanced during incubation at 41°C. Lastly, no change in the sedimentation coefficient of the cytosolic glucocorticoid receptor or in its translocation into the nucleus occurred during incubation at 41°C. Thus, the enhancement of T₃-induced GH production in GC cells by heat stress appeared independent of the effect of glucocorticoids and not mediated through glucocorticoid receptors.

We have previously studied the effects of the Walker 256 carcinoma implanted in rats (tumour rats) as a model of the altered responses to thyroid hormones which occur in nonthyroidal diseases (1). We have found in tumour rats that specific hepatic responses to thyroid hormone can be either enhanced or diminished (2). Most recently, we have found an enhanced stimulation of T₃-induced GH and GH mRNA content in pituitaries of hypothyroid tumour rats (3). It was unclear whether these changes were a primary effect of the stress of nonthyroidal diseases on thyroid hormone responses. Since glucocorticoids are known to enhance T₃-induced GH synthesis in vivo (4), one possible explanation was that increased plasma glucocorticoid concentrations mediated the effects of nonthyroidal diseases on GH production.

CG cells are a biologically relevant model for thyroid hormone regulation of the GH gene (5). We have previously suggested that heat stress of cultured GC cells was a useful model for studies of the effects of nonthyroidal diseases on thyroid hormone action (6,7). This hypothesis was supported by our report that heat stress of cultured GC cells at 41°C resulted in the enhanced appearance of T₃-induced GH and GH mRNA in a manner similar to the effect of nonthyroidal diseases in rat pituitaries (3,7). We concluded that the effect of heat stress on T₃-induced GH synthesis might be part of a general stress response in GC cells. Heat and other stresses elicit similar stress responses in all living tissues including cultured cells (8). In addition, the 90 kD HS protein (HSP 90) is part of the cytoplasmic protein complex containing the inactive, unoccupied form of the cytosolic glucocorticoid receptor (9). We have now studied the interrelationship between glucocorticoids and the enhanced T₃-induced GH response in cultured GC cells incubated at 41°C.
Materials and Methods

Cell culture reagents including animal sera were purchased from Grand Island Biological Company (Grand Island, NY). Carrier-free sodium $[^{131}]$iodide, $[^{3}H]$leucine (60 Ci/μmol), $[^{3}H]$triamcinolone acetone (42.5 Ci/μmol) were from New England Nuclear Corporation (Boston, MA). $[^{3}P]$dCTP (3000 Ci/μmol) was from Amersham Corporation (Arlington Heights, IL). A 27-base oligonucleotide probe for rat α-tubulin was purchased from Clontech Lab Inc, Palo Alto, CA. Cell culture flasks were from Falcon Plastics, Oxnard, CA. Immunoassay for rat GH was performed using reagents and methods provided by the National Hormone and Pituitary Program, Baltimore, MD. Scintillation counting was performed in a Packard TriCarb spectrometer and a Packard Autogamma spectrometer both from United Technologies (Sterling, VA). Opti-Fluor scintillation fluid was from Packard Instrument Company Incorporated (Downers Grove, IL). Other reagents were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Company (Pittsburgh, PA).

Cell Culture

Stock cultures of GC cells were maintained as previously described (10) using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% normal animal sera (calf:horse = 2:1). Prior to each experiment, medium was changed to DMEM-containing resin-treated calf (RT) serum processed to deplete endogenous thyroid hormones (11). In some experiments medium was supplemented with RT serum additionally treated with powdered charcoal (Malbrinckrodt Chemical Works, St. Louis, MO) to diminish endogenous levels of glucocorticoids (12). Experimental flasks were routinely incubated in RT medium for 3 days prior to experiments and hydrocortisone, when employed, was present for 2 days prior to addition of $T_3$. Incubations at 37°C and 41°C were carried out in separate water jacketed incubators (NAPCO, Portland, OR) in a humidified atmosphere of 95% air and 5% CO$_2$.

Hormone-induced GH production at 37°C and 41°C

Experiments were performed using medium supplemented with calf serum treated to remove thyroid hormone (11) and in some experiments, glucocorticoid (12). Replicate 6-cm diameter dishes were plated with 125 000 GC cells and subsequently cultured in RT medium for 2 days. Medium was then exchanged with fresh hormone-depleted medium to which 100 nmol/l hydrocortisone could be added. After 2 days incubation in the presence or absence of glucocorticoid, media were exchanged with fresh medium to which 5 nmol/l $T_3$ could be added. In some experiments 0.5 μCi $[^{3}H]$leucine was added to estimate the level of amino acid incorporation occurring in each experimental condition (7). Preliminary studies determined that cellular content of trichloroacetic acid (TCA) soluble $[^{3}H]$leucine/cell protein was unaffected by the hormonal manipulations used. This suggested that $[^{3}H]$leucine uptake was not rate-limiting in the cellular accumulation of TCA precipitable cpm. After 18 h at 37°C or 41°C, media were collected for analysis of GH by RIA and the GC cells were analysed for $[^{3}H]$leucine incorporation (7), protein content (13), cell number (10), DNA content (14), GHmRNA (7), and α-tubulin mRNA (15).

Hormone-induced GH and GH mRNA production during incubation at 37°C and 41°C

The amount of GH secreted into media and the content of rat GH mRNA and rat α-tubulin mRNA was determined in GC cell lysates exactly as previously described (7,15). Media GH by RIA was assayed in multiple dilutions in duplicate tubes. The results in each of duplicate determinations varied by less than 5%. The intra-assay variation was <7% and the inter-assay variation was <5%. GH mRNA and α-tubulin mRNA determinations were by dot-blot analysis (7,15) of duplicate dots for each of 3 dilutions of each cell lysate. Triplicate culture dishes per experimental point were used.

Determination of $[^{3}H]$triamcinolone acetone binding to GC cells incubated at 37°C and 41°C.

Methods of studying glucocorticoid binding were adapted from those used by Raaka & Samuels in GH1 cells (16). To determine the effect of incubation at 41°C on cytosol glucocorticoid receptor, ten 175-cm$^2$ tissue culture flasks were plated, each with 10$^6$ GC cells. On the following day, growth medium was exchanged for RT medium to deplete endogenous thyroid hormones. On the next day, medium was exchanged for RT medium supplemented with 5 nmol/l $T_3$ and 5 flasks were incubated at either 37°C or 41°C. After 18 h of incubation, the cells were harvested by trypsin-EDTA solution, chilled to 4°C, and collected by centrifugation. The cells were resuspended in ice-cold 0.15 mol/l NaCl and centrifuged twice. Each cell pellet from 5 flasks was suspended in 13 ml of lysis buffer (20 μmol/l TRIS, 5 μmol/l sodium molybdate, 0.02% Triton, 2 μmol/l dithiothreitol). After vigorous vortex mixing the lysate was centrifuged (1000 × g, 30 min). The supernatant was considered "cytosol" and the nuclear pellet was saved for DNA determination. Scatchard analysis (17) of cytosol glucocorticoid receptor binding was carried out using 0.3 ml of cytosol in a total reaction volume of 0.4 ml. Each tube contained 0.2 nmol/l of $[^{3}H]$triamcinolone acetone (approximately 6000 cpm). Total triamcinolone acetone concentration ranged from 0.2 nmol/l to 100 nmol/l. After 18 h of incubation in an ice bath, bound and free triamcinolone acetone was separated by protamine sulphate precipitation (16).

To determine the effect of incubation at 41°C on the sedimentation coefficient of the glucocorticoid receptor, replicate 75-cm$^2$ flasks were plated with 375 000 GC cells and, the following day, incubated in RT medium. After 2
days incubation in RT medium, the cells were incubated with 5 nmol/l T₃ at 37°C and 41°C. After 18 h, flasks were chilled to 4°C and incubated in serum-free DMEM containing 10 nmol/l [³H]triamcinolone acetonide in the presence or absence of 10 μmol/l triamcinolone acetonide. After 5 h, media were removed and cells were washed with 0.15 mol/l NaCl (4°C) 2 times and then 0.25 ml of lysis buffer was added. The nuclear fraction was separated by centrifugation and cytosol was sampled for radioactivity, protein, and sucrose density gradient ultracentrifugation (16). The nuclear pellet was washed 2 times with lysis buffer and then assayed for DNA content and radioactivity. In these experiments specific glucocorticoid binding was calculated from the radioactivity in each fraction of cells incubated with 10 nmol/l [³H]triamcinolone acetonide minus the corresponding radioactivity derived from incubations containing 10 μmol/l triamcinolone acetonide.

Statistics
Each experiment was performed at least 2 times with similar results. The data are expressed as the mean ±so of triplicate or quadruplicate experimental replicates. The significance of differences between mean values was determined by Student's t-test or analysis of variance (18).

Results

The effects of T₃, hydrocortisone, and incubation at 41°C on GH production in GC cells
Incubation of GC cells at 41°C might have enhanced T₃-induced GH production (7) by the same mechanism that glucocorticoids affected the T₃ response during incubations at 37°C (19). If this were so, the maximum effect of glucocorticoid on T₃-induced GH production during incubation at 37°C would not be further increased by incubation at 41°C. To study this, we determined the effect of 100 nmol/l hydrocortisone on GH production induced by 5 nmol/l T₃ in GC cells incubated at either 37°C or 41°C. These hormone concentrations have been reported to elicit maximum GH responses in cultured GH-producing cells (19). Table 1 shows a representative of three similar experiments. Results are expressed as total medium GH content/per 10⁶ cells. As reported previously for GH₁ cells (19), during incubation of GC cells at 37°C hydrocortisone had little effect alone but enhanced T₃-induced GH production. The enhanced T₃-induced GH production due to incubation at 37°C in the presence of hydrocortisone was further increased by incubation at 41°C. Intracellular GH was in all cases less than 12% of the total (cellular plus medium GH) so that GH production was the result of de novo synthesis and could not have been due to leakage from damaged cells.

The results of GC cell GH mRNA analysis in this experiment is shown in Fig. 1. In incubations without added T₃, there was a small but significant enhancement of cellular GH mRNA levels due to incubation at 41°C. In dishes containing added T₃, incubation at 41°C resulted in a cellular GH mRNA content which was 1.6-fold (5 nmol/l T₃) and 2.0-fold (5 nmol/l T₃ + 100 nmol/l hydrocortisone) the levels present in 37°C incubations. The analysis of GC cell α-tubulin mRNA showed no significant (p>0.05) effect due to incubation at 41°C under

<table>
<thead>
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<th>Hormone(s) added</th>
<th>GH produced ng/10⁶ cells</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td>41°C</td>
<td>P</td>
</tr>
<tr>
<td>No addition</td>
<td>561±85.2</td>
<td>818±176</td>
<td>NS</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>884±36</td>
<td>933±30</td>
<td>NS</td>
</tr>
<tr>
<td>T₃</td>
<td>2035±299</td>
<td>4764±196</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>T₃ + hydrocortisone</td>
<td>8849±1125</td>
<td>13955±2132</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 1:
Effect of incubation at 41°C on hormone-induced GH production.

Triplicate dishes of GC cells were equilibrated with thyroid hormone-depleted medium prior to 48 h incubation with or without 100 nmol/l hydrocortisone. At this time, 5 nmol/l T₃ was added for an 18-h incubation at either 37°C or 41°C. Media were assayed for GH by RIA and expressed as the mean ± so of GH secreted during each incubation. Intracellular GH was 9-12% of total GH in dishes without added T₃ and 3-8% of total GH in dishes to which 5 nmol/l T₃ was added. Mean of cell numbers per flask for each hormonal condition was decreased during 41°C incubation by 0 to 20% compared to 37°C control incubations. "p" is the probability that the effect of different incubation temperatures occurred by chance.
any hormonal condition (not shown). Thus, the effects on GHmRNA were specific and were not due to variation in mRNA yields.

These results indicated that both hydrocortisone and incubation at 41°C enhanced T₃-induced GH production at the mRNA level. However, the effect of a maximal hydrocortisone dose is further enhanced by incubation at 41°C suggesting that heat stress acts through a mechanism distinct from that of glucocorticoid action.

Effect of glucocorticoid depletion on T₃-induced GH production

It was possible that the heat stress effect on T₃-induced GH production was due to an enhanced GC cell response to glucocorticoid contained in the medium calf serum supplement. For this reason, an experiment was carried out using media depleted of glucocorticoid. Replicate dishes of GC cells were first equilibrated in thyroid hormone-deficient medium. Two days before the experiment, medium was exchanged either with fresh RT medium or with RT medium additionally treated to deplete glucocorticoids. Groups of 3 dishes were incubated at 37°C or 41°C in the presence of 5 nmol/l T₃. In order to estimate nonspecific effects of the experimental conditions on total protein synthesis, all experimental media contained 0.5 μCi per ml of [³H]leucine. After 18 h of incubation, media were assayed for GH content and the cells were assayed for [³H]leucine protein. The results of the experiment are shown in Table 2. The effect

Table 2.
Effect of glucocorticoid depletion on T₃-induced GH production.

|                      | 37°C       | 41°C       | p<  
|----------------------|------------|------------|---
| Control              |            |            |   
| GH (ng/µg protein)   | 4.69±0.42  | 8.83±1.3   | 0.005  
| cpm/µg protein       | 93.1±3.90  | 123±17.5   | 0.05   
| Protein µg/dish      | 322±15.7   | 217±21.7   | 0.005  
| Glucocorticoid-depleted|          |            |   
| GH (ng/µg protein)   | 8.25±0.67  | 13.7±0.14  | 0.001  
| cpm/µg protein       | 70.2±1.87  | 56.4±2.40  | 0.005  
| Protein µg/dish      | 89.05±5.18 | 79.6±2.05  | 0.05   

Replicate dishes of GC cells were cultured in either thyroid hormone-depleted (Control) or thyroid and glucocorticoid hormone-depleted media. 5 nmol/l T₃ and 0.5 μCi/ml [³H]leucine were added to all dishes and incubation was carried out for 18 h at 37°C and 41°C. "cpm" indicates [³H]leucine incorporation into cellular protein during incubation. "p" indicates the probability that the difference in results of incubations at 37°C and 41°C occurred by chance. Data are the mean ± sd for triplicate dishes per point.
of charcoal treatment of serum was to diminish significantly cellular protein and $[^3]$Hleucine content as well as medium GH. However, a significant and specific increase in $T_3$-induced GH production after incubation at 41°C occurred even when medium was depleted of glucocorticoid. Thus, the augmentation of $T_3$-induced GH production during incubation at 41°C did not require the presence of glucocorticoid.

The effect of incubation at 41°C on glucocorticoid receptor
To determine whether incubation at 41°C altered glucocorticoid binding, GC cells were first equilibrated in thyroid hormone-deficient conditions. $T_3$ (5nmol/l) was then added for 18 h incubation at 37°C and 41°C. Cells at each temperature were pooled for the determination of triamcinolone acetone binding in cytosol. Fig. 2 illustrates a representative experiment suggesting similar binding affinity in cells cultured at 37 and 41°C. There was a decrease in $[^3]$Htriamcinolone acetone binding capacity in lysates from cells incubated at 41°C (155 fmol/l 100 µg DNA) compared with cells incubated at 37°C (174 fmol/100 µg DNA). This small decrease was seen in experiments in which cytosol binding was measured in vitro, but not in experiments in which binding was determined in intact cells (see below).

$$\text{Fig. 2.}$$

Effect of incubation at 41°C on $[^3]$Htriamcinolone acetone binding to GC cell cytosol. GC cells were equilibrated with thyroid hormone-depleted medium prior to 18 h incubation with 5 nmol/l $T_3$ at 37°C (○) or 41°C (□). Cytosol was prepared for in vitro determination of triamcinolone acetone binding. Each point is the mean of triplicate determinations.

It was possible that incubation at 41°C shifted the distribution of glucocorticoid receptor in cytosol from the inactive (10S) form to the activated (4S) form and thus enhanced the translocation of receptor into the nucleus. To test this hypothesis, GC cells were equilibrated in thyroid hormone-depleted conditions prior to incubation with 5 nmol/l $T_3$ at 37°C and 41°C. After 18 h incubation at 41°C or 37°C, the flasks were chilled on an ice tray and $[^3]$Htriamcinolone acetone binding was determined in intact GC cell monolayers in a cold room (4°C). $[^3]$Htriamcinolone acetone binding at 4°C prevents activation of the cytosolic GC receptor (16) and thus reflects the cellular distribution of the glucocorticoid receptor which occurred during the previous experimental incubation. After 5 h of incubation, nuclear and cytosol fractions were prepared. Estimates of binding capacity for $[^3]$Htriamcinolone acetone were made in aliquots from each fraction. An additional aliquot of cytosol from three flasks incubated at each temperature was then subjected to sucrose density gradient analysis to determine the size distribution of cytosol receptor. There was no significant difference in receptor numbers or in the distribution of specifically bound cytosol or nuclear triamcinolone acetone (Table 3). Fig. 3 shows representative sucrose density gradient analyses demonstrating that the cytosol glucocorticoid receptor was apparently only of the high molecular weight (inactive form) after incubation at either 41°C or 37°C. Thus, in-

<table>
<thead>
<tr>
<th>Temperature of incubation</th>
<th>Triamcinolone acetone binding (fmol/100 µg DNA)</th>
<th>Nuclear binding % total cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Nucleus</td>
</tr>
<tr>
<td>37°C</td>
<td>129±36</td>
<td>31.9±7.8</td>
</tr>
<tr>
<td>47°C</td>
<td>133±26</td>
<td>30.0±2.8</td>
</tr>
</tbody>
</table>

Replicate dishes of GC cells were equilibrated in thyroid hormone-depleted conditions before 18 h incubation with 5 nmol/l $T_3$ at 37°C and 41°C. After cooling to 4°C incubation of intact cells with 10 nmol/l $[^3]$Htriamcinolone acetone was performed. Specific glucocorticoid binding in cytosol and nuclear fractions were determined and expressed as fmol per 100 µg DNA. Data are the mean ± s.d for triplicate dishes per point.
cubation at 41°C did not increase glucocorticoid receptor levels, activation, or nuclear translocation.

Discussion

Glucocorticoids are stress-related hormones and have been shown to enhance T₃-induced GH synthesis both in cell culture and in vivo. The purpose of this study was to determine the role of glucocorticoids in the enhancement of T₃-induced GH production in GC cells incubated at 41°C. Our results indicate that the effect of incubation at 41°C is not mediated through a cellular glucocorticoid response; it is neither due to glucocorticoid contained in the medium serum supplement nor to an enhancement of the glucocorticoid receptor function.

It was previously reported that the induction of GH synthesis by T₃ in somatotropic GH₁ cells cultured at 37°C was markedly enhanced by glucocorticoid (19). Similarly, glucocorticoid was reported to enhance the degree by which GH deficiency was reversed by T₃ in hypothyroid rats (4). We determined that this hormonal relationship existed in GC cells incubated either at 37°C or 41°C by measuring the GH response to maximally effective doses of T₃ and hydrocortisone at each temperature. Since the maximally stimulated response to glucocorticoid in cells cultured at 37°C was further enhanced by incubation at 41°C, it was suggested that the effect of heat stress occurs through a mechanism distinct from the glucocorticoid response pathway.

While it was likely that the effects of glucocorticoid and heat stress were, mechanistically, unique at maximum levels of hydrocortisone, it was possible that the results of incubation at 41°C were due to enhanced responses to lower levels of glucocorticoids present in tissue culture media. Calf serum contains approximately 20 nmol/l of cortisol and the method used to deplete thyroid hormones has been estimated to remove 30% of endogenous cortisol (11). It was possible that incubation at 41°C resulted in enhanced receptor association of the glucocorticoid present in media. To test this hypothesis, RT serum was treated with charcoal in a manner previously shown to lower endogenous cortisol level 15-20 fold (12), to a level too low to have a measurable effect on GH production (19). We found that incubation at 41°C resulted in an enhanced T₃-induced GH response in glucocorticoid-depleted medium, indicating that the effect was not mediated through glucocorticoid contained in the culture media.

It was possible that incubation at 41°C affected glucocorticoid receptor function in a manner which resulted in a cellular glucocorticoid response. The cellular response to glucocorticoid is initiated by steroid binding to its unoccupied cytosol receptor which is part of a 10S protein complex also containing HSP 90 (9). Steroid binding results in a conformational change in which the 4S receptor dissociates from HSP 90 and becomes a specific DNA binding protein and a ligand-dependent transcription factor for glucocorticoid responsive genes (20). Since activation of the glucocorticoid

Fig. 3.
Effect of incubation at 41°C on sedimentation velocity of glucocorticoid receptor in GC cell cytosol. Cytosol from GC cells incubated with 10 nmol/l [³H]triamcinolone acetonide at 37°C (●) or 41°C (○) was analysed by discontinuous sucrose gradient centrifugation. Arrows indicate migration of BSA (3.5 S) and activated glucocorticoid receptor (4.0 S).
receptor in vitro can be induced by increased temperature, it was possible that the GC cell glucocorticoid receptor might be activated by incubation at 41°C, even in the absence of added glucocorticoid. To test if this occurred, we determined the effect of incubation at 41°C on glucocorticoid receptor binding, cellular distribution, and sucrose density sedimentation. We found no evidence to support this hypothesis.

The response to heat stress is a laboratory prototype of the universal response of cells to many forms of environmental stress. The induction of HS proteins has been correlated with the acquisition of an increased tolerance to the inducing stress (21) and is believed to play a protective role in nature. Since the induction of HS proteins occurs in animal models of diseases (22-24), the HS response possibly determines the altered tissue responses to thyroid hormone found in animals with nonthyroidal diseases.

Incubation at 41°C is stressful to GC cells and results in cell growth arrest after 24 h and decreased cell viability (7). However, incubation at 41°C enhances the synthesis of HSP 70 and HSP 90 and T3-induced GH production (7). Since T3-induced GH synthesis is similarly affected by the stress of tumour implantation in rats, it is possible that incubation of GC cells at 41°C creates a cellular stress similar to the stress in vivo caused by nonthyroidal diseases. Thus, this system represents a unique model in which to investigate the mechanisms by which stress distorts cellular responses to thyroid hormone in the tissues of animals with nonthyroidal disease. We plan studies to determine whether the effect of incubation at 41°C is due to enhanced GH gene transcription or to GH mRNA stabilization.

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References


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