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Somatotropin Antagonism of Insulin-Stimulated Glucose Utilization in 3T3-L1 Adipocytes

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It is well established that somatotropin (GH) antagonizes insulin action in vivo and that supraphysiologic concentrations of GH frequently result in insulin resistance and glucose intolerance. However, the demonstration of an anti-insulin activity by GH in vitro has been difficult. This study, therefore, set out to determine whether cultures of 3T3-L1 adipocytes could be used to examine the anti-insulin activity of GH. The ability of insulin to stimulate glucose utilization by 3T3-L1 adipocytes increases approximately five-fold during the first 4 days following treatment of the cells with a differentiation medium. It was found that glucose utilization in 3T3-L1 adipocytes is regulated in a reciprocal fashion by insulin and GH. Bovine or human GH directly inhibit up to 50% of insulin-stimulated [^{14}C]-glucose incorporation into lipids in a concentration-dependent manner. The 3T3-L1 sensitivity to GH appears to be at the maximum (50% inhibition of an insulin response) immediately following removal of the cells from the differentiation medium and remains essentially constant during the subsequent 4 days. The GH inhibition of insulin action does not appear to be due GH enhancement of cellular degradation of insulin, competitive binding of GH to the insulin receptor, or GH-induced decrease in cell number. The 3T3-L1 adipocyte system appears to be a sensitive and reliable in vitro model with which to study the molecular mechanisms involved in both GH antagonism of insulin action and development of hormone responsiveness during cellular differentiation into adipocytes.

Key words: growth hormone, adipocytes, lipid synthesis, differentiation

It is well established that somatotropin (GH) alters glucose and lipid metabolism in vivo [1–4]. For example, chronic elevation of circulating GH levels in certain animals results in diabetes [3] or insulin resistance [2,5,6]. In addition, clinical treatment of short stature with human somatotropin (hGH) is complicated by GH induction of glucose intolerance in some patients [1]. Two possibly overlapping mechanisms have been suggested for GH-stimulated diabetes: 1) GH suppression of non-insulin regulated glucose uptake and/or utilization [7]; and 2) GH interference or modification of the cellular response(s) to insulin action [3]. However, elucidation of the mechanism(s) involved in the diabetogenic effects of GH has been difficult because of a lack of appropriate in vitro systems that are responsive to GH. One problem is that, in vivo, the diabetogenic

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action of GH is evident only after prolonged GH treatment [1,2,8]. Therefore, it is possible that an in vitro demonstration of the diabetogenic effects of GH also will involve cellular responses that are not acutely apparent following addition of GH. It has been shown that specific cellular functions of primary adipocyte and adipose tissue cultures are responsive to GH in vitro [5,7–13]. One of the better characterized effects of GH on primary adipose cultures, for example, is that glucose utilization is stimulated by GH immediately following GH treatment in vitro. Typically, however, these cultures are limited by being viable for only short periods of time, making it difficult to reproducibly demonstrate a diabetogenic-like effect by GH in these systems [5,8–11].

It has recently been reported that cultured 3T3-F442A adipocytes are viable for long periods of time and, therefore, may represent a useful alternative to primary adipose cultures for studying the metabolic effects of GH on glucose utilization [14–16]. The 3T3-F442A cells are a subclone of the 3T3-L1 preadipocyte cell line. The 3T3-F442A cells were initially isolated for their enhanced ability to convert into adipocytes as compared to the parental cell line. Findings for 3T3-F442A cells paralleled well-established observations with primary rat adipocytes: it was shown that GH stimulated [^{14}C]-glucose utilization by 3T3-F442A adipocytes during the first 4 hr of incubation with GH. However, unlike primary adipoctye cultures, suppression of glucose utilization by GH was observed when the 3T3 adipocytes were treated with GH for 24 to 48 hr. Both the transient and long-term GH effects on glucose utilization in 3T3-F442A adipocytes appear to be due to direct action of GH on the cells and not due to action of intermediates such as IGF-I and IGF-II [16]. However, none of the previous studies analyzed the action of GH on 3T3-F442A adipocytes in the presence of insulin, a state that is typical under in vivo conditions.

Similar to the results with 3T3-F442A adipocytes, the present results show that GH suppresses glucose utilization in 3T3-L1 adipocytes. In addition, it is shown that GH antagonizes insulin-stimulated glucose utilization in 3T3-L1 adipocyte cultures. Data presented clearly illustrate that 3T3-L1 adipocytes are a sensitive and reliable in vitro model to study GH suppression of both insulin-stimulated and unstimulated glucose incorporation into lipids. It is further shown that glucose incorporation into lipids in 3T3-L1 adipocytes is regulated by a balance between insulin and GH concentrations, with elevated concentrations of either hormone capable of overcoming the effects of the other hormone.

MATERIALS AND METHODS

Materials

Insulin (Regular Iletin, 100 Units/cc) was purchased from Eli Lilly, Inc., Indianapolis, IN, and bovine pituitary somatotropin from Dr. A. F. Parlow, Harbor-UCLA Medical Center, Torrance, CA. Recombinant human (rhGH) and bovine somatotropin (rbGH), were obtained from Genentech, Inc., South San Francisco, CA, and the Animal Sciences Division of the Monsanto Agriculture Co., St. Louis, MO, respectively.

Cell Culture

All procedures were performed under sterile conditions and represent modifications to the procedures originally described by Reed and Lane [17] and Schwartz [14]. Stock cultures of 3T3-L1 cells (American Type Culture Collection, Rockville, MD, ATCC CCL 92.1) were grown in 100-mm tissue-culture dishes (Falcon, Oxnard, CA), in culture

medium [Dulbecco-Vogt modified Eagle's medium with 4.5g of glucose per liter (high-glucose DME) containing 10% (v/v) bovine calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all five from Gibco, Grand Island, NY)], and in a humidified atmosphere composed of 7.5% CO_2 and 92.5% air at 37°C. Exponentially growing stock cultures of cells were subcultured every 3 to 4 days to prevent them from becoming confluent. To subculture the cells, medium was removed by aspiration, and the cell layer was rinsed twice with Ca^{2+}-and Mg^{2+}-free Dulbecco's phosphate-buffered saline. The 3T3-L1 cells were incubated with a solution of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in isotonic buffer (Gibco) for 5 to 10 min at 37°C to remove them from the plastic dish. Dispersed cells were rinsed from the dish into centrifuge tubes with the use of culture medium and subjected to centrifugation at 150*g* for 5 min at 22°C. The cell pellet was resuspended in fresh culture medium and inoculated into either fresh 100-mm dishes at 3.8 to 7.6 × 10^2 cells/cm^2/ in 10 ml of medium, or into 60-mm dishes (Falcon) at 3.0 to 5.0 × 10^3 cells/cm^2 in 4 ml of medium for experiments. Conversion of 3T3-L1 cells to adipocytes was initiated by adding 2.5 ml of differentiation medium, which was high-glucose DME containing 2 μg/ml insulin, 0.5 mM 1-methyl-3-isobutylxanthine (Sigma, St. Louis. MO), 25 nM dexamethasone (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% (v/v) fetal bovine serum (Gibco), to 48- to 72-hr postconfluent cultures. Differentiation medium was removed by aspiration after 48 hr of incubation at 37°C and, unless otherwise indicated, incubation of the cells was continued in 2.5 ml culture medium containing 10% (v/v) fetal bovine serum in place of bovine calf serum for an additional 72 hr. Phase contrast microscopic examination of the cultures routinely showed 70% to 95% conversion of the cells into adipocytes. The culture medium was replaced with 2.5 ml serum-free medium, which was low-glucose DME (1 g/L glucose) containing 1% (w/v) bovine serum albumin (Sigma #A-6003), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, 20 to 24 hr prior to experiments. Cell number following conversion of confluent cultures of 3T3-L1 cells into adipocytes was measured in duplicate with the use of a Coulter electronic particle counter in four separate experiments and found to consistently be 1.9 ± 0.1 × 10^6 cells per 60-mm dish.

Lipid Accumulation Experiments

The indicated concentrations of hormones (e.g., insulin and somatotropin) were added to monolayers of converted cells in 2.5 ml of serum-free medium. At 6 hr after addition of hormones to the cells, 0.25 μCi of uniformly labeled D-[^{14}C]glucose (Amersham, Arlington Heights, IL) was added to each culture in 100 μl of serum-free medium, and the cells were further incubated at 37°C for 18 hr. The amount of [^{14}C] – lipid synthesized by 3T3-L1 adipocytes in response to insulin increased linearly over at least 24 hr (data not shown), and it was found that the most consistent results were obtained by using incubation periods of greater than 8 hr. Therefore, a total incubation period of 24 hr was chosen as a reliable and convenient interval for these studies. Incorporation of D-[^{14}C]glucose into lipids was stopped by complete aspiration of the medium, followed immediately by addition of 2.0 ml of Dole's reagent, [i.e., 78% (v/v) isopropyl alcohol, 20% (v/v) HPLC grade *n*-heptane, and 2% (v/v) 1.0 N H_2SO_4]-(18), and incubated at 22°C for 15 min to dissolve the cell layer. The solubilized cell layer was suspended by repeated pipetting of the extraction buffer over the surface of the dish with a glass Pasteur pipette. This mixture was transferred to 16 × 100 mm borosilicate glass screw-capped

tubes, and an additional 2.0 ml Dole's reagent was used to rinse each plate and pooled with the first extract. To each tube of extract, 1.75 ml water and 1.75 ml *n*-heptane were added, and the tubes were vortex mixed. Following separation of the organic and aqueous solvent phases, 2.0 ml of the upper organic phase was transferred to a scintillation vial, 5.0 ml Ready-Solv (Beckman Instruments, Inc., Palo Alto, CA) was added to each vial, and radioactivity in each sample was measured using a Beckman liquid scintillation counter. Unless noted otherwise, each observation discussed in this study was made in at least two, and typically three or more, independent replicate experiments.

Insulin Binding

Converted 3T3-L1 adipocytes, cultured in serum-free medium for 24 hr, were rinsed once in binding buffer, 100 mM HEPES, pH 7.6; 120 mM NaCl, 15 mM sodium bicarbonate, 10 mM glucose, 2.5 mM KCl, 1.2 mM $MgSO_4$, 1 mM Na_2EDTA, and 10 mg/ml BSA (Sigma #A9647). The indicated amount of [^{125}I]-insulin, 127 μCi/μg, generously provided by Dr. Gavin (Washington University, St. Louis, MO), either in the absence or presence of unlabeled insulin (10 μg/ml) or unlabeled rbGH (10 μg/ml) was added to triplicate plates in 2.0 ml amounts of binding buffer per plate. Cells were incubated for 90 min at 16°C, the buffer was removed by aspiration, and the cell layer was rinsed free of unbound [^{125}I]-insulin by rapid dipping of each culture dish in three successive beakers containing 500 ml of ice-cold PBS. The cell layer was dissolved by incubation at 37°C for 1 hr in 0.5 ml 1 N NaOH. The cell lysate, plus a 0.5-ml rinse of the plate with 1 N NaOH, were pooled, and cell-bound [^{125}I]-insulin was measured in a Beckman gamma counter.

TCA Precipitation of [^{125}I]-Insulin

Approximately 200,000 cpm of [^{125}I]-insulin was added per milliliter of DME containing 1% (w/v) BSA and the indicated concentration of hormone(s). Three 100-μl aliquots of each medium was removed for an accurate quantitation of the initial amount of [^{125}I]-insulin per milliliter, and 2.5-ml aliquots of each hormone-supplemented medium were added to each of triplicate 60-mm dishes of 3T3-L1 adipocytes and incubated for 24 hr at 37°C. Following the 37°C incubation period, 100-μl aliquots were transferred from each plate into 1.5-ml microcentrifuge tubes, and the tubes were placed on ice. Ice-cold 20% (v/v) TCA (100 μl) was mixed with the aliquots of medium in each tube and incubated on ice for 15 min. An additional 800 μl of ice-cold 10% (v/v) TCA was added to each tube and mixed, and the tubes were subjected to centrifugation for 10 min at 20,000*g*. The supernatant was removed by aspiration, and the radioactivity of the pellet-containing tubes was measured with the use of a Beckman gamma counter. The values are expressed as a percentage of the number of DPMs of [^{125}I]-radioactivity present in each TCA precipitate tube, relative to the initial number of total DPMs of [^{125}I]-insulin present in each medium.

RESULTS

Somatotropin Suppression of Insulin-Stimulated Glucose Incorporation Into Lipids

It has previously been shown that glucose utilization for lipid synthesis by 3T3-F442A adipocytes is stimulated by insulin and inhibited by GH [14–16]. However, none of those studies showed the action of GH in the presence of insulin, therefore the experiment shown in Table I was designed to address this question. Untreated 3T3-L1

adipocytes incorporated approximately 5,800 dpm (1.73 nmoles) of [^{14}C]-glucose per 1×10^6 cells over the 18-hr incubation period. Approximately 40% of this unstimulated amount of glucose utilization by 3T3-L1 adipocytes was inhibited by a concentration of 500 ng/ml rbGH and by rhGH to a level of 3,200 and 3,600 dpm per 1×10^6 cells over 18 hr, respectively. By comparison, a 500 ng/ml concentration of pituitary bGH inhibited 20% of the unstimulated amount of glucose incorporation into lipids to a level of 4,700 DPM per 1×10^6 cells. Insulin (50 μUnits/ml) stimulated utilization of [^{14}C]-glucose for lipid synthesis to approximately 45,000 DPM (13.42 nmoles) per 1×10^6 cells, approximately an eight-fold increase over the level of glucose utilization exhibited by untreated 3T3-L1 adipocytes. All of the tested forms of somatotropin (bGH, rbGH, and rhGH), at 500 ng/ml levels, inhibited insulin-stimulated incorporation of [^{14}C]-glucose into lipids to a level of 23,000 to 25,000 DPM per 1×10^6 cells over the 18-hr incubation period, approximately a 45% inhibition. Therefore, regardless of the amount of stimulation of [^{14}C]-glucose incorporation into lipids achieved with or without insulin in 3T3-L1 adipocytes, GH appears to inhibit up to approximately 50% of that level.

However, those earlier reports also showed that GH significantly stimulated [^{14}C]-glucose incorporation into lipids in 3T3-F442A adipocytes during the first 4 hr of hormone treatment [14]. This observation suggests that simultaneous addition of [^{14}C]-glucose, insulin, and GH over short incubation periods would result in no apparent GH antagonism of insulin action, since GH would be exhibiting insulin-like activity during the early hours of treatment. Indeed, a 10-hr treatment of 3T3-L1 adipocytes with [^{14}C]-glucose, 15 μUnits/ml insulin, and concentrations of bGH up to 72 ng/ml showed no significant inhibition of insulin action by bGH. However, addition of [^{14}C]-glucose to the cells during only the last 5 hr of a 10-hr hormone treatment should result in GH antagonism of insulin action, since GH would not be exhibiting insulin-like activity during those last 5 hr of the experiment. Consistent with this hypothesis, it was observed in the present studies that bGH significantly inhibited [^{14}C]-glucose incorporation into lipids stimulated by insulin. For example, 6 ng bGH per milliliter inhibited $52.7 \pm 2.1\%$ of [^{14}C]-glucose incorporation into lipids stimulated by insulin at levels of 15 μUnits/ml. Additionally, this result shows that long incubation periods are not required for GH antagonism of insulin action to occur. However, all of the subsequent experiments described in this report were performed over a 24-hr time period as described in Materials and Methods for ease of experimental design and to allow incorporation of sufficient levels of [^{14}C]-glucose into lipids to ensure statistically significant results.

The relationship between bGH concentration and its level of inhibition of insulin-stimulated glucose utilization is shown in greater detail in Figure 1. As low a concentration

TABLE I. Effect of Somatotropin and Insulin on [^{14}C]-Glucose Incorporation Into Lipids in 3T3-L1 Adipocytes

Type of GH (500 ng/ml)	No insulin	50 μUnits/ml insulin
None	5748 ± 706[a]	44907 ± 577
Human rGH	3578 ± 247	24403 ± 905
Bovine GH	4713 ± 106	23819 ± 1808
Bovine rGH	3248 ± 237	25421 ± 1683

[a]Values given are DPMs [^{14}C]-lipids/1×10^6 cells/18 hr of the means of triplicate samples ±95% confidence levels as explained in Materials and Methods.

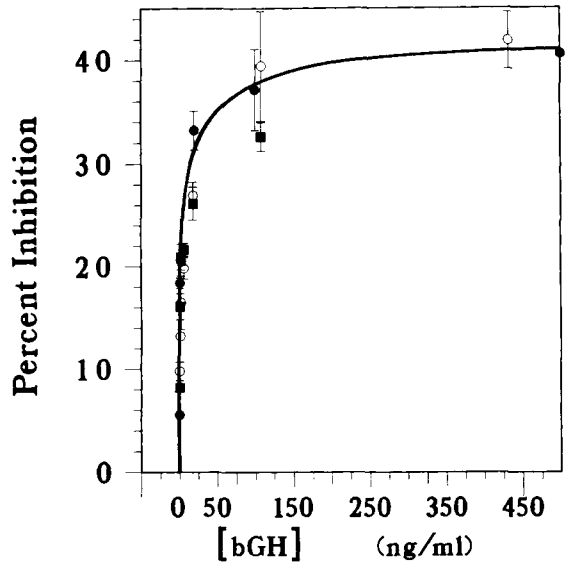

Fig. 1. Bovine somatotropin inhibition of insulin-stimulated [^{14}C]-glucose incorporation into lipids in 3T3-L1 adipocytes. The results of three separate experiments (○ = 1; ● = 2; ■ = 3) are shown. The experiments were performed as described in Materials and Methods. The insulin concentration in all three experiments was 50 μUnits/ml. The values shown are the mean of triplicate samples expressed as the percent inhibition of the insulin response by the indicated concentration of somatotropin relative to parallel insulin-treated cultures without somatotropin. The error bars indicate ± 95% confidence values for each data point.

of rbGH as 0.2 ng/ml reproducibly inhibited 8% to 10% of the amount of [^{14}C]-glucose incorporation into lipids stimulated by insulin. Concentrations of rhGH greater than or equal to 100 ng/ml were maximally effective by inhibiting approximately 40% of the 3T3-L1 adipocyte response to insulin.

One explanation for somatotropin inhibition of insulin-stimulated utilization of glucose by 3T3-L1 adipocytes is somatotropin-induced cellular degradation of insulin. To address this particular issue, two different types of experiments were performed. One experiment measured the amount of intact [^{125}I]-insulin that can be precipitated by 10% (v/v) TCA following incubation with 3T3-L1 adipocytes in the presence of various hormone treatments. Approximately 89.1% of the [^{125}I]-insulin used for this study was precipitable by 10% TCA in the presence of 1% (w/v) BSA. The small amount of TCA-soluble [^{125}I]-insulin may represent either radiolysis of the [^{125}I]-insulin during the 10 days of storage at −20°C prior to this study or an inherent problem of precipitating a small protein such as insulin with TCA. In the absence of cells, incubation of approximately 500,000 dpm of [^{125}I]-insulin in 2.5 ml of DME containing 1% BSA at 37°C for 24 hr resulted in 76.9% of the [^{125}I]-insulin remaining intact and TCA precipitable. As shown in Table II, incubation of [^{125}I]-insulin with 3T3-L1 adipocytes at 37°C for 24 hr resulted in approximately 41% or 44% of the initial amount of [^{125}I]-insulin remaining TCA precipitable in the absence or presence of additional insulin, respectively. Approximately 45% to 50% of the [^{125}I]-insulin remained TCA precipitable in the presence of either a low (4.5 ng/ml) or high (243 ng/ml) concentration of rbGH. Under experimental conditions identical to those used to show that rbGH treatment of 3T3-L1

TABLE II. Effect of Insulin and Somatotropin on 3T3-L1 Adipocyte Degradation of [^{125}I]-Insulin Over 24 hr

Insulin concentration (μUnits/ml)	Somatotropin concentration (ng/ml)		
	0	4.5	243
0	40.9 ± 0.9[a]	45.6 ± 2.0	44.7 ± 0.4
15	44.1 ± 2.4	48.6 ± 2.0	49.9 ± 1.2
50	44.4 ± 0.7	49.1 ± 2.4	49.0 ± 0.9

[a]Values given are percent of DPM of [^{125}I]-insulin that can be precipitated by 10% TCA following a 24 hr incubation with 3T3-L1 adipocytes at 37°C in DME containing 1% BSA relative to the initial DPM of [^{125}I]-insulin initially added to the cells (as described in Materials and Methods) expressed as the means of triplicate samples ±95% confidence levels.

adipocytes did not stimulate cellular degradation of [^{125}I]-insulin, it was shown with parallel cultures of 3T3-L1 adipocytes that rbGH at concentrations of 4.5 ng/ml and 243 ng/ml inhibited 26% and 61%, respectively, the amount of glucose incorporation into lipids stimulated by 15 μUnits of insulin per milliliter.

A second experiment was designed to determine whether the 45% of the insulin which remained TCA precipitable following incubation with the adipocytes was biologically active. This experiment involved preincubation of media containing various hormone mixtures with 3T3-L1 adipocytes for 24 hr at 37°C prior to transfer of the media to fresh 3T3-L1 adipocyte cultures for measurement of hormone regulation of [^{14}C]-glucose utilization for lipid synthesis. Two sets of triplicate plates of 3T3-L1 adipocytes were incubated either without hormones, or with 15 μUnits of insulin per milliliter in the presence of 0, 4.5 or 243 ng of rbGH per milliliter. One set of plates received [^{14}C]-glucose 6 hrs later in order to measure hormone regulation of glucose utilization during the initial 24 hour incubation with 3T3-L1 adipocytes. As shown in Figure 2, 15 μUnits/ml concentration of insulin stimulated incorporation into lipids of approximately 29,000 cpm of [^{14}C]-glucose per 1×10^6 cells over 18 hr, and 8.4-fold increase in glucose utilization, compared to that in untreated control cell cultures. Approximately 24,000 and 14,500 dpm of [^{24}C]-lipid were synthesized in response to 15 μUnits of insulin per milliliter in the presence of 4.5 and 243 ng/ml concentrations of rbGH, respectively. Media conditioned by the second set of plates for 24 hr was tranferred to fresh 3T3-L1 adipocytes for measurement of hormone regulation of glucose utilization for lipid synthesis. Conditioned medium containing insulin at a 15 μUnits/ml concentration stimulated approximately 18,000 DPM of [^{14}C]-glucose incorporation into lipids per 1×10^6 cells over 18 hr, or a 4.0-fold increase in glucose utilization over parallel untreated control cultures. Approximately 14,000 and 11,000 DPM of [^{14}C]-lipid was synthesized by 3T3-L1 adipocytes in response to conditioned medium containing 15 μUnits of insulin per milliliter and 4.5 or 243 ng rbGH per milliliter, respectively. The increase in stimulation of glucose utilization by insulin plus or minus the two concentrations of rbGH in conditioned medium was approximately 51 ± 8% of the increase in stimulation exhibited by fresh medium composed of the corresponding insulin and rbGH composition. Therefore, it appears that the majority of the 45% of the insulin previously shown to be undegraded and precipitable by TCA was also biologically active regarless of the amount of rbGH present. Additionally, it appears that a significant percentage of rbGH survived preincubation with 3T3-L1 adipocytes, since the amount of inhibition was similar for the conditioned and fresh media (e.g., 18% and 21% inhibition of the insulin-stimulated

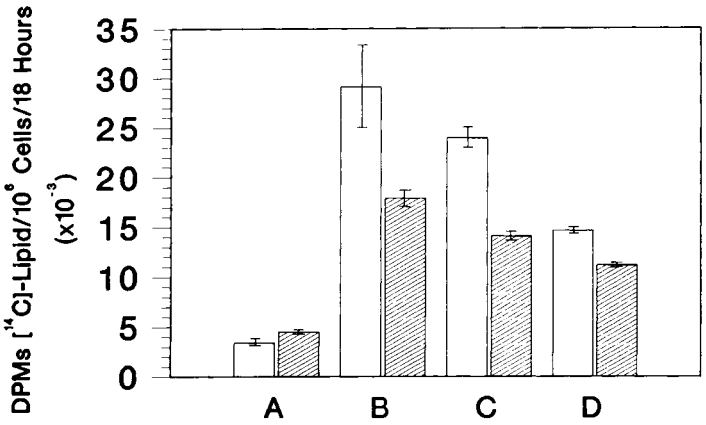

Fig. 2. Effect of conditioning by 3T3-L1 adipocytes on medium containing insulin and somatotropin. Cells incubated with fresh medium containing either: no hormones (**A**); insulin alone, 15 μUnits/ml (**B**); or insulin, 15 μUnits/ ml plus rbGH at either 4.5 ng/ml (**C**) or 243 ng/ml (**D**) concentration as described in Materials and Methods are indicated by open bars. Cells incubated with medium conditioned by 3T3-L1 adipocytes for 24 hr containing: no hormones (**A**): insulin alone, 15 μUnits/ml (**B**); insulin, 15 μUnits/ ml plus rbGH at either 4.5 ng/ml (**C**) or 243 ng/ml (**D**) concentrations as described in Materials and Methods are indicated by crosshatched bars. The values shown in the main panel are the mean values of [^{14}C]-glucose incorporated into lipids in triplicate 3T3-L1 andipocyte cultures. The error bars represent ± 95% confidence levels for each data point.

response by 4.5 ng/ml concentrations of rbGH, before and after conditioning by the cells, respectively).

Somatotropin-induced loss of cells during the 24-hour incubation period is a second possible explanation for somatotropin inhibition of insulin-stimulated glucose utilization by 3T3-L1 adipocytes for lipid synthesis. However, no significant change in cell number was observed on triplicate plates of 3T3-L1 adipocytes before and after 24-hour treatment with various hormone regimens. Cell number prior to addition of hormones to the cells was 1.95 ± 0.03 × 10^6 per 60-mm dish. Following treatment with 0, 15, or 50 μUnits/ ml concentrations of insulin and/or 0, 4.5 and 243 ng/ml concentrations of rbGH for 24 hours, cell number was 1.99 ± 0.06 × 10^6 for all plates regardless of the hormone treatment.

A third explanation for somatotropin suppression of insulin-stimulated incorporation of glucose into lipids is that GH interferes with insulin binding to the insulin receptor. However, rbGH at 10 μg/ml, a concentration that is 25- to 100-fold higher than the maximal inhibitory somatotropin concentrations, showed no ability to compete for binding of [^{125}I]-insulin at 2.55 or 5.1 μUnits/ml (30,000 or 60,000 DPM) to 3T3-L1 adipocytes (Table III). In contrast, 254 mUnits of unlabeled insulin per milliliter competed for 56% to 69% of the [^{125}I]-insulin binding, indicating the presence of functional, specific insulin receptors on the adipocytes.

Insulin and Somatotropin Reciprocally Regulate Glucose Utilization by 3T3-L1 Adipocytes in a Concentration-Dependent Manner

As shown in Figure 3, glucose incorporation into lipids in 3T3-L1 adipocytes was reciprocally regulated by insulin and somatotropin concentrations. In the absence of GH, 11 and 33 μUnits/ml insulin stimulated 3.5- and 9.3-fold increases in [^{14}C]-glucose

TABLE III. Effect of Somatotropin on Binding of [^{125}I]-Insulin to 3T3-L1 Adipocytes

	[^{125}I]-Insulin	
Competitive ligand	30,000 DPM (2.55 μUnits/ml)	60,000 DPM (5.1 μUnits/ml)
None	1360 ± 202[a]	2839 ± 6
10 μg/ml rbGH	1479 ± 110	2734 ± 110
254 mUnits/ml Insulin	417 ± 55	1236 ± 59

[a]Values given are the mean DPM values of triplicate samples ±95% confidence levels of [^{125}I]-insulin bound to 1 × 10^6 3T3-L1 adipocytes as explained in Materials and Methods.

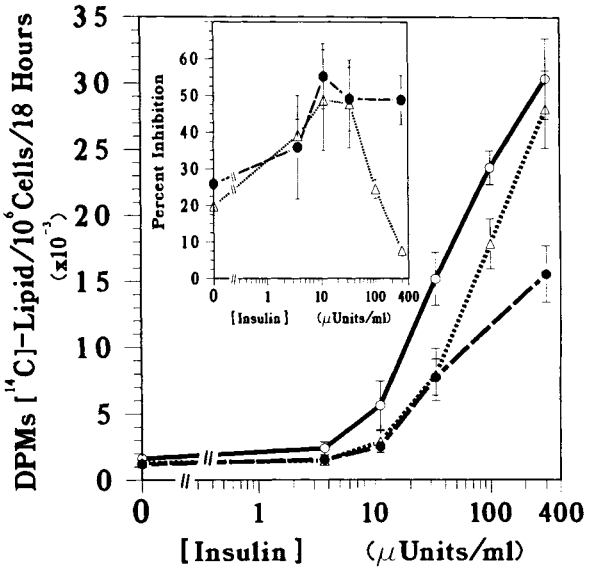

Fig. 3. Interrelationship of insulin and somatotropin concentrations on regulation of glucose incorporation into lipids in 3T3-L1 adipocytes. The cells were incubated with the indicated concentration of insulin alone (○), or with rbGH at either 10 ng/ml (△) or 1000 ng/ml (●) concentrations, as described in Materials and Methods. The values shown in the main panel are the mean values of [^{14}C]-glucose incorporated into lipids in triplicate 3T3-L1 adipocyte cultures. The values of the data in the inset panel were derived from the data shown in the main panel as explained for Figure 1. The error bars represent ± 95% confidence levels for each data point.

incorporation into lipids, respectively. However, in the presence of 10 and 1000 ng/ml rbGH, 11 and 33 μUnits/ml insulin stimulated only a 2- and 6-fold increase in [^{14}C]-glucose utilization for lipid synthesis, respectively. These results indicate that GH altered the sensitivity of the 3T3-L1 adipocytes to insulin. Further support of this conclusion is highlighted by the replotting of the data from the main panel of Figure 3 as shown in the inset. Recombinant bGH, at 1000 ng/ml, suppressed approximately 50% of the [^{14}C]-glucose utilization in the presence of 33, 100, and 300 μUnits/ml insulin despite the fact that the amount of [^{14}C]-glucose incorporation into lipids stimulated by insulin increased approximately 2-fold over the range of insulin concentrations. Figure 3 inset also shows that somatotropin exerts a greater percentage suppressive effect on glucose utilization in the presence of low insulin concentrations (up to 50%) than in its absence

(~ 25%). Preincubation of 3T3-L1 adipocytes with various concentrations of rbGH for up to 24 hr prior to insulin addition did not significantly modify the amount of GH-mediated inhibition of the insulin response (data not shown).

Just as GH altered the sensitivity of 3T3-L1 adipocytes to insulin, the data in Figure 3 also show that the effect of GH can be overcome by increases in the insulin concentration. For example, 10 ng rbGH per milliliter is equivalent to 1,000 ng rbGH per milliliter at inhibiting insulin at 5 to 30 μUnits/ml levels. However, 10 ng rbGH per milliliter has little, if any, inhibitory effects on the response to 300 μUnits insulin per milliliter while 1,000 g rbGH per milliliter still inhibited approximately 50% of that response.

Relationship Between the Degree of 3T3-L1 Differentiation into Adipocytes and the Ability of Insulin and Somatotropin to Regulate Glucose Utilization

Concomitant with the appearance of an adipocyte phenotype in 3T3-L1 fibroblasts treated with differentiation medium (including a rounded morphology and accumulation of intracellular lipid vesicles) is an increase in responsiveness to insulin. As shown in Figure 4, an insulin concentration of 50 μunits/ml stimulated a four-fold increase in glucose utilization in the 3T3-L1 cells 24 hr after their treatment with differentiation medium. By comparison, 96 hr following treatment with differentiation medium, an insulin concentration of 50 μunits/ml stimulated greater than an 18-fold increase in

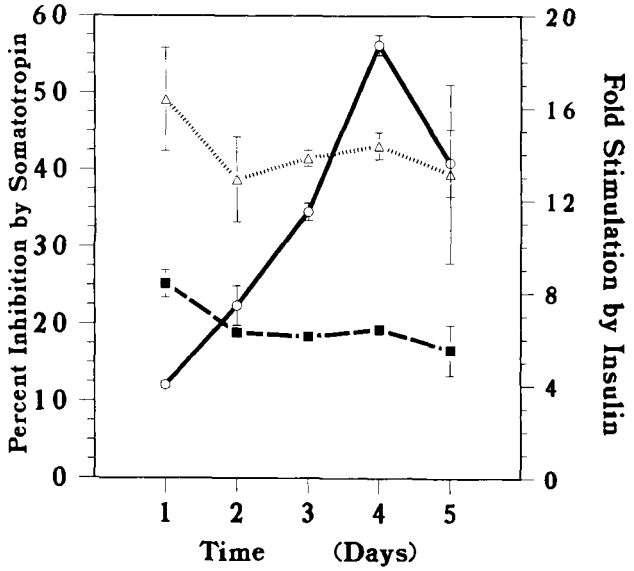

Fig. 4. Effect of 3T3-L1 differentiation on insulin and somatotropin regulation of glucose incorporation into lipids. At the indicated times following treatment of the 3T3-L1 cells with differentiation medium, triplicate cell cultures were incubated with insulin: 50 μUnits/ml (○), or 50 μUnits/ml plus rbGH at either 5 ng/ml (■) or 500 ng/ml (△) as described in Materials and Methods. The values for the cultures treated with insulin alone are expressed as the relative increase in [^{14}C]-glucose incorporation into lipids relative to untreated control cultures. The value of the data for the GH-treated cultures are the mean of triplicate samples expressed as the percent inhibition of the insulin response by the indicated concentration of somatotropin relative to parallel insulin-treated cultures without somatotropin. The error bars indicate ± 95% confidence values for each data point.

glucose incorporation into lipids. Therefore, the sensitivity of 3T3-L1 cells increased dramatically during cellular differentiation into adipocytes. In contrast to the observation with insulin, the percent suppression by rbGH of the 3T3-L1 response to insulin was not dependent upon the degree of differentiation of 3T3-L1 cell into adipocytes (Fig. 4). Approximately 40% to 49% of the 3T3-L1 response to insulin was suppressed by 500 ng/ml levels of rbGH throughout the 5 days following treatment of the cells with differentiation medium. Similarly, 5 ng rbGH per milliliter suppressed 17% to 24% of the insulin response regardless of the degree of differentiation of the cells. The difference in the amount of inhibition by 5 ng rbGH per milliliter seen at days 1 and 5 in Figure 4 was not significant.

DISCUSSION

Somatotropin antagonism of insulin-stimulated glucose utilization in vivo is well documented [1-3,8]; however, it has been difficult to demonstrate similar results in vitro [5,8–11]. The present study demonstrates that GH is a potent antagonist of insulin-stimulated glucose incorporation into lipids in 3T3-L1 adipocyte cultures. Somatotropin antagonism of insulin action was shown not simply to be attributable to 1) GH enhancement of cellular degradation of insulin, 2) GH-induced decrease in viable cell number during the 24 hr incubation period with hormones, or 3) GH interference with insulin binding to its receptor. Several of the observations in this report closely parallel in vivo observations [2,5,6]. Therefore, it appears that the 3T3-L1 adipocyte system is a sensitive and reliable in vitro model for studying the mechanism of the anti-insulin activity of GH.

It has been shown that the elevated GH levels of acromegalic patients frequently correlate with glucose intolerance that arises as a result of a decrease in sensitivity to insulin [3,19]. In addition, chronic elevation of circulating GH levels in certain animals results in diabetes [3] and insulin resistance [2,5,6]. Similar to these in vivo results, the present experiments show that the sensitivity of 3T3-L1 adipocytes to insulin is significantly reduced by GH. Insulin, at all tested concentrations, stimulated nearly twice as much [^{14}C]-glucose incorporation into lipids in the absence of GH than in the presence of 1 μg rbGH per milliliter. Since the range of insulin concentrations that were tested stimulated nearly a 16-fold increase in glucose utilization by the 3T3-L1 adipocytes, GH must be able to suppress the specific metabolic processes stimulated by insulin.

However, the results here contrast with the majority of previously reported in vitro studies with either human adipose cultures [20] or primary rat adipocytes [7] which showed that GH did not alter cellular sensitivity to insulin. In those studies, the diabetogenic effect of GH was evident primarily as a reduction in the basal rate of [^{14}C]-glucose utilization. The stimulatory effects of insulin at any particular insulin concentration were unaffected by GH. Several potential reasons for the experimental differences between the early in vitro adipose studies and the present results with 3T3-L1 adipocytes include: 1) poor in vitro viability of primary adipocytes [5,8–11]; 2) 10- to 100-fold higher insulin and GH concentrations used in the earlier studies [20] (clearly outside the physiologically relevant range for these hormones); 3) the rat adipocyte cultures involved collagenase dispersal of the cells immediately prior to the experiments [7], which may have reduced responsiveness to GH by enzymatic degradation of the GH receptor [9]; and 4) fundamental differences between 3T3-L1 adipocytes and the rat adipose cultures that are as yet uncharacterized, for example, the distribution of insulin receptors on the

plasma membrane of 3T3-L1 and primary rat adipocytes has been reported to be different [21].

Unlike the majority of previously reported in vitro studies with primary adipocytes, one recent study with primary porcine adipose cultures described an anti-insulin effect by porcine GH that is similar in many respects to the present 3T3-L1 results [13], such as 1) GH alteration of cellular sensitivity to insulin, and 2) the percent suppression of glucose utilization for lipid synthesis by GH was greater in the presence of insulin than in its absence. However, several significant differences between the two in vitro systems suggest that 3T3-L1 adipocytes may be a more accessible model system to study the mechanism of the anti-insulin effects of GH. For example, primary porcine adipose tissue cultures were viable and retained hormone responsiveness only when maintained in the presence of both insulin and dexamethasone. In contrast, 3T3-L1 adipocytes remain hormone responsive under serum-free culture conditions. In addition, it can be noted from the report on the porcine adipose cultures that very particular conditions were necessary. Such specific conditions included the diet, site of biopsy, and physiology of the pigs prior to obtaining explants, as well as the exact lot of bovine serum albumin used during the culturing of the tissues. By comparison, 3T3-L1 adipocytes are derived from a cloned cell line that is well adapted to culture conditions and, as shown in this report, are able to provide very reproducible results (Fig. 1).

A second important parallel between the in vivo situation and the present results is that many features of the 3T3-L1 experiments utilize conditions that are similar to the environment experienced by adipocytes in vivo. One example is that the concentrations of GH and insulin which were effective at regulating glucose utilization in 3T3-L1 adipocytes are comparable to the circulating concentration of these hormones [1,3]. A second example is that GH inhibition of [^{14}C]-glucose utilization in 3T3-L1 adipocytes was greater in the presence of insulin than in its absence. The previous experiments on GH suppression of glucose utilization in 3T3-L1 adipocytes were performed in the absence of insulin [14–16], a nonphysiologic situation. In vivo, the diabetogenic effects of GH occur in the presence of insulin. Therefore, the present observation that GH exerts an even greater suppressive effect in the presence of insulin supports the notion that the GH effect on 3T3-L1 adipocytes reflects a physiologically relevant process.

Apart from the potential utility of the 3T3-L1 adipocytes as a convenient and reliable in vitro system to study GH suppression of insulin-stimulated glucose utilization, the 3T3-L1 culture system, in contrast to primary adipocyte cultures, has been shown to permit experiments focused on the development of hormone responsiveness during cellular differentiation into adipocytes. It has been shown that regulation of responsiveness to GH during 3T3-L1 differentiation into adipocytes is considerably different from regulation of insulin responsiveness. In the case of insulin, there is a marked increase in the ability of insulin to stimulate glucose utilization by 3T3-L1 adipocytes during the differentiation process. The relative amount of increase in 3T3-L1 responsiveness to insulin over the first 2 days following treatment of the cells with differentiation medium is essentially identical to previously published results [4,22]. Furthermore, stimulation by insulin is maximal by the third to fourth day following initiation of 3T3-L1 differentiation into adipocytes. It has previously been suggested that the rise in sensitivity of 3T3-L1 cells to insulin during differentiation is due in large part to a greater than 10-fold increase in insulin receptor number [23]. In contrast to the observations with insulin, it appears that the sensitivity of 3T3-L1 cells to the anti-insulin effects of GH is at its maximal level as early as the first 24 hr following removal of the cells from the differ-

entiation medium. These results are consistent with a previous report that GH receptor number and affinity on 3T3-L1 cells is unchanged before and after conversion into adipocytes [15,24].

In summary, the results of the present studies using 3T3-L1 cells provide in vitro evidence that GH directly suppresses insulin stimulation of glucose utilization in adipocytes. This in vitro system provides a sensitive and reliable means to study GH suppression of insulin action that may be useful in the study of GH-induced diabetes. Most importantly, this culture system should permit experiments designed to elucidate the biochemical mechanism by which GH interferes with insulin regulation of glucose metabolism. In addition, experiments focused on the regulation of homone responsiveness during differentiation of cells into adipocytes should be possible with the use of 3T3-L1 cells.

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