Growth Hormone Alters Lipolysis and Hormone-Sensitive Lipase Activity in 3T3-F442A Adipocytes

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While growth hormone (GH) has long been known as a lipolytic hormone, it has been difficult to study the cellular mechanisms for this effect. Since cultured 3T3-F442A adipocytes have recently proven to be useful to study chronic effects of GH on adipocyte metabolism, we examined the effects of GH on lipolysis. In these cells, GH alone produced a dose-dependent increase in the release of glycerol after 24 to 48 hours. The maximum increase occurred with 10 ng/mL human GH. The effect of GH was similar in the presence and absence of dexamethasone. Under each condition, the stimulation of glycerol release was accompanied by a GH-induced increase in the activity of hormone-sensitive lipase (HSL), a key lipolytic enzyme. The increase in HSL required 24 hours with GH and lasted at least 48 hours. The increase in HSL activity by epinephrine, like glycerol release, was potentiated by GH. Although GH potently simulates the activity of the lipogenic enzyme glycerol phosphate dehydrogenase (GPD) in differentiating 3T3-F442A preadipocytes, GH had a negligible effect on GPD activity in the differentiated adipocytes with chronic or short-term incubation. However, in contrast to the chronic effect of GH, short-term (30-minute) incubation with GH inhibited epinephrine-stimulated glycerol release, a characteristic transient antilipolytic effect of GH. These studies indicate that chronic GH treatment is lipolytic in cultured 3T3-F442A adipocytes, and document that lipolytic responses to GH involve an increase in the activity of HSL.

IT HAS LONG BEEN recognized that growth hormone (GH) treatment decreases carcass lipid.[1] Chronic exposure to GH in vivo has been shown to increase plasma free fatty acids, to inhibit the conversion of glucose into lipid, and to decrease the lipid content of adipose tissue.[2] However, the in vitro effects of GH on lipid metabolism, particularly on lipolysis, have been controversial. Pituitary GH was shown to be lipolytic in isolated adipose tissue or adipocytes, but this required the presence of dexamethasone.[3,4] Incubation with GH for 1 to 4 hours was subsequently shown to potentiate other lipolytic stimuli in rat adipose tissue, such as epinephrine or theophylline,[5,6] suggesting that GH modulated other lipolytic stimuli, rather than acting as an initiator of lipolysis.[2] Another challenge to the idea that GH was lipolytic was raised by the reported failure of highly purified pituitary GH preparations or human GH (hGH) of recombinant DNA origin to show lipolytic activity,[7-9] leading to the suggestion that contaminants in pituitary GH preparations possessed the lipolytic activity attributed to GH. However, subsequent studies by Goodman and Grichting indicated that these same GH preparations were lipolytic in vitro in rat adipose tissue in the presence of dexamethasone.[10,11] Pituitary and biosynthetic GH also had comparable lipolytic activity in chicken adipose tissue.[12] Moreover, pituitary and biosynthetic GH were reported to be lipolytic when administered to hypopituitary children,[13] attesting not only to the intrinsic lipolytic activity of hGH, but also to the importance of this action of GH in overall metabolic regulation in humans.

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With this background of GH as a lipolytic agent, we undertook studies to examine the cellular basis for this action. As a cellular model, we used cultured 3T3-F442A adipocytes. We had found that chronic incubation of these cells with GH inhibited various indicators of glucose metabolism, including glucose uptake and conversion to lipid or carbon dioxide.[14-16] The object of the present study was to determine first whether chronic incubation with GH also affected lipolysis in this adipocyte model, and then to evaluate whether changes in the activity of hormone-sensitive lipase (HSL) contributed to the observed lipolytic responses to chronic GH treatment. Since GH also increases the activity of glycerol phosphate dehydrogenase (GPD), a lipogenic enzyme, in association with promoting differentiation of 3T3-F442A preadipocytes to adipocytes, we examined the effect of GH on GPD activity in the differentiated adipocytes. GH of recombinant DNA origin was used throughout to insure that the activity observed was intrinsic to the GH molecule.

MATERIALS AND METHODS

Materials

3T3-F442A cells were provided by Dr H Green (Harvard University, Cambridge, MA). Sera and media were purchased from Irvine Scientific (Santa Ana, CA) or Hyclone (Logan, UT). Methionyl hGH (lot K9016AX) was provided by Genentech (South San Francisco, CA). Crystalline porcine insulin and dexamethasone were gifts from Eli Lilly (Indianapolis, IN) and Merck (Rahway, NJ), respectively. D-[^{14}C]-glucose (uniformly labeled) and [^{14}C]-triolein were purchased from New England Nuclear (Boston, MA). Fatty acid-poor bovine serum albumin (BSA), dihydroxyacetone phosphate, NADH, adenosine triphosphate (ATP), and 3-isobutyl-1-methylxanthine were purchased from Sigma (St Louis, MO). NAD was purchased from Calbiochem (San Diego, CA). GPD and glycerokinase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other reagents were of the highest grade available.

Cell Culture

3T3-F442A cells were grown to confluence in 100-mm dishes (Corning, Corning, NY) in an atmosphere of 10% CO_2/90% air,

using Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 10% bovine serum. Streptomycin (100 μg/mL), penicillin (100 U/mL), fungizone (0.25 μg/mL), and glutamine (2 mmol/L) were added to all formulations of DMEM. The confluent cells were converted to adipocytes in the presence of 10% fetal bovine serum, dexamethasone (0.25 μmol/L), insulin (2 μg/mL) and 3-isobutyl-1-methyl-xanthine (0.5 mmol/L) for 48 hours. The cells were then maintained in DMEM with 10% fetal bovine serum until 70% to 80% conversion was achieved. Before experimentation, the cells were deprived of serum for 18 to 24 hours by incubation with DMEM (1 g/L glucose) containing 2% BSA.

Lipolysis

Lipolysis was measured as the release of glycerol into the incubation medium.[17] Adipocyte monolayers were incubated in 4.0 mL of Krebs-Ringer bicarbonate buffer (pH 7.4) with 4% BSA, 1 mmol/L glucose, and hormones or vehicle. Preliminary experiments indicated that the increase in glycerol release due to epinephrine, and cell viability, were greater in cells incubated with 1 mmol/L glucose than in cells incubated without glucose. At specified times, the incubation medium was removed and assayed for glycerol concentration by the method of Weiland.[18] The cells were washed with phosphate-buffered saline (PBS), scraped with a rubber policeman into 1N NaOH and assayed for protein content. Glycerol output is expressed as μmol glycerol released per mg protein.

Hormone-Sensitive Lipase

After incubation with and without hGH and other hormones, adipocyte monolayers were scraped in 250 μL of 200 mmol/L MOPS with 10 mmol/L $MgCl_2$ and homogenized by 10 passages through a 25-gauge needle. Assays optimized for HSL activity[19] were performed on total homogenate, since preliminary experiments in which pellet and supernatant were assayed separately indicated no major difference in lipase activity or in hormone response in the fractionated material as compared with homogenate. This difference from previous reports may reflect methodological differences related to using monolayers as opposed to suspended cells.[19,20] The lipase assay was performed in a total volume of 200 μL, containing 50 mmol/L sodium phosphate (pH 7.0), 2.8% BSA, 150 mmol/L emulsified triolein, and 120 μL of cell homogenate. [^{14}C]-triolein was emulsified with unlabeled triolein[19] to provide 2,500 cpm/nmol triolein. After a 1-hour incubation at 37°C, cleaved fatty acids were extracted by the addition of 1.0 mL of a mixture of chloroform-methanol-benzene (2:2.4:1) containing 50 μg/mL carrier oleic acid, followed by 67 μL of 0.5N NaOH. Samples were vortexed vigorously for 20 seconds, and centrifuged at 1,000 × *g* for 5 minutes to separate the lower organic layer and the upper aqueous layer containing extracted fatty acids. An aliquot of the upper layer was removed, suspended in ACS (Amersham) scintillation cocktail and radioactivity was measured by liquid scintillation counting. Remaining cell homogenate was assayed for protein content by the method of Lowry et al.[21] Enzyme activity is expressed as U/mg protein. One unit of enzyme activity corresponds to the release of 1 nmol oleic acid per hour. The assay conditions used are optimal for HSL and are relatively incompatible with assays for lipoprotein lipase,[22] another lipase present in cultured adipocytes.

Lipid Accumulation

Monolayers of 3T3-F442A adipocytes were incubated at 37°C in DMEM (1 g/L glucose) with 1% BSA, D-[^{14}C]-glucose (0.1 μCi/mL), and hormones or vehicle as indicated for 48 hours. The reaction was terminated by removal of the incubation medium and scraping of cells in Dole's reagent (isopropyl alcohol:heptane:1N H_2SO_4; 40:10:1).[23] The lipids were extracted, the amount of lipid was determined gravimetrically, and radioactivity of the lipids was measured. Incorporation of glucose into lipid was expressed as cpm per mg of lipid.

Glycerol Phosphate Dehydrogenase

Monolayers of 3T3-F442A adipocytes were incubated with or without hormones for specified lengths of time. Cells were washed three times with PBS and scraped with a rubber policeman into 0.5 mL of 50 mmol/L Tris buffer (pH 7.5) with 1 mmol/L EDTA and 1 mmol/L β-mercaptoethanol. Cell suspensions were disrupted on ice for 5 seconds at 80% maximum output with the microtip of a Branson (Danbury, CT) model 350 Sonifier. Samples were centrifuged for 20 minutes at 30,000 × *g* at 4°C. Fat cakes were removed and the infranatants were stored at −70°C. Assays for GPD used a modification[24] of the method of Kozak and Jensen.[25] Briefly, assays were performed in a total volume of 1 mL, containing 100 mmol/L triethanolamine, 2.5 mmol/L EDTA, 0.12 mmol/L NADH, 0.1 mmol/L β-mercaptoethanol, 0.2 mmol/L dihydroxyacetone-phosphate, and 0.25 mL of infranatant containing solubilized enzyme. The change in absorbance was followed at 340 nm on a Beckman model 24 spectrophotometer (Fullerton, CA). Infranatant protein concentration was determined by the method of Lowry et al.[21] Enzyme activity is expressed as U/mg protein. One unit of enzyme activity corresponds to the oxidation of 1 nmol NADH per minute.

Statistics

Values are shown as mean ± SE. Data expressed as percent of maximum refer to the percent of the maximum increment produced by the hormone(s) in that experiment. Data were analyzed by Student's *t* test when two values were compared, and by ANOVA for repeated measures (StatView 512) followed by Scheffe F test when multiple comparisons were made. A value of $P < .05$ was considered significant.

RESULTS

GH Stimulates Lipolysis

To determine whether GH increased lipolysis in cultured murine 3T3-F442A adipocytes, glycerol release was measured after a 48-hour incubation with hGH of recombinant DNA origin. GH at 1 to 100 ng/mL significantly increased glycerol release (Fig 1A). This lipolytic response was dose-dependent, reaching a maximum at 10 ng/mL. The extent of the increase, approximately 40%, corresponds well with the extent of the increase observed with GH in adipose tissue and in human subjects.[10-13]

Because dexamethasone has been shown to potentiate the lipolytic effect of GH in rat adipose tissue and adipocytes,[3,4] the effect of GH was also examined in the presence of this glucocorticoid. Dexamethasone alone significantly increased glycerol release (Fig 1A). However, the increase in glycerol release with increasing concentrations of GH was similar in the presence and absence of dexamethasone. The similarity is especially evident when the responses are expressed as percent of the maximum increment produced by the hormones, as shown in Fig 1B.

GH Stimulates HSL

HSL is a major control point in the lipolytic pathway in adipocytes.[26,27] To determine if stimulation of HSL plays a

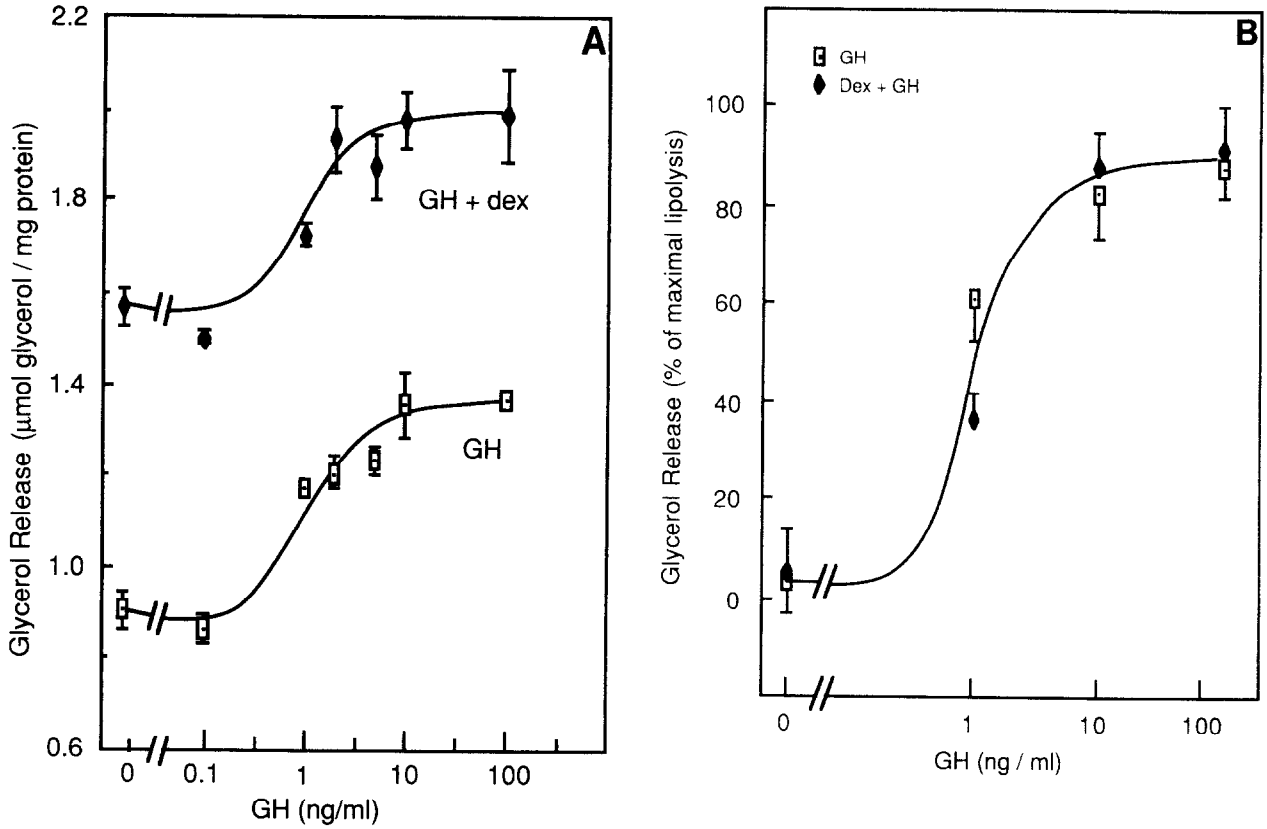

Fig 1. GH stimulates lipolysis in the presence and absence of dexamethasone after a 48-hour incubation. (A) Comparison of the effect of GH on glycerol release in the absence and presence of dexamethasone. Each point represents the mean ± SE of quadruplicate observations in a representative experiment. In the absence of dexamethasone, GH at concentrations of 1 ng/mL and higher produced significant ($P < .01$) increases in glycerol release. In the presence of 41 nmol/L dexamethasone, GH at concentrations of 2, 10, and 100 ng/mL produced significant ($P < .05$) increases over dexamethasone alone. (B) Each point represents the mean ± SE of 12 observations in three different experiments. Alone, GH significantly ($P < .001$) increased glycerol release at all concentrations tested from 1 ng/mL and higher. In the presence of dexamethasone, GH increased ($P < .001$) glycerol release at concentrations of 10 ng/mL and higher.

role in the lipolytic response to GH in the 3T3-F442A adipocytes, cells were incubated with and without GH (50 ng/mL) for 24 hours and assayed for HSL activity. Under these conditions, GH increased HSL activity, from 31 ± 0.5 to 39 ± 0.6 U/mg protein ($P < .005$) in a representative experiment. The effect of GH on HSL activity was dose-dependent (Fig 2). An increase in lipase activity was evident with 1 ng/mL hGH, and continued to increase with increasing GH concentrations to 100 ng/mL of GH ($P < .001$). A highly reproducible increase of similar magnitude was observed in every experiment, averaging 19% ± 2% (n = 6) above control values. Figure 3 shows the comparison in HSL activity at various times over a 48-hour period in adipocytes incubated in the presence and absence of GH. For the first 20 hours, there was no consistent difference in lipase activity between GH-treated and control cells, although lipase activity increased gradually in both groups. By 24 hours, lipase activity was significantly greater in GH-treated cells than in controls. The increase due to GH was maintained for at least 24 hours longer. The elevation at the 48-hour time point reflects the fact that GH treatment prevented a decrease in lipase activity observed in the controls.

GH is known to potentiate the lipolytic response to other hormones such as epinephrine.[2,3,5] To determine whether the potentiation reflected regulation at the level of HSL, 3T3-F442A adipocytes were incubated with GH for 23 hours, and then were challenged with epinephrine (1 ng/mL) for a final hour. The effectiveness of epinephrine in stimulating HSL activity was increased significantly in the cells previously incubated with GH (Fig 4). Thus, potentiation by GH is evident in the activity of HSL, as well as in glycerol release.

GPD Activity

GPD activity is markedly increased by GH in differentiating 3T3-F442A preadipocytes, and is often used as a marker for GH-induced differentiation.[28-30] Since GPD is a lipogenic enzyme, it was of interest to determine whether GH altered the activity of GPD in the differentiated adipocytes, and if so, whether such changes corresponded to the effects of GH on incorporation of ^{14}C-glucose into lipid (lipid accumulation).[14,15] This might show whether decreased lipogenesis, as well as increased lipolysis, contributed to the inhibition of lipid incorporation due to chronic GH treatment in these cells. Changes in GPD activity did not correspond to changes in lipid accumulation (Table 1). After 48 hours, when GH inhibited lipid accumulation, GH

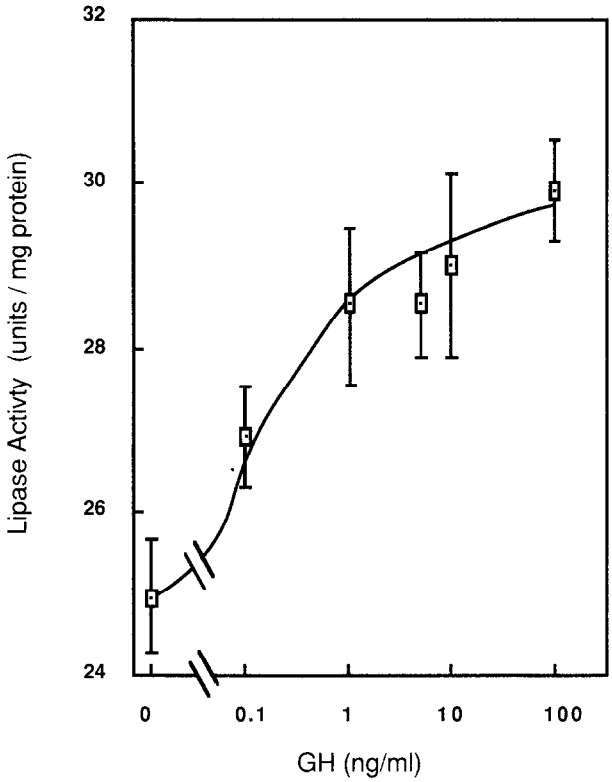

Fig 2. GH stimulates the activity of hormone-sensitive lipase after 24-hour incubation. Each point represents the mean ± SE of quadruplicate observations in a representative experiment. The response to GH is significant ($P < 0.001$) at 100 ng/mL. Similar results were obtained in five other experiments.

increased GPD activity. In the same experiment, insulin increased both the incorporation of glucose into lipid and GPD activity after 48 hours (not shown). Thus, although GPD is susceptible to hormonal regulation in the 3T3-F442A adipocytes, its regulation by GH does not appear to contribute in a major way to the GH-induced decrease in the incorporation of glucose into lipid. This is consistent with our recent observations that in the cultured adipocytes, GH does not alter the expression or transcription of the gene for GPD (G. Gurland et al, unpublished observations).

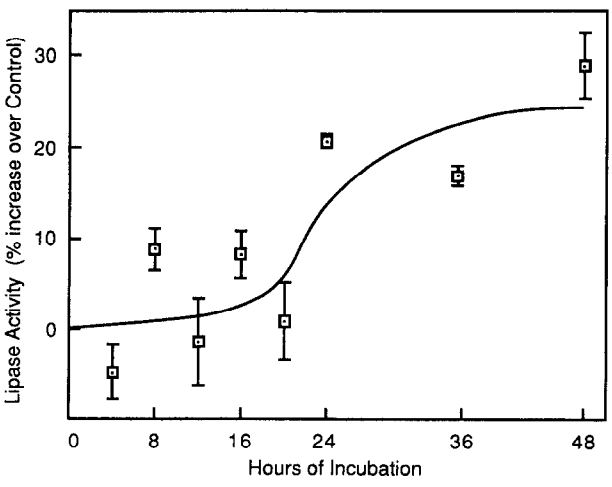

Fig 3. Time course of the increase in HSL activity due to GH. The increment due to GH (50 ng/mL) is shown as percent above the control at each time point, and was significant at 24 ($P < 0.01$), 36 ($P < .05$), and 48 ($P < .001$) hours. The initial value for lipase activity was 13.1 ± 0.81 U/mg protein. Each point represents the mean ± SE for three to four observations in a representative experiment. Similar results were obtained in two other experiments.

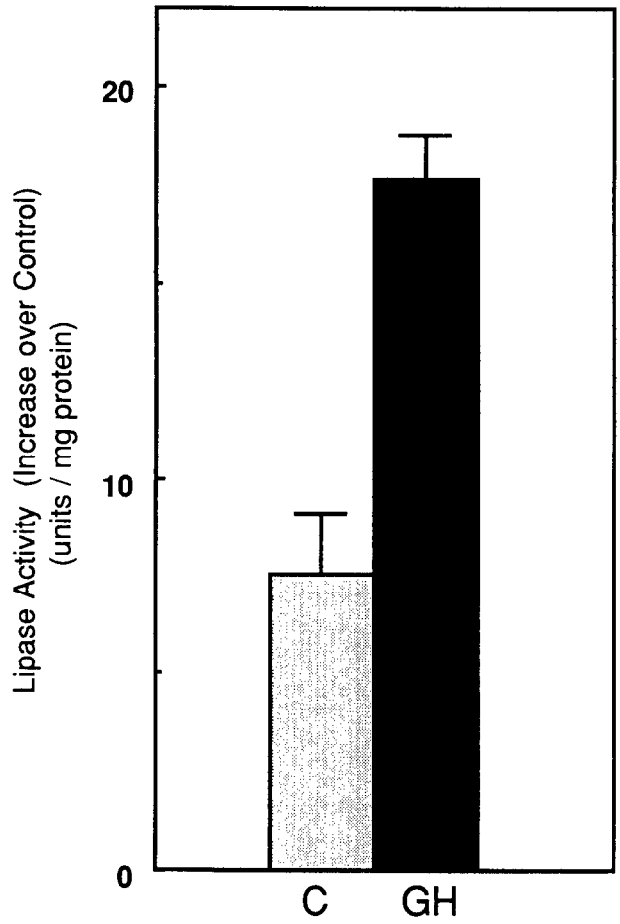

Fig 4. GH potentiates epinephrine-stimulated HSL activity after 24 hours. The increment in lipase activity due to epinephrine (1 ng/mL) is shown. Cells incubated with (■) or without (□) GH (50 ng/mL) for 23 hours. During the final hour, the response to epinephrine was measured. Control values were 32.2 ± 1.0 in the absence of GH, and 39.8 ± 1.5 U/mg protein in the presence of GH ($P < .005$).

Short-Term Responses to GH

A hallmark of GH action in adipocytes is that short-term responses (eg, 30-minute) are characteristically opposite to the delayed responses (eg, after several hours). In GH-deficient preparations, the delayed responses to GH, which are insulin-antagonistic, are typically preceded by transient insulin-like responses.[2,6] One example of such a response is inhibition by GH of epinephrine-stimulated lipolysis in adipocytes from hypophysectomized rats.[2,6,31-33] Figure 5 shows that in the 3T3-F442A adipocytes, GH inhibited epinephrine-stimulated lipolysis. Comparing Figs 4 and 5 indicates that the inhibition of epinephrine-stimulated lipolysis is transient, since after 24 hours, GH potentiated epinephrine-stimulated glycerol release and HSL activity.

Table 1. Noncorrespondence of GH-Induced Changes in Lipid Accumulation and GPD Activity

Incubation Time	Lipid Accumulation (cpm/mg lipid)		GPD Activity (U/mg protein)	
	Control	GH	Control	GH
48-hour	23,064 ± 604	17,136 ± 302	24.8 ± 0.3	28.0 ± 0.8
Percent change due to GH	26% decrease		13% increase	
4-hour	3,242 ± 138	4,619 ± 260	35.8 ± 2.6	30.8 ± 1.0
Percent change due to GH	50% increase		14% decrease	

NOTE. Lipid accumulation is expressed as the incorporation of ^{14}C-glucose into lipid. GPD activity is evaluated as the oxidation of NADH. Values are presented as means ± SE of quadruplicate observations in a representative experiment. After 48 hours, GH (1 μg/mL) produced a significant ($P < .01$) decrease in lipid accumulation and increase ($P < .05$) in GPD activity. After 4 hours, GH produced a significant ($P < .05$) increase in lipid accumulation, and had no significant effect on GPD activity. Similar observations were made in two other experiments.

Another insulin-like response evident after short-term incubation with GH is increased, rather than decreased, lipid accumulation (Table 1). However, GH had a negligible or even slightly inhibitory effect on GPD in the same cells. This indicates that GPD, which is so sensitive to GH in the preadipocytes in association with differentiation, does not contribute substantially to modulation of lipid synthesis by GH in the differentiated adipocytes.

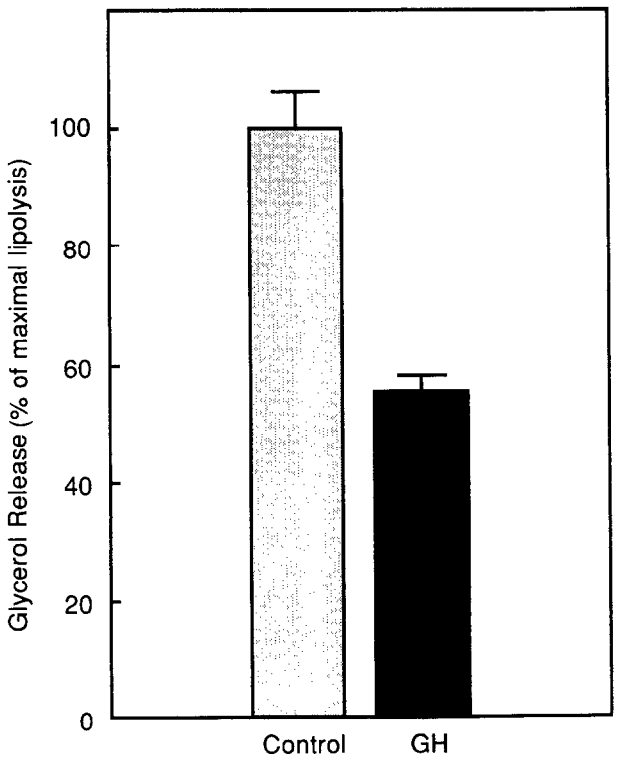

Fig 5. GH inhibits epinephrine-stimulated lipolysis with 30 minutes incubation. The increase in glycerol release due to epinephrine is shown. Each bar represents the mean ± SE of quadruplicate observations in a representative experiment. Epinephrine, 1 ng/mL, increased glycerol release from a control value of .88 to 1.08 μmol/mg protein in this experiment. This was designated maximal lipolysis and corresponds to 100% (designated control). When epinephrine and GH (500 ng/mL) were added simultaneously (■), GH reduced the change in glycerol release by 45%. The decrease was statistically significant ($P < .01$). Similar observations were made in three other experiments.

DISCUSSION

GH Is Lipolytic and Stimulates the Activity of HSL With Chronic Incubation

Recombinant DNA-derived hGH was lipolytic after long-term incubation, in that it stimulated glycerol release in the 3T3-F442A adipocytes. This is significant since it has been reported that highly purified hGH lacked lipolytic activity in closely related 3T3-L1 cells.[34] Differences in handling of the cells (eg, duration of incubation, prior serum deprivation period, glucose concentration in the medium), and possibly in the GH preparation used, may account for this discrepancy. Since the recombinant DNA-derived hGH is necessarily free of pituitary contaminants, the present findings are in agreement with other reports that stimulation of lipolysis is intrinsic to the GH molecule.[10-13] The magnitude of the response observed is characteristically modest,[35] in keeping with observations of 40% stimulation of glycerol release by biosynthetic GH in rat adipose tissue,[10,11] in chicken adipose tissue[12] and in human subjects.[13] It should be noted that the inhibition of lipolysis by adenosine, which potently antagonizes GH-stimulated lipolysis,[36] was unchecked in these experiments, which were performed in the absence of adenosine deaminase. If this enzyme were present, the effect of GH would be expected to be greater, as observed for other hormones.[37]

It is of note that GH alone was lipolytic in the cultured adipocytes. This contrasts with the requirement for dexamethasone in rat adipocytes to detect lipolytic activity of GH,[2-4] and underscores the fact that GH has direct lipolytic activity. The reason for the difference in interaction between GH and dexamethasone in the two types of adipocytes is not clear, especially since GH potentiated the lipolytic effect of epinephrine or theophylline in the 3T3-F442A adipocytes, consistent with observations in rat adipocytes.[2,5]

These studies further indicate that the GH-induced increase in lipolysis is due, at least in part, to stimulation of the activity of HSL when GH is added to cultured adipocytes. The involvement of HSL is also evident in the potentiation by GH of epinephrine-stimulated lipolysis. HSL, which plays a key role in hormone-regulated lipolysis, is activated by phosphorylation stemming from a cyclic adenosine monophosphate (cAMP)-mediated event.[20,38] In fact, the antilipolytic effect of GH in noradrenaline-

stimulated rat adipocytes is associated with dephosphorylation of HSL.[39] With prolonged incubation, GH may increase the activity of HSL by favoring the phosphorylation of the enzyme, although this has not been demonstrated. In addition, the lag period required for detecting the stimulation of HSL by GH in 3T3-F442A adipocytes suggests that synthesis of the enzyme, possibly induction of the gene for HSL,[40,41] may be involved.

GPD Is Insensitive to GH in 3T3-F442A Adipocytes

In differentiated 3T3-F442A adipocytes, GPD activity was relatively insensitive to GH, particularly as compared with preadipocytes. Changes in GPD activity thus do not correspond to, and do not appear to contribute substantially to, the changes in lipid accumulation produced by GH in 3T3-F442A adipocytes. The lack of effect of GH on GPD may be related to the relatively high levels of GPD activity in differentiated 3T3-F442A adipocytes, as compared with the preadipocytes. Hence, subtle modulation of GPD by GH might be obscured by high basal levels in the adipocytes, while regulation by GH is evident in the preadipocytes, which have low basal levels of GPD activity. This is in marked contrast to sensitivity of GPD to GH in the preadipocytes, in which this enzyme is used as a marker of differentiation, since its activity increases as much as 2,000-fold with GH-induced differentiation and its synthesis and expression of its mRNA increase similarly.[27-29,42,43]

While GPD appears to be insensitive to GH in the adipocytes, other lipogenic enzymes, for example those involved in fatty acid synthesis (fatty acid synthetase, malic enzyme, ATP citrate lyase, acetyl CoA carboxylase, pyruvate carboxylase) may be modulated by GH. All of these enzymes, except pyruvate carboxylase, have been reported to be inhibited by GH in hepatocytes or rat adipocytes.[44,45] Like GPD, they have all been observed to increase with differentiation of 3T3-F442A or the closely related 3T3-L1 adipocytes,[45-48] suggesting that they might be regulated by a differentiation-promoting agent such as GH. However, activities of the various lipogenic enzymes can be regulated independently of each other.[49] Furthermore, changes in lipogenic enzymes do not have to be invoked to account for GH-dependent changes in lipid accumulation in these cells, since GH-induced changes in glucose transport may be sufficient to account for these responses to GH in the 3T3-F442A adipocytes.[16]

In conclusion, using an in vitro model for studying chronic metabolic effects of GH, which appear to be representative of its in vivo physiological actions, we have examined cellular mechanisms for effects of GH on lipid metabolism. Our findings indicate that GH alone is lipolytic in 3T3-F442A adipocytes, and that changes in the activity of HSL contribute to its lipolytic activity. GPD is not substantially regulated by GH in the differentiated cells, although this lipogenic enzyme is highly sensitive to GH in 3T3-F442A preadipocytes. It will be of great interest to pursue mechanisms for these direct effects of GH at the molecular level.

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