Long-Term Regulation of Lipolysis and Hormone-Sensitive Lipase by Insulin and Glucose

Leida M. Botion and Allan Green

Adipocyte glucose transport can be impaired by prolonged hyperglycemic conditions. However, at the whole body level, lipolysis is quantitatively a more important function of adipocytes than glucose uptake. We have therefore investigated the effect of prolonged high glucose and insulin on adipocyte lipolysis in basal conditions or with maximal concentrations of adenosine deaminase (ADA), dibutyryl cyclic-AMP (dbcAMP), or isoproterenol (ISO). Neither insulin nor glucose alone affected basal or maximally stimulated lipolysis. However, insulin plus glucose increased the rate of ADA-, dbcAMP-, and ISO-stimulated lipolysis by 40–65%, and the effect was maximal by 8 h. When insulin was kept constant, the half-maximally effective concentration (EC_{50}) of glucose was ~2.5 mmol/l. We also demonstrated that the effect is not glutamine-dependent and does not induce insulin resistance of lipolysis. Because the effect of insulin and glucose was evident whether lipolysis was stimulated by ADA, dbcAMP, or ISO, we hypothesized that the expression of the rate-limiting enzyme for lipolysis, hormone-sensitive lipase (HSL), was increased. Our results show that insulin plus glucose-treated cells contain ~40% more HSL protein than control cells, in good agreement with the increase in maximally stimulated lipolysis. We conclude that hyperglycemic-hyperinsulinemic conditions increase basal and maximal adipocyte lipolysis by a mechanism that is not glutamine-dependent and involves maintenance of cellular concentrations of HSL. The results also provide evidence that factors other than translocation of HSL to the lipid droplet are necessary to activate the enzyme. *Diabetes* 48:1691–1697, 1999

It is well established that adipocyte glucose transport can be impaired by prolonged hyperglycemic conditions (1–5), which can induce or worsen insulin resistance (6). Adipocytes incubated in primary culture in media containing glucose, insulin, and amino acids develop glucose dose–dependent insulin resistance of glucose transport (3,7–9), by mechanisms that include impairment of insulin signaling and responsiveness and recruitment of fewer glucose transporters to the plasma membrane in response to insulin (3,8).

Although these are important findings, it raises the question of whether other functions of adipocytes are impaired. At the whole body level, lipolysis is quantitatively a more important function of adipocytes than glucose uptake because adipose tissue is the major regulator of the supply of lipid energy to tissues during fasting.

The rate-limiting step of adipose tissue lipolysis is the hydrolysis of triacylglycerol by hormone-sensitive lipase (HSL). Acute activation of HSL has been shown to be controlled by phosphorylation/dephosphorylation events, which are regulated by the respective activities of cAMP-dependent protein kinase (PKA) and protein phosphatase (10). Lipolytic hormones, such as catecholamines, cause an activation of PKA that phosphorylates and activates HSL (11,12). The enzyme is subject to complex activity regulation involving multisite phosphorylation. Phosphorylation of Ser-563 and Ser-565 in HSL have been shown to be mutually exclusive, and phosphorylation of Ser-565 has been shown to inhibit subsequent phosphorylation and activation by PKA (13). Most recently, it was reported that phosphorylation sites other than that previously identified are phosphorylated in HSL in response to isoproterenol stimulation of rat adipocytes (14). Using site-directed mutagenesis, it was possible to elucidate that sites Ser-659 and Ser-660 are critical activity-controlling sites in the activation of HSL upon phosphorylation with PKA, whereas Ser-563 plays a minor role in direct activation of HSL (14). They also showed that the phosphorylation state of Ser-660 is presumably more important in adipocytes at maximal lipolysis (14). Insulin, in contrast, prevents HSL phosphorylation and activation by lowering cAMP levels and activity of cAMP-dependent protein kinase (12,15,16). In adipocytes, insulin-mediated reduction of cAMP/cAMP-dependent protein kinase is mainly mediated through phosphorylation of Ser-302 (17) and activation of phosphodiesterase 3B (PDE 3B) (18). Recent studies strongly suggest that the insulin-stimulated kinase that phosphorylates PDE 3B in vitro is protein kinase B (19). It has also been observed that on lipolytic stimulation, HSL translocates from the cytosol to the lipid droplet (20,21) by a mechanism involving redistribution of perilipins (a family of proteins found tightly bound to the limiting surface of the lipid droplet) from fat cake to cytosol (22,23). Conversely, insulin redistributes HSL back to the cytosol and promotes a shift of perilipin from the cytosol back to fat cake (22,23).

Although it is well established that glucose and insulin can chronically regulate the glucose transport system in isolated

From the Department of Physiology and Biophysics (L.M.B.), Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil; and the Bassett Research Institute (A.G.), The Mary Imogene Bassett Hospital, Cooperstown, New York.

Address correspondence and reprint requests to Allan Green, PhD, Bassett Research Institute, The Mary Imogene Bassett Hospital, One Atwell Rd., Cooperstown, NY 13326-1394. E-mail: allan.green@bassett.org.

Received for publication 5 October 1998 and accepted in revised form 19 May 1999.

ADA, adenosine deaminase; BSA, bovine serum albumin; dbcAMP, dibutyryl cyclic-AMP; DMEM, Dulbecco's modified Eagle's medium; 2-DOG, 2-deoxy-[^{3}H]glucose; HSL, hormone-sensitive lipase; ISO, isoproterenol; PBS, phosphate-buffered saline; PDE 3B, phosphodiesterase 3B; PKA, protein kinase A.

adipocytes (3,24,25), little information exists about regulation of lipolysis by these agents. It has been shown that at least some of the regulatory effects of glucose on cellular metabolism are mediated by the hexosamine biosynthesis pathway, in which fructose-6-phosphate is converted to glucosamine-6-phosphate with glutamine acting as the donor of its amido group (8). Other studies strengthened the hypothesis and currently support the idea that glucosamine flux results in transcriptional regulation of a number of genes relevant to glucose homeostasis in hepatocytes (26,27). Thus, we decided to investigate the effect of prolonged high glucose and insulin on adipocyte lipolysis and the effect of glutamine on this metabolic process.

RESEARCH DESIGN AND METHODS

Materials. Porcine monocomponent insulin from Eli Lilly (Indianapolis, IN); bovine serum albumin (BSA) (Bovuminar Reagent Pure Powder; Intergen, Purchase, NY); collagenase from Worthington (Freehold, NJ); [U-^{14}C]glucose, 2-deoxy-[^{3}H]glucose from NEN-DuPont (Boston, MA); Dulbecco's modified Eagle's medium (DMEM) from Gibco (Grand Island, NY); fetal bovine serum from Hyclone (Logan, UT); Safety-Solve from Research Products International (Mount Prospect, IL); and other reagent grade chemicals from Sigma (St. Louis, MO) were used in this study.

Animals. Male Sprague-Dawley rats weighing 200–270 g were purchased from Harlan (Indianapolis, IN) and maintained on a 12-h light-dark cycle.

Primary culture of adipocytes. Adipocytes were isolated under sterile conditions from epididymal fat pads by the method of Rodbell (28) and maintained in primary culture according to the method of Marshall and colleagues (1,3,7) for various times (0–24 h) in DMEM formulated without glucose and containing 1 mmol/l sodium pyruvate, 25 mmol/l HEPES, 2% fetal bovine serum, 20 U/ml penicillin, 20 mg/ml streptomycin, and 1% BSA. Isolated adipocytes were diluted (1:120 wt/vol) and placed in sterile airtight polypropylene tubes with cells floating on top of the medium in a thin cell layer and incubated in glucose-free DMEM (control) or 25 ng/ml insulin (I), 20 mmol/l glucose (G), or both (I + G). Insulin and glucose concentrations used in these experiments were reported to be maximally effective in the induction of desensitization of the glucose transport system (3). In some experiments, the cells were incubated in DMEM with or without glutamine. After incubation, cells were washed three times with cold buffer containing 137 mmol/l NaCl, 5 mmol/l KCl, 4.2 mmol/l $NaHCO_3$, 1.3 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l KH_2PO_4, 20 mmol/l HEPES (pH 7.4), and 1% BSA.

Lipolysis assay. Lipolysis was measured by following the rate of glycerol release, as described previously (29). At the end of the incubation period, cells were washed as described above, incubated at 37°C for 30 min and treated as indicated in RESULTS.

Determination of hydrolysis of newly synthesized triacylglycerol. After preincubating cells for 16 h in DMEM containing 5 mmol/l [U-^{14}C]glucose (10 nCi/ml) and 25 ng/ml insulin, cells were washed to remove radioactivity, and lipolysis was measured for various times (0–2 h) in the presence of 1 µmol/l isoproterenol. At the end of the incubation period, 400 µl of cell suspension was transferred to plastic microtubes containing silicone oil (100 µl) and centrifuged for 30 s. The tubes were cut through the middle of the oil layer, and the radioactivity present in the incubation medium (aqueous phase) was determined by the addition of Safety-Solve (aqueous cocktail with high counting efficiency) and used as a lipolysis index. After stimulating adipocytes with isoproterenol, the lipolytic products released in the incubation medium are glycerol and free fatty acids. Using aqueous cocktail, we have detected mainly [^{14}C]glycerol released into the incubation media.

Quantification of HSL. Adipocytes were incubated in glucose-free DMEM (control) or DMEM containing insulin plus glucose (I + G) for 0 or 16 h and then washed with phosphate-buffered saline (PBS) and dissolved in Laemmli sample buffer (30). To determine the subcellular distribution of HSL, adipocytes were incubated in glucose-free DMEM (control) or DMEM containing insulin plus glucose (I + G) for 0 or 16 h. At the end of the incubation period, the cells were washed in cold buffer (137 mmol/l NaCl, 5 mmol/l KCl, 4.2 mmol/l $NaHCO_3$, 1.3 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l KH_2PO_4, 20 mmol/l HEPES, and 1% BSA) and incubated at 37°C in basal conditions or in the presence of 1 µmol/l isoproterenol. After 30 min, cells were washed with PBS and homogenized, and cell fractions were extracted according Holm et al. (31). The extracts were resolved on SDS-PAGE (8% gels) and Western blotted with antiserum against a recombinant HSL fusion protein, as described previously (32). Densitometric analysis of enhanced chemiluminescence–detected Western blots allowed a quantitative measurement of the enzyme and its subcellular distribution. A previous study (32) demonstrated that the amount of extract added is within the linear range.

Glucose transport assay. After incubation, adipocytes were washed and suspended in the buffer containing 137 mmol/l NaCl, 5 mmol/l KCl, 4.2 mmol/l $NaHCO_3$, 1.3 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 0.5 mmol/l $MgSO_4$, 0.5 mmol/l KH_2PO_4, 20 mmol/l HEPES (pH7.4), and 1% BSA and uptake of 2-deoxy-[^{3}H]glucose (2-DOG) was used as an assay to determine the effect of insulin on the rate of glucose transport (33).

Lipogenesis. After incubation of adipocytes for 16 h in DMEM containing 5 mmol/l [U-^{14}C]glucose (10 nCi/ml) and 25 ng/ml insulin, incorporation rates in total lipids were measured by transferring 400 µl of the cell suspension to plastic microtubes containing 100 µl of silicone oil. After centrifuging for 30 s, the tubes were cut through the middle of the oil layer, and the extraction and counting of the lipids was done by adding a toluene-based scintillation fluid [5 g of diphenyloxazol, 50 mg of 1,4-bis-2.2 (4 methyl-5-phenoloxazolyl)benzene per liter of toluene] to the cells. Radioactivity detected by this method was used as an index of triacylglycerol synthesis (34). This very simple technique is possible because the toluene extracts the total lipids, which are therefore counted with high efficiency, whereas the water-soluble metabolites remain in the water phase and are not counted to a significant degree (34). The rate of conversion of [U-^{14}C]glucose to ^{14}C-labeled lipids is very similar to total radioactivity associated with the cell pellet in the presence of 5 mmol/l glucose (35).

Statistical analysis. All experiments were repeated at least three times on different days. Statistical analysis was, as appropriate, by paired *t* test or one-way analysis of variance followed by the Tukey test for pair-wise comparisons of treatment groups with the use of SigmaStat 2.0 (Jandel).

RESULTS

To determine the effect of prolonged incubation with insulin and glucose on lipolysis, adipocytes were incubated in primary culture for up 16 h in glucose-free media (control) or in the presence of 25 ng/ml insulin (I) or 20 mmol/l glucose (G) or both (I + G). After incubation, the cells were washed, and lipolysis (glycerol release) was measured over 30 min with no additions, or with maximal concentrations of adenosine deaminase (10 µg/ml), isoproterenol (1 µmol/l), or dbcAMP (2 mmol/l). As shown in Fig. 1, neither insulin nor glucose alone had any effect on maximally stimulated lipolysis when compared with the control group. However, combined treatment with insulin plus glucose led to a significant increase by 43% in adenosine deaminase, 40% in isoproterenol, and 65% in dbcAMP-stimulated lipolysis in relation to the control group. When compared with insulin- or glucose-pretreated groups, the increase was 70 and 14% in adenosine deaminase, 75 and 27% in isopro-

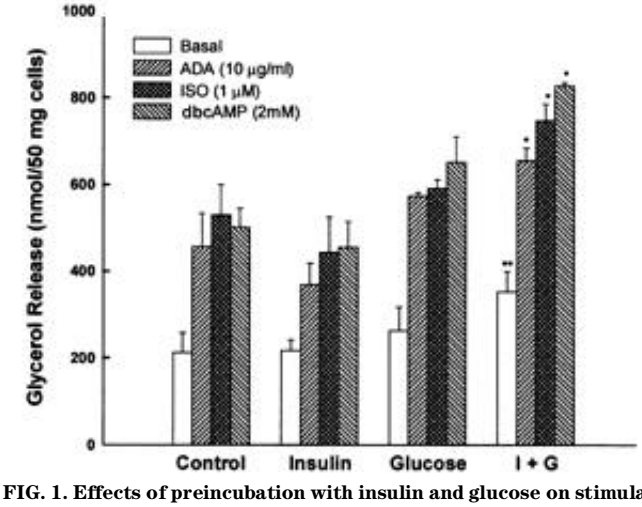

FIG. 1. Effects of preincubation with insulin and glucose on stimulated lipolysis. Adipocytes were preincubated for 16 h in glucose-free media (control) or in the presence of 25 ng/ml insulin (I), 20 mmol/l glucose (G), or both (I + G). Cells were then washed in glucose and insulin-free buffer, and basal, ADA, ISO, or dbcAMP-stimulated lipolysis was measured for 30 min. Data represent means ± SE. *P < 0.05, I + G versus control and insulin; **P < 0.05, I + G basal versus control basal.

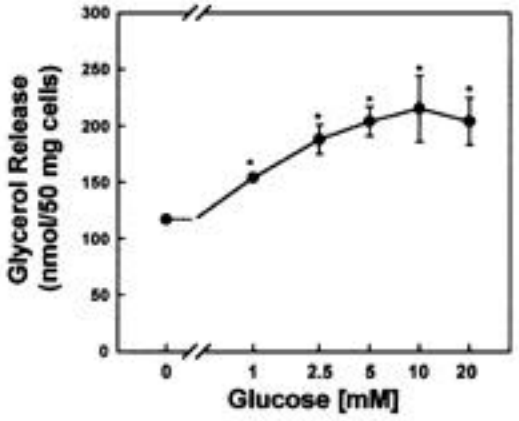

FIG. 2. Dose-response curve for glucose effects on adenosine deaminase–stimulated lipolysis. Cells were preincubated in media containing 25 ng/ml insulin and the indicated D-glucose concentrations for 6 h. Then the cells were washed in glucose and insulin-free buffer, and adenosine deaminase–stimulated lipolysis was measured for 30 min. Data represent means ± SE. *$P < 0.05$.

terenol, and 70 and 26% in dbcAMP-stimulated lipolysis, respectively. The simultaneous presence of insulin plus glucose during 16 h of incubation increased the basal lipolytic rate by 66% compared with that found in control and insulin-treated cells. Taking into account the increase in basal lipolysis, there is a difference in the level of stimulation of I + G compared with controls only in adipocytes stimulated with dbcAMP. These findings demonstrate that the combination of insulin and glucose leads to an increase in the rate of basal and stimulated lipolysis.

To determine the effective concentration of glucose, we incubated cells with insulin plus various concentrations of glucose for 6 h, and then glycerol release was measured in the presence of adenosine deaminase, whose effect on lipolysis is similar to that observed with other stimulating agents (Fig. 1). As can be seen in Fig. 2, increasing glucose concentrations led to a progressive increase in glycerol release and the half-maximally effective concentration (EC_{50}) values were ~2.5 mmol/l glucose.

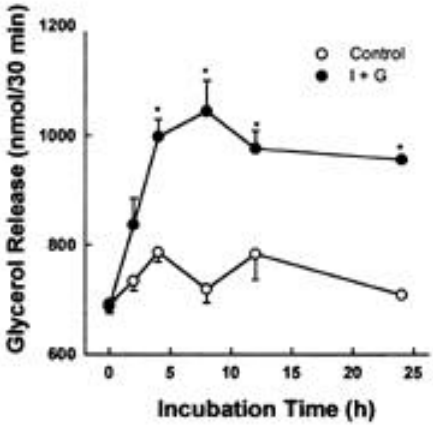

FIG. 3. Time course for the effect of insulin plus glucose on lipolysis. Adipocytes were preincubated in glucose-free media (control) or with 25 ng/ml insulin and 20 mmol/l glucose (I + G) for the times indicated. Then the cells were washed in glucose and insulin-free buffer, and dbcAMP-stimulated lipolysis was determined. Data represent means ± SE. *$P < 0.05$, control versus I + G.

To investigate the time course of the effect of insulin plus glucose on lipolysis, adipocytes were cultured for various times in glucose-free media (control) or in the presence of 25 ng/ml insulin plus 20 mmol/l glucose (I + G). After various times, the cells were washed, and maximal lipolytic rates were measured by acutely stimulating the cells with 2 mmol/l dbcAMP. As can be seen in Fig. 3, a 43% increase in glycerol release in treated cells is apparent at 4 h of incubation and remained constant up to ~24 h. In control cells, the rate of glycerol release did not change appreciably over the 24-h incubation.

Cells incubated with insulin plus glucose for prolonged periods would be expected to have high rates of triacylglycerol synthesis. Therefore, a possible explanation for the increased rate of lipolysis after these treatments may be that newly synthesized triacylglycerol is more readily hydrolyzed than existing triacylglycerol. To investigate this possibility, we performed the experiments illustrated in Fig. 4. Adipocytes were incubated with insulin (25 ng/ml) and 5 mmol/l glucose plus 10 nCi/ml [U-^{14}C]glucose for 16 h. The cells were then

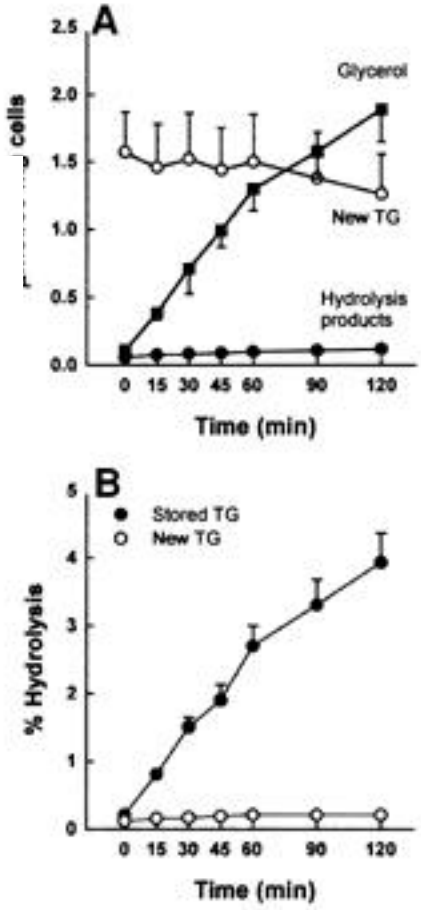

FIG. 4. Relationship between newly synthesized triacylglycerol (TG) and lipolysis (*A*) and percent of hydrolysis of newly synthesized and stored triacylglycerol (*B*). Adipocytes were incubated with insulin (25 ng/ml) and 5 mmol/l glucose, plus 10 nCi/ml [U-^{14}C]glucose for 16 h, and the rate of incorporation in triacylglycerol and hydrolysis products was measured as described in METHODS. Glycerol release was determined at intervals after incubation of adipocytes with 1 µmol/l isoproterenol. Percent of hydrolysis of newly synthesized triacylglycerol was calculated in relation to radioactive hydrolysis products, and percent of stored triacylglycerol was calculated in relation to glycerol release. Data represent means ± SE.

washed and incubated with 1 µmol/l isoproterenol, and at intervals, radioactivity released into incubation medium was determined (see METHODS). This allowed us to calculate the percent hydrolysis of newly synthesized triacylglycerol. Percent of stored triacylglycerol was calculated in relation to total glycerol release. Total cellular triacylglycerol was estimated from the weight of the cells, assuming that 90% of the cells are triacylglycerol and the average molecular weight of triacylglycerol is 885.4. This experiment demonstrated that the rate of hydrolysis of newly synthesized triacylglycerol is lower than the rate of hydrolysis of stored triacylglycerol and, therefore, that the newly synthesized triacylglycerol is not preferentially hydrolyzed. Therefore, there must be another explanation for the effect of insulin plus glucose on maximal lipolytic rate.

Previous studies (7–9) have demonstrated a regulatory role for glutamine in desensitization of the glucose transport system in adipocytes incubated with high insulin and glucose. Therefore, further studies were performed to examine whether this amino acid was involved in the alterations observed on lipolytic rates. Adipocytes were cultured for 16 h in glucose-free media (control) or in the presence of insulin plus glucose, with or without glutamine. Consistent with previous reports, insulin plus glucose resulted in desensitization of the glucose transport system only when glutamine was present in the incubation medium (Fig. 5*A*). To determine if the effect of insulin plus glucose on lipolysis is dependent on glutamine, we performed the experiments illustrated in Fig. 5*B*. Unlike the glucose transport system where glutamine induced a fall in maximal responsiveness, glutamine had no effect on the ability of insulin plus glucose to regulate lipolysis, i.e., insulin plus glucose increased the rate of lipolysis independently of the presence of glutamine in the media.

To examine the insulin sensitivity of lipolysis and glucose uptake, adipocytes were incubated in media with or without glutamine in the presence of insulin plus glucose for 16 h. The cells were then washed, and the effect of insulin on lipolysis (Fig. 6*A*) and on glucose transport (Fig. 6*B*) were measured. As shown in Fig. 6*A*, the insulin sensitivity of lipolysis was not altered by the presence or absence of glutamine. Conversely, the presence of this amino acid in the incubation media decreased the insulin sensitivity of adipocyte glucose transport, as revealed by a decrease in basal and in the maximally stimulated rate, as has been reported by others (8,9).

Because the effect of insulin plus glucose was evident whether lipolysis was stimulated by adenosine deaminase, dbcAMP, or isoproterenol, we hypothesized that expression of the rate-limiting enzyme for lipolysis, HSL, was increased. Adipocytes were incubated for 0 or 16 h in glucose-free media (control), or in the presence of insulin plus glucose, and

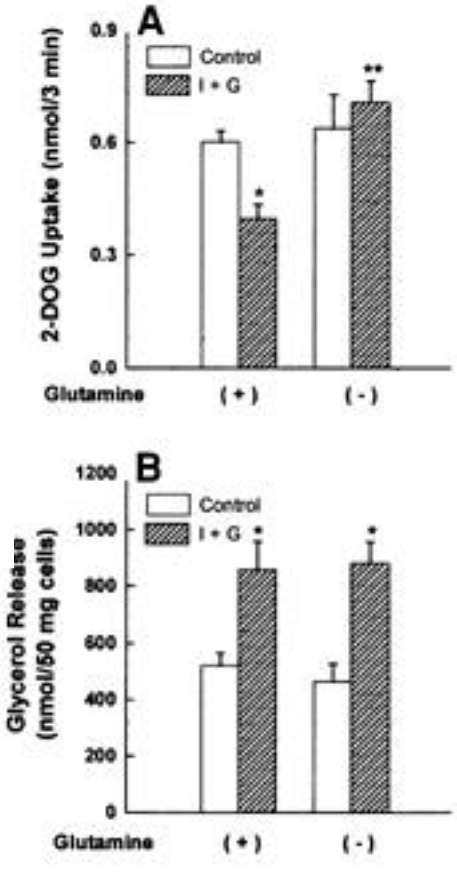

FIG. 5. Effects of L-glutamine on glucose transport (*A*) and lipolysis (*B*). Adipocytes were cultured in media containing 25 ng/ml insulin plus 20 mmol/l glucose in the absence or presence of L-glutamine. After 16 h, cells were washed and dbcAMP-stimulated lipolysis was measured for 30 min. Rates of glucose transport were determined by acutely restimulating cells with 25 ng/ml insulin for 30 min and then measuring the 2-DOG uptake during a 3-min assay. The data represent means ± SE. **P* < 0.05, control versus I + G; ***P* < 0.05, I + G (+) glutamine versus I + G (–) glutamine.

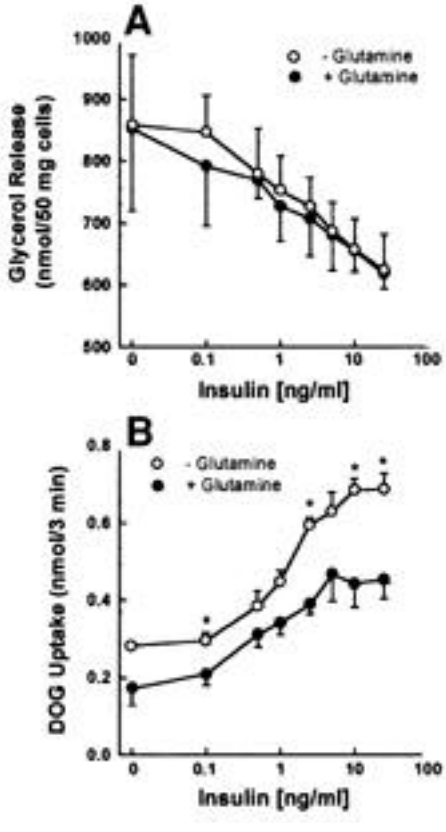

FIG. 6. Effects of L-glutamine on insulin sensitivity of the lipolytic (*A*) and glucose transport (*B*) systems. Adipocytes were treated with insulin plus glucose, with or without glutamine, as in Fig. 5 and washed. The effects of insulin on dbcAMP-mediated lipolysis and 2-DOG uptake were determined by constructing dose-response curves using 0–25 ng/ml insulin. The data represent means ± SE. **P* < 0.05, I + G (+) glutamine versus I + G (–) glutamine.

then extracted and analyzed on Western blots using an antiserum against a rat HSL fusion protein (32). As can be seen in Fig. 7, two immunoreactive bands were detected in all groups. In addition, no change in HSL levels was observed after 16 h of incubation in cells treated with insulin plus glucose in relation to preincubation levels (C_0 and I + G_0 groups), while control cells showed a decrease by 40% when compared with preincubation levels.

Next we investigated the subcellular distribution of HSL, since it has been demonstrated that the enzyme translocates from the cytosol to the fat cake upon lipolytic stimulation (20,21). The results in Fig. 8 show that in control adipocytes without preincubation ($control_0$) in basal conditions, the distribution of HSL is similar in supernatant and fat cake, but after stimulation with 1 µmol/l isoproterenol, an increased amount of HSL was found associated with the fat cake. Similar results were obtained for treated cells without preincubation (data not shown). After 16 h of incubation, the amount of the enzyme decreased in control cells, both in unstimulated and stimulated conditions, and showed an increased amount of HSL associated with the fat cake. In relation to treated cells, the total levels of enzyme (supernatant plus fat cake) did not change when compared with preincubation levels, and a predominant distribution of enzyme in the fat cake was observed in basal and stimulated conditions.

DISCUSSION

In the present study, chronic regulation of lipolysis has been investigated in rat adipocytes maintained in primary culture. The findings demonstrated that prolonged treatment of adipocytes with high glucose and insulin concentrations increases basal and stimulated lipolysis. The mechanisms responsible for the increased rate of lipolysis in adipocytes treated with high glucose and insulin concentrations are not clear. However, hyperglycemia and hyperinsulinemia are frequent findings in obesity and in type 2 diabetes, which are also associated with increased rates of lipolysis (36,37). One possibility is glycosylation of proteins involved in the lipolytic cascade because it has been reported that high concentrations of glucose damage cells through nonenzymatic glycosylation of proteins and accumulation of advanced glycosylation end products (38,39).

Considering that treated cells were incubated in the presence of high glucose and insulin concentrations while control cells were not, the content of newly synthesized triacylglycerol in treated cells was possibly higher than that in control cells, providing more substrate for HSL and consequently increased lipolysis. Our data indicated that stored triacylglycerol is preferentially hydrolyzed by HSL, excluding the possibility that increased triacylglycerol content in treated cells could promote an increase in lipolytic rate.

Many studies (3,8,24,25) have demonstrated that high levels of glucose and insulin induce insulin resistance of the glucose transport system and that this effect requires the simultaneous presence of glutamine (3,7–9). This is believed to be caused by the routing of incoming glucose through the hexosamine biosynthesis pathway (8,9). Therefore, we decided to investigate whether glutamine is required for the effect of glucose and insulin on lipolysis. Our results demonstrate that the insulin plus glucose-induced lipolysis is not glutamine-dependent. At the whole body level, lipolysis is quantitatively much more important in adipose tissue than glucose transport. Therefore, we felt that it was important to determine whether these mechanisms are operative in development of cellular resistance to the antilipolytic effect of insulin. We found that the presence of glutamine in the culture medium did not alter the ability of insulin to inhibit lipolysis. This finding indicates that the well-documented effect of the hexosamine pathway on insulin sensitivity is not universal to all insulin actions and that the effect of insulin on lipolysis is unaffected.

Because the lipolytic response to insulin plus glucose was similar in basal or stimulated conditions, we hypothesized that

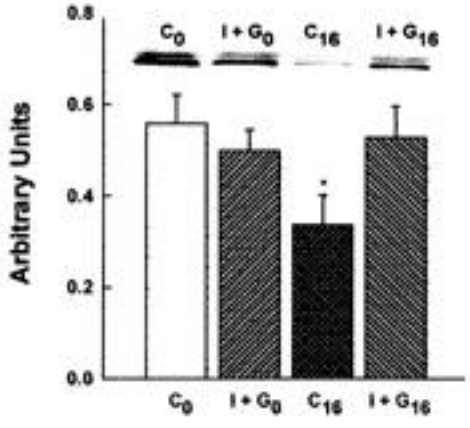

FIG. 7. Adipocyte HSL. Adipocytes were incubated in glucose-free media (C) or in the presence of insulin plus glucose (I + G) for 0 or 16 h. Cells were then washed, and relative concentrations of HSL were measured by Western blot and analyzed by laser densitometry. Densities of both bands together were reported. The inset above the bars shows representative examples of immunoblots of HSL from control or experimental groups. C_0, control at time zero; I + G_0, treated group at time zero; C_{16}, control after 16 h of incubation; I + G, treated group after 16 h of incubation. The data represent means ± SE. *$P < 0.05$.

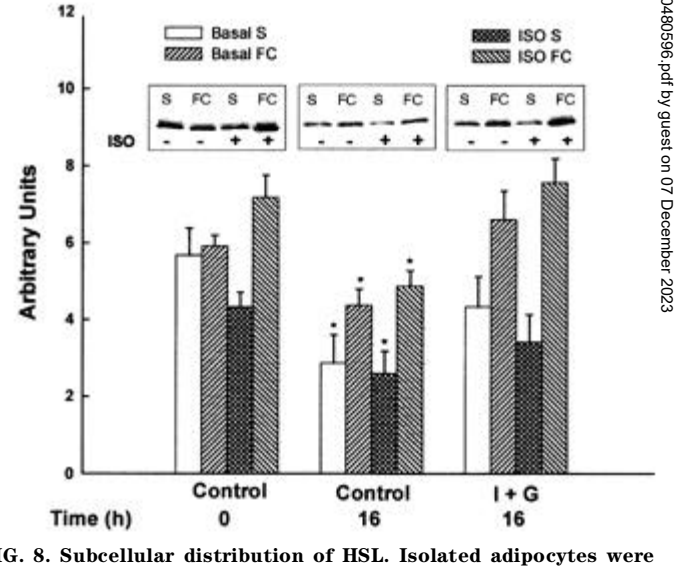

FIG. 8. Subcellular distribution of HSL. Isolated adipocytes were incubated for 0 or 16 h in glucose-free DMEM (control) or in the presence of insulin plus glucose (I + G). HSL levels were measured in basal conditions or in the presence of 1 µmol/l isoproterenol. After 30 min of incubation, cells were washed and homogenized, cell fractions were extracted, and relative concentrations of HSL were measured by Western blot and analyzed by laser densitometry. The insets above the bars are representative immunoblots of subcellular fractions of HSL. S, supernatant; FC, fat cake; ISO, isoproterenol. The data represent means ± SE. *$P < 0.05$, $control_0$ versus $control_{16}$.

expression of HSL, the rate-limiting enzyme for lipolysis, was increased. Our results show two immunoreactive bands: a major band previously identified at ~84 kDa and a minor upper band also previously identified (33,40,41). The nature of the two different forms of the enzyme in the rat adipocytes detected in these studies is unknown. However, they do not appear to represent lipase at different states of phosphorylation (40). Our results also show a decrease in HSL levels in control cells after 16 h of incubation, with no change in treated cells, when compared with levels of the enzyme before incubation. Relevant to our studies is a recent report (42) indicating that exposure of 3T3-F442A adipocytes for 32 h to a medium without glucose led to a decrease in HSL mRNA and HSL total activity. The molecular mechanisms underlying the effect of glucose on HSL gene expression are unknown. It has been demonstrated that glucose can increase gene expression by increasing mRNA stability and by the rate of transcription (42). Alternatively, Kang et al. (43) have shown that chronic exposure of adipose tissue to high concentrations of insulin increases the subsequent lipolytic response of this tissue to β-agonists. Other studies show that insulin has no effect on HSL gene expression (44). Thus, our results and that from Raclot et al. (42) suggest that glucose could participate in the regulation of adipose tissue lipolysis by modulation of HSL gene expression.

The lower levels of HSL in control cells after 16 h of incubation contrast with the rates of lipolysis in intact cells, which were constant over 24 h of incubation. In previous studies (20,21), it was proposed that HSL gains increased access to substrate upon phosphorylation by translocating from the cytosol to the lipid storage droplet by a mechanism involving the translocation of perilipins away from the fat cake (22,23). Other studies demonstrate that expression of perilipins is closely linked to the storage of neutral lipids in adipocytes and steroidogenic cells, and inhibition of triacylglycerol deposition concomitantly inhibits perilipin accumulation in differentiating 3T3-L1 adipocytes; fatty-acid supplementation restores triacylglycerol synthesis and perilipin accumulation (45). Considering that our control cells were incubated without glucose, an explanation for the findings may be that these cells have low rates of triacylglycerol synthesis and consequently perilipin accumulation, which could increase HSL access to the lipid droplet and promote lipid hydrolysis. Our results also show an increased amount of HSL associated with the fat cake after lipolytic stimulation, as previously described (20–23), and more HSL was found associated with the fat cake in basal groups after 16 h of incubation, in relation to control preincubation. The data suggest a translocation of HSL from the cytosol to the lipid droplet during 16 h of preincubation. The distribution of HSL in control cells stimulated with isoproterenol at time zero is similar to insulin plus glucose–treated cells at 16 h, which also contrasts with the rates of lipolysis. The exact activation mechanism of HSL is poorly understood, but it presumably involves both translocation of HSL (20,21) and conformational changes in the HSL molecule. Current evidence suggests that the perilipins not phosphorylated by PKA provide a barrier against hydrolysis by neutral lipid lipases, and this barrier is removed upon phosphorylation by PKA (22). It is possible that different phosphorylation sites in HSL and the degree of perilipin phosphorylation play different roles in the process of translocation and increase in specific activity of HSL (14).

In conclusion, we observed that long-term exposure of adipocytes to high insulin and glucose concentrations increases the rate of lipolysis, which is independent of the presence of glutamine in the incubation media. This effect involves maintenance of cellular concentrations of HSL and provides evidence that factors other than translocation of HSL to the lipid droplet are necessary to activate the enzyme. In addition, we have found that the well-established effect of glucose and insulin to induce resistance of glucose transport does not alter the insulin sensitivity of lipolysis.

ACKNOWLEDGMENTS

L.M.B. was the recipient of a Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) fellowship.

We thank Universidade Federal de Minas Gerais and Fundação de Amparo a Pesquisa de Minas Gerais for financial support.

REFERENCES

1. Marshall S, Garvey WT, Geller M: Primary culture of isolated adipocytes: a new model to study insulin receptor regulation and insulin action. *J Biol Chem* 259: 6376–6384, 1984
2. Unger RH, Grundy S: Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance: implications for the management of diabetes. *Diabetologia* 28:119–121, 1985
3. Garvey WT, Olefsky JM, Matthaei S, Marshall S: Glucose and insulin coregulate the glucose transport system in primary cultured adipocytes: a new mechanism of insulin resistance. *J Biol Chem* 262:189–197, 1987
4. Rosseti L, Giaccari A, DeFronzo RA: Glucose toxicity. *Diabetes* 3:610–630, 1990
5. DeFronzo RA, Bonadonna RC, Ferrannini E: Pathogenesis of NIDDM: a balanced overview. *Diabetes Care* 15:318–368, 1992
6. Baron AD, Zhu JS, Zhu JH, Weldon H, Maianu L, Garvey WT: Glucosamine induces insulin resistance in vivo by affecting GLUT 4 translocation in skeletal muscle. *J Clin Invest* 96:2792–2801, 1995
7. Marshall S, Monzon R: Amino acid regulation of insulin action in isolated adipocytes. *J Biol Chem* 264:2039–2042, 1989
8. Marshall S, Bacote V, Traxinger RR: Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system: role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem* 266:4706–4712, 1991
9. Traxinger RR, Marshall S: Role of amino acids in modulating glucose-induced desensitization of the glucose transport system. *J Biol Chem* 264: 20910–20916, 1989
10. Sztalryd C, Kraemer FB: Regulation of hormone-sensitive lipase in streptozotocin-induced diabetic rats. *Metabolism* 44:1391–1396, 1995
11. Stralfors P, Belfrage P: Phosphorylation of hormone-sensitive lipase by cyclic AMP-dependent protein kinase. *J Biol Chem* 258:15146–15152, 1983
12. Stralfors P, Bjorgell P, Belfrage P: Hormonal regulation of hormone-sensitive lipase in intact adipocytes: identification of phosphorylation sites and effects on the phosphorylation by lipolytic hormones and insulin. *Proc Natl Acad Sci U S A* 81:3317–3321, 1984
13. Garton JA, Yeaman SJ: Identification and role of the basal phosphorylation site on hormone-sensitive lipase. *Eur J Biochem* 191:245–250, 1990
14. Anthonsen MW, Ronstrand L, Wernstedt C, Degerman E, Holm C: Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties *in vitro*. *J Biol Chem* 273:215–221, 1998
15. Londos C, Honnor RC, Dilhon GS: cAMP-dependent protein kinase and lipolysis in rat adipocytes. III. Multiple modes of insulin regulation of lipolysis and regulation of insulin responses by adenylate cyclase. *J Biol Chem* 260: 15239–15145, 1985
16. Smith CJ, Vasta V, Degerman E, Belfrage P, Manganiello VC: Hormone-sensitive cyclic GMP-inhibited cyclic AMP phosphodiesterase in rat adipocytes: regulation of insulin-dependent and cAMP-dependent activation by phosphorylation. *J Biol Chem* 266:13385–13390, 1991
17. Rahn T, Ronnstrand L, Leroy MJ, Wernstedt C, Tornqvist H, Manganiello VC, Belfrage P, Degerman E: Identification of the site in the cGMP-inhibited phosphodiesterase phosphorylated in adipocytes in response to insulin and isoproterenol. *J Biol Chem* 271:11575–11580, 1996
18. Degerman E, Belfrage P, Manganiello VC: Structure, localization and regulation of cGMP-inhibited phosphodiesterase (PDE 3). *J Biol Chem* 272: 6823–6826, 1997

19. Wijkander J, Landstrom TR, Manganiello VC, Belfrage P, Degerman E: Insulin-induced phosphorylation and activation of phosphodiesterase 3B in rat adipocytes: possible role for protein kinase B but not mitogen-activated protein kinase or p70 S6 kinase. *Endocrinology* 139:219–227, 1998
20. Hirsh AH, Rosen OM: Lipolytic stimulation modulates the subcellular distribution of hormone-sensitive lipase in 3T3-L1 cells. *J Lipid Res* 25:665–677, 1984
21. Egan JJ, Greeberg AS, Chang MK, Wek SA, Moos MC Jr, Londos C: Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. *Proc Natl Acad Sci U S A* 89:8537–8541, 1992
22. Clifford GM, McCormick DKT, Vernon RG, Yeaman SJ: Translocation of perilipin and hormone-sensitive lipase in response to lipolytic hormones. *Biochem Soc Trans* 25:S672, 1997
23. Christie AW, McCormick DKT, Emmison N, Kraemer FB, Alberti KGMM, Yeaman SJ: Mechanism of anti-lipolytic action of acipimox in isolated adipocytes. *Diabetologia* 39:45–53, 1996
24. Garvey WT, Olefsky JM, Marshall S: Insulin induces progressive insulin resistance in cultured rat adipocytes: sequential effects at receptor and multiple postreceptor sites. *Diabetes* 35:258–267, 1986
25. Lima FB, Thies RS, Garvey WT: Glucose and insulin regulate insulin sensitivity in primary cultured adipocytes without affecting insulin receptor kinase activity. *Endocrinology* 128:2415–2426, 1991
26. Decaux JF, Antoine B, Kahn A: Regulation of the expression of the L-type pyruvate kinase gene in adult rat hepatocytes in primary culture. *J Biol Chem* 264: 11584–11590, 1989
27. Cifuentes ME, Espinet C, Lange AJ, Pilkis SJ, Hod Y: Hormonal control of 6-phosphofructo-2-kinase/frutose-2,6-biphosphatase gene expression in rat hepatoma cells. *J Biol Chem* 266:1557–1563, 1991
28. Rodbell M: Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* 239:375–380, 1964
29. Gasic S, Green A: G_i down-regulation and heterologous desensitization in adipocytes after treatment with the α_2-agonist UK 14304. *Biochem Pharma - col* 49:785–790, 1995
30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
31. Holm C, Langin D, Manganiello V, Belfrage P, Degerman E: Regulation of hormone-sensitive lipase activity in adipose tissue. *Meth Enzymol* 286:45–67, 1997
32. Green A, Dobias SB, Walters DJA, Brasier AR: Tumor necrosis factor increases the rate of lipolysis in primary cultures of adipocytes without altering levels of hormone-sensitive lipase. *Endocrinology* 134:2581–2588, 1994
33. Green A: Adenosine receptor down-regulation and insulin resistance following prolonged incubation of adipocytes with an A_1 adenosine receptor agonist. *J Biol Chem* 262:15702–15707, 1987
34. Moody AJ, Stan MA, Glieman J: A simple free fatty cell bioassay for insulin. *Horm Metab Res* 6:12–16, 1973
35. Gliemann J, Rees WD, Foley JA: The fate of labelled glucose molecules in the rat adipocyte: dependence on glucose concentration. *Biochim Biophys Acta* 804:68–76, 1984
36. Bjorntorp P, Bergman H, Varnauskas E: Plasma free fatty acid turnover in obesity. *Acta Med Scand* 185:351–356, 1969
37. Jensen MD, Haymond MW, Rizza RA, Cryer PE, Miles JM: Influence of body fat distribution on free fatty acid metabolim in obesity. *J Clin Invest* 83: 1168–1173, 1989
38. Brownlee M, Vlassara H, Cerami A: Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Inter Med* 101:527–537, 1984
39. Brownlee M, Vlassara H, Cerami A: Advanced glycosylation and products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318: 1315–1321, 1988
40. Holm C, Belfrage P, Fredrikson G: Immunological evidence for the presence of hormone-sensitive lipase in rat tissues other than adipose tissue. *Biochem Biophys Res Commun* 148:99–105, 1987
41. Holm C, Belfrage P, Fredrickson G: Human adipose tissue hormone-sensitive lipase: identification and comparison with other species. *Biochim Biophys Acta* 1006:193–197, 1989
42. Raclot T, Dauzats M, Langin D: Regulation of hormone-sensitive lipase expression by glucose in 3T3-F442A adipocytes. *Biochem Biophys Res Commun* 245: 510–513, 1998
43. Kang ES, Betts D, Fain JN, Suleiman WB, Myers L: Chronic exposure of rat fat cells to insulin enhances lipolysis and activation of partially purified hormone-sensitive lipase. *Diabetes* 42:1415–1424, 1993
44. Plee-Gautier E, Grober J, Duplus E, Langin D, Forest C: Inhibition of hormone-sensitive lipase gene expression by cAMP and phorbol esters in 3T3-F442A and BFC-1 adipocytes. *Biochem J* 318:1057–1063, 1996
45. Brasaemle DL, Barber T, Kimmel AR, Londos C: Post-translational regulation of perilipin expression: stabilization by stored intracellular neutral lipids. *J Biol Chem* 272:9378–9387, 1997