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GLYCEROL-3-PHOSPHATE DEHYDROGENASE ACTIVITY IN HUMAN LYMPHOCYTES: EFFECTS OF INSULIN, OBESITY AND WEIGHT LOSS

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SUMMARY: Insulin exposure stimulates an increase in glycerol-3-phosphate dehydrogenase (G3PDH) activity in isolated human lymphocytes that correlates to an increase in G3PDH mRNA and requires new protein synthesis. Synthetic diacylglycerol or phorbol ester can mimic the effect of insulin on G3PDH activity, suggesting that protein kinase C may be involved in regulation of G3PDH levels. In addition, lithium chloride, an inositol phosphate phosphatase inhibitor, and calcium uptake inhibitors can abolish insulin stimulation of G3PDH activity. For obese subjects in whom insulin resistance *in vitro* can be demonstrated, the extent of insulin stimulation of G3PDH activity is decreased compared to normal weight individuals, and treatment by a very low calorie diet restores insulin stimulation of G3PDH activity. Thus, insulin stimulation of G3PDH activity is dependent upon the metabolic state of the subject from whom the cells are obtained.

Glycerol-3-phosphate dehydrogenase (G3PDH) occupies the branch point between the glycolytic pathway and triglyceride biosynthesis (1-3). It has been reported in murine adipocytes that G3PDH activity and mRNA levels can be regulated by insulin and that enzyme activity also serves as a late marker of differentiation (4-6). Because obesity appears to be correlated to malfunctions of metabolism as well as insulin utilization (7-10), we hypothesized that alterations in metabolism or insulin response might be found in all cells of obese subjects. Given the role of G3PDH in regulating nutrient flow between carbohydrate and triglyceride metabolism (2,3), we have examined insulin effects on the activity of this enzyme in human lymphocytes from normal weight individuals and obese subjects. Because lymphocytes share insulin responses and many metabolic pathways in common with cells from other tissues (11-13) and can be isolated readily, lymphocyte responses may be of use as an indicator and monitor of cellular metabolism. In particular, studies over long periods of time are possible with regular analysis of blood samples.

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Abbreviations used are:G3PDH, glycerol-3-phosphate dehydrogenase; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PMA, phorbol-12β-myristate-13α-acetate; VLCD, very low calorie diet.

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MATERIALS AND METHODS

Subjects and diet treatment. Normal-weight healthy donors were recruited from Rice University as control subjects. A group of obese patients (17 women and 6 men) that were progressing through the diet program were recruited from the Optifast Program at Memorial City General Hospital, Houston. All subjects provided written, voluntary, informed consent following a detailed explanation of the procedure. On the basis of laboratory examinations, all obese patients had normal liver, kidney, and thyroid function. The mean age of patients was 41.9 ± 10.7 y, range from 20 to 65 years old. Mean initial body weight was 96.7 ± 22.3 kg, range from 70.0 - 153.7 kg. A subset of the patients in the weight program (n = 11) that were treated with a very low calorie diet (VLCD) program were examined throughout the period of diet treatment (10 weeks) and refeeding (6 weeks). During the first 10 week period, all of the calories (800 cal/day) were supplied by a special formula diet, Optifast-800.

Lymphocyte culture. Peripheral blood mononuclear cells were isolated from human blood obtained by venipuncture using Ficoll-Hypaque discontinuous density gradient centrifugation. The cells isolated were washed free of plasma and cultured in CFBI 1000 medium (14) as described previously (15). Cells were pulsed with 0.17 μCi [^{3}H]thymidine/well 24 h before harvesting on day 5. All assays were carried out in triplicate.

G3PDH assay and effects of inhibitors. Lymphocytes were cultured in CFBI 1000 media with 1% heat-inactivated fetal calf serum at a glucose concentration of 14.4 μg/ml in 200 ml flasks at 150,000 cells/ml. Insulin (0.01 unit/ml) was added to the medium in half of the flasks at the beginning of the culture. The cells were cultured in 5% CO_2 at 37°C for 5 days, resuspended by scraping, and then centrifuged for 10 minutes at 270 x g. The cell pellets were washed twice with phosphate-buffered saline (PBS, containing 10 mM sodium phosphate, 1 mM EDTA, and 5 mM DTT, pH 7.5). After washing, cell pellets were resuspended in 150 μl of PBS and sonicated three times for fifteen seconds at a setting of 4 by an Ultrasonics-Heat System instrument. The sonicate was centrifuged in a microcentrifuge at the highest speed for 10 minutes at 4°C, and the supernatants were used for G3PDH assay. The G3PDH assay was a modification of the method of McGinnis and DeVellis (16); reaction mixture (1 ml) contained 1 mM dihydroxyacetone phosphate as substrate, 0.1 mM NADH as coenzyme and spectrophotometric indicator in PBS, and 50 μl of cell extract containing G3PDH. One unit of G3PDH activity was defined as the enzyme required to catalyze the oxidation of 1 nmole NADH per minute, quantitated by the decrease in NADH absorbance at 340 nm. Protein content in the cell extracts was determined by Bio-Rad assay using lysozyme as a standard (17). Inhibitors of RNA synthesis (actinomycin D, 10 μg/ml) or protein synthesis (cycloheximide, 10 μg/ml) were added to the cultures on day 5 coupled with insulin addition (0.01 unit/ml) for 30 minutes, then the cells were scraped and collected. The amounts of other compounds added were OAG at 1 μg/ml, PMA at 100 pg/ml, lithium chloride at 10 mM, nickel chloride at 1 mM, or manganese chloride at 0.6 mM.

RNA isolation and analysis. Human lymphocytes were cultured as described for 5 days with half of the cells exposed to insulin (0.01 unit/ml) for 30 min at day five; then cells were collected. RNA was isolated using a Stratagene RNA isolation kit. The quantity of RNA in samples was determined by measuring absorbance at 260 nm. The purity of RNA samples was determined by the ratio of OD_{260}/OD_{280} (>1.9 for all samples). RNA samples were resuspended in buffer containing formamide, formaldehyde and bromphenol blue, then RNAs (10 μg/lane) were loaded in a 1% agarose gel with 6.7% formaldehyde and run at 50 V. RNA was transferred to a nylon membrane (18) by capillary blotting overnight and immobilized by crosslinking the membrane with UV light in a UV Stratalinker. The membrane was prehybridized at 42°C for 24 hr in a Hybridization Incubator (Robbins Scientific) with buffer containing 50% formamide, 0.5% SDS, 10 x Denhardt's solution, 6 x SSPE solution (containing 3 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M Na_2EDTA), and 50 μg/ml salmon sperm DNA. Radiolabeled DNA probe was added to membrane with hybridization solution containing 50% formamide, 0.5% SDS, and 6 x SSPE solution at 42°C for 24 hr. The nylon membrane was then washed in low stringency solution (2 x SSPE and 0.1% SDS) at room temperature for 3 hours and in high stringency solution (0.2 x SSPE and 0.1% SDS) at 65°C for 15 min to remove non-specific binding. The nylon membrane was exposed to a phosphorimager plate. The exposure time may vary from 2 hr (β-actin probe) to 12 hr (G3PDH probe) for phosphorimager plates.

Probe preparation and RNA quantitation. G3PDH probe was generously provided by Dr. Birkenmeier and colleagues (Jackson Laboratories) and was a 3.5 kb Hind III-Bam HI DNA

fragment of clone C of human genomic G3PDH DNA (19). The β-actin probe was a 2.0 kb cDNA fragment obtained from Clontech Lab. The probe fragments were labeled with [α-^{32}P] dCTP using a Pharmacia Oligolabeling Kit. Radiolabeled probe was passed through a NICK column (Pharmacia) to remove all unbound radioactive materials. After Northern blotting, the RNA levels of β-actin or G3PDH in samples were quantified by phosphorimager. Levels of β-actin mRNA in different samples were used to standardize the RNA levels.

Statistical analysis. The Student's *t* test was used for statistical analysis, and values in the text are presented as mean ± standard error of the mean. Paired Student's *t* test was used to analyze effects of inhibitors on G3PDH activity.

RESULTS AND DISCUSSION

Insulin effect on G3PDH activity for normal weight subjects. Since G3PDH occurs at the branch point between the glycolytic and triglyceride biosynthetic pathways, G3PDH activity in lymphocytes under varying conditions was examined (Table 1). A 64% increase ($p = 0.011$) in G3PDH activity is observed in the presence of insulin. This stimulation is similar to that observed for G3PDH mRNA levels or activity in murine adipocytes and other cell types (4-6,20-22). G3PDH is therefore among the enzymes regulated by insulin to alter utilization of substrates by the cell, presumably to alter production of glycerol-3-phosphate for triglyceride synthesis (9,23,24).

Effects of inhibitors. To distinguish among the mechanisms by which insulin regulates enzymatic activities (i.e., phosphorylation/dephosphorylation, shifts in protein trafficking, and increased protein expression) (9,24-26), we have added inhibitors in culture. Table 1 shows that RNA and protein synthesis inhibitors both inhibit the response of G3PDH activity to insulin. In order to measure the effect of inhibitors on insulin stimulation of G3PDH activity, insulin and inhibitor were added simultaneously on day 5 of the culture for a period of 30 minutes (Table 1).

TABLE 1

G3PDH ACTIVITY[a]

Glycerol-3-phosphate dehydrogenase activity	**Normal weight subjects**	**Obese subjects**
Units/mg protein	(n = 7)	(n = 15)
Basal activity	1.29 ± 0.09	1.25 ± 0.13
Insulin-stimulated activity (5 day exposure)	2.12 ± 0.26[b]	1.55 ± 0.17
Insulin-stimulated activity (30 minute exposure)	2.22 ± 0.29[b]	
Insulin plus actinomycin D	1.77 ± 0.36[b]	
Insulin plus cycloheximide	1.47 ± 0.41[b]	

[a]The values are indicated with standard error of the mean.

[b]Significant differences are as follows: ± insulin, $p = 0.011$ (5 days or 30 min); insulin ± actinomycin D, $p = 0.027$; insulin ± cycloheximide, $p = 0.033$. No significant difference was noted from the activity in the presence of inhibitors compared to basal value in the absence of insulin ($p > 0.25$), for basal activities of control *vs* obese subjects ($p > 0.7$), or for basal *vs* insulin-stimulated activities for obese subjects ($p = 0.13$).

The difference in exposure time did not have a significant effect on the stimulation of enzyme activity by insulin. With addition of insulin and inhibitors, G3PDH activity decreased to levels indistinguishable from the unstimulated basal level. These results indirectly indicate that stimulation of G3PDH activity by insulin is derived from increased synthesis of new protein in the lymphocyte.

Insulin effect on G3PDH kinetic parameters and G3PDH mRNA level. To further establish that insulin stimulation of G3PDH activity in human lymphocytes is due to increased synthesis of new enzyme, G3PDH kinetics and mRNA levels have been examined. Monitoring the reaction in the direction of dihydroxyacetone phosphate reduction at a single NADH concentration, the values of apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were obtained from Lineweaver-Burke plots of the results. In the absence of insulin, apparent V_{max} for G3PDH activity in lymphocytes was 6.1 ± 1.1 min^{-1}, whereas with insulin addition, V_{max} was increased by ~2-fold to 12.7 ± 1.9 min^{-1} ($p = 0.03$). In contrast, no significant difference in apparent K_m was observed in the absence of insulin (K_m = 10.2 ± 1.8 mM) *vs* in the presence of insulin (K_m = 13.8 ± 2.1 mM) ($p > 0.2$). This result is consistent with an increase in G3PDH enzyme levels in lymphocytes upon insulin stimulation. This interpretation is supported by analysis of mRNA production; a low level of G3PDH mRNA (~1 kb) can be detected in non-insulin-stimulated lymphocytes by Northern blot. The apparent smaller size for G3PDH mRNA found in human lymphocytes *versus* mouse adipocytes (3.2 kb) may reflect different processing or may derive from existence of extensive secondary structure in this mRNA, as observed in other cases (27). An average increase of 36% in G3PDH mRNA was observed in insulin-stimulated lymphocytes compared to unstimulated lymphocytes (Fig. 1). The level of stimulation for G3PDH mRNA from human lymphocytes was lower than the 3-fold increase found in insulin-stimulated mouse adipocytes. It may be that not only different forms of mRNA and/or enzyme but also differences in extent of insulin response may exist in different tissues or in different species (28).

Effects of other inhibitors on G3PDH activity. We have examined the influence of synthetic diacylglycerol (OAG) and phorbol ester (PMA), both of which affect protein kinase C activity (29,30), on insulin stimulation of G3PDH activity. Addition of OAG or PMA to lymphocytes in culture replicates the effect of insulin on G3PDH activity (Fig. 2). These data suggest possible involvement of diacylglycerol and protein kinase C in signal transduction to generate insulin regulation of G3PDH. Both RNA and protein synthesis inhibitors abolish the diacylglycerol effect on G3PDH activity, as observed for insulin. To evaluate inositoltriphosphate or calcium participation in regulating G3PDH activity, a series of inhibitors was examined. Lithium chloride (LiCl), which inhibits inositol phosphate phosphatase and limits the supply of inositol (31), alone did not alter G3PDH activity ($p > 0.35$ for ± LiCl). In the presence of LiCl and insulin, the G3PDH activity is indistinguishable from the basal level ($p > 0.46$ for +insulin ± LiCl). Similarly, calcium uptake inhibitors (nickel chloride and manganese chloride) inhibit insulin stimulation of G3PDH activity ($p > 0.46$ for +insulin ± inhibitors), while these compounds in the absence of insulin have no effect on G3PDH activity ($p > 0.35$ for ± inhibitors). These results suggest the possibility that both inositol phosphate pathways and calcium mobilization might play a

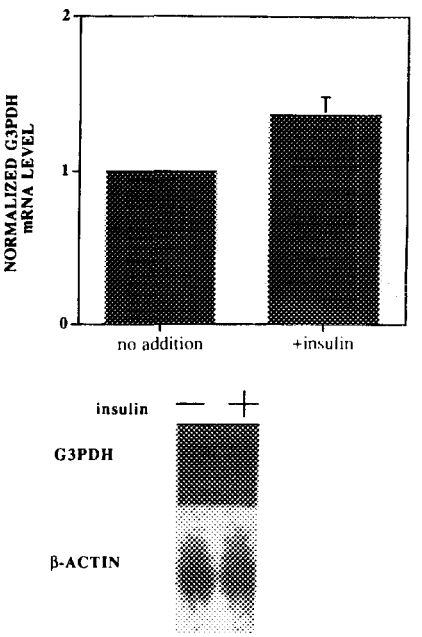

Figure 1. Insulin effect on G3PDH mRNA level. Lymphocytes isolated from normal weight subjects were cultured in CFBI 1000 medium with 14.4 μg/ml of glucose. After five days of culture, insulin (0.01 unit/ml) was added in half of the culture flasks for 30 min, then cells were collected. Total RNAs from different samples were isolated using a Stratagene RNA isolation kit according to *Materials and Methods*. The averages of intensities of the autoradiographic bands from five experiments are shown in upper panel and are presented as normalized G3PDH mRNA level, which is [G3PDH mRNA (in insulin-treated cells)]/[G3PDH mRNA (basal level)]/[β-actin mRNA (in insulin-treated cells)]/[β-actin mRNA (basal level)]. A statistically significant difference for G3PDH mRNA levels was observed in cells ± insulin ($n = 5$, $p = 0.002$). Autoradiograms of a representative experiment are shown in the bottom panel.

role in regulation of G3PDH activity, but further study will be required to delineate the detailed mechanism by which insulin stimulates G3PDH activity.

Insulin effect and dietary effect on G3PDH activity in obese subjects. Because several enzymes are altered in obese/diabetic animals (9) and insulin stimulation of pyruvate dehydrogenase was found to be lower in lymphocytes from obese/diabetic humans (25), we have examined lymphocytes from obese subjects for any alterations in insulin-regulated G3PDH activity. No difference in the basal level of G3PDH (without insulin stimulation) was found between normal weight and obese subjects (Table 1). However, insulin induced only a 24% increase for obese subjects, and this increase did not attain statistical significance. This impaired stimulation of G3PDH by insulin in obese subjects is consistent with the insulin resistance found in obesity (reviewed in 9,32) and correlates to the general state of insulin resistance for lymphocytes from these obese patients observed by other measures (15).

Dietary restriction can alter the homeostatic mechanisms, including decreasing plasma glucose level and regulating insulin secretion and utilization (15,33-36). We have examined G3PDH activities, which can be regulated by insulin in normal weight individuals, during VLCD treatment and refeeding periods. Before dietary treatment, the slight insulin stimulation observed was not of statistical significance in lymphocytes isolated from obese subjects. Both basal and

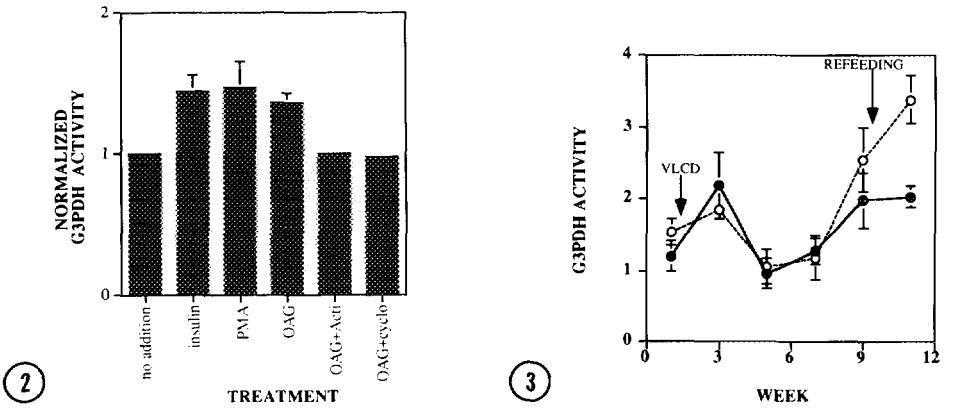

Figure 2. The effect of insulin, 1-oleoyl-2-acetyl-sn-glycerol and 4-phorbol-12-myristate-13-acetate on G3PDH activity. Lymphocytes isolated from normal weight subjects were cultured as described for five days, after which OAG, PMA, OAG + actinomycin D, or OAG + cycloheximide were added to the cultures for 30 minutes; the cells were scraped and collected by centrifugation for G3PDH assay. Data are presented as normalized G3PDH activity, which is [G3PDH activity in units/mg protein (various treatment)]/[G3PDH activity in units/mg protein (basal level)]. Statistical significance of the difference observed was as follows: no addition *vs* insulin addition ($p = 0.010$); no addition *vs* OAG addition ($p = 0.027$); no addition *vs* PMA addition ($p = 0.006$); actinomycin + OAG addition *vs* OAG addition ($p = 0.009$); cycloheximide + OAG addition *vs* OAG addition ($p = 0.007$). No difference was observed between control *vs* actinomycin + OAG addition ($p = 0.883$) or control *vs* cycloheximide + OAG addition ($p = 0.478$). The error bars indicate the standard error of the mean. The error bars in OAG + Acti and OAG + cyclo are smaller than could be shown.

Figure 3. G3PDH activity during diet and refeeding period for obese subjects. Blood was collected from obese subjects (n = 11) on alternate weeks during the study. Lymphocytes were isolated and cultured with (open circle) or without (closed circle) insulin addition at a concentration of 0.01 unit/ml as described in *Materials and Methods*. After 5 days of culture, cells were collected and sonicated to generate cell extracts; subsequently G3PDH assays were performed as described in *Materials and Methods*. The only significant difference between basal and insulin-stimulated G3PDH activities is observed for week 11 ($p = 0.044$). The error bars indicate the standard error of the mean.

insulin-stimulated levels of G3PDH were found to vary during the dietary period (Fig. 3); however, the insulin-stimulated levels do not differ significantly from the basal levels. The variation in basal levels suggests that there may be adjustments in metabolic activity during the diet treatment that involve conversion between lipogenic and glycolytic pathways. G3PDH activity in lymphocytes normalized during the refeeding period to a level comparable to that found in normal weight subjects. Simultaneously, insulin stimulation of G3PDH activity increased to a value comparable to that for normal weight subjects ($p = 0.044$ for ± insulin at week 11) in lymphocytes isolated from obese subjects after diet treatment; it should be noted that these subjects no longer meet the standards for obesity due to weight loss during the period of fasting. These results indicate that caloric restriction to generate weight loss followed by a period of establishing a modified caloric intake results in restoration of the G3PDH pattern to normalcy, a process that can be followed *in vitro* by examining responses in lymphocytes.

CONCLUSION

We have demonstrated the presence of G3PDH in human lymphocytes as well as the stimulation of G3PDH mRNA levels and enzyme activity by insulin. Based on data from inhibitor

studies, the mechanism by which insulin stimulates G3PDH activity requires new protein synthesis and may involve protein kinase C and other intracellular signalling pathways. The altered metabolic state characteristic of obesity diminishes insulin stimulation of G3PDH activity, and normalization of body weight following a very low calorie diet results in restoration of G3PDH activity stimulation by insulin. Insulin stimulation of G3PDH activity and mRNA levels in human lymphocytes suggests that these cells, which are readily obtained and cultured *in vitro*, may prove useful in human studies aimed at understanding metabolic pathways, in particular those regulated by insulin.

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