Role of Hexosamine Biosynthesis in Glucose-Mediated Up-Regulation of Lipogenic Enzyme mRNA Levels:

EFFECTS OF GLUCOSE, GLUTAMINE AND GLUCOSAMINE ON GLYCEROPHOSPHATE DEHYDROGENASE, FATTY ACID SYNTHASE, AND ACETYL-COA CARBOXYLASE mRNA LEVELS

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Running Title: Regulation of Lipogenic Enzyme mRNA Levels by the Hexosamine Biosynthesis Pathway

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Regulation of Lipogenic Enzyme mRNA Levels by the Hexosamine Biosynthesis Pathway

SUMMARY

Glucose uptake into adipose and liver cells is known to up-regulate mRNA levels for various lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). To determine whether the hexosamine biosynthesis pathway (HBP) mediates glucose regulation of mRNA expression, we treated primary cultured adipocytes for 18 hrs with insulin (25 ng/ml) and either glucose (20 mM) or glucosamine (2 mM). A ribonuclease protection assay was used to quantitate mRNA levels for FAS, ACC and glycerol-3-P dehydrogenase (GPDH). Treatment with insulin and various concentrations of d-glucose increased mRNA levels for FAS (280%), ACC (93%), and GPDH (633%) in a dose-dependent manner (ED₅₀ 8-16 mM). Mannose similarly elevated mRNA levels, but galactose and fructose were only partially effective. L-glucose had no effect. Omission of glutamine from the culture medium markedly diminished the stimulatory effect of glucose on mRNA expression. Since glutamine is a crucial amide donor in hexosamine biosynthesis, we interpret this data to mean that glucose flux through the HBP is linked to regulation of lipogenesis through control of gene expression. Further evidence for hexosamine regulation was obtained using glucosamine, which is readily transported into adipocytes where it directly enters the HBP. Glucosamine was 15-30 times more potent than glucose in elevating FAS, ACC, and GPDH mRNA levels (ED₅₀ ~ 0.5 mM). In summary: 1) GPDH, FAS, and ACC mRNA levels are up-regulated by glucose; 2) glucose-induced up-regulation requires glutamine; and 3) mRNA levels for lipogenic enzymes are up-regulated by glucosamine. Hyperglycemia is the hallmark of diabetes mellitus and leads to insulin resistance, impaired glucose metabolism and dyslipidemia. We postulate that disease pathophysiology may have a common underlying factor – excessive glucose flux through the HBP.
INTRODUCTION

It is known that mRNA levels for various lipogenic enzymes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), are up-regulated by glucose in liver and adipose tissue (1); however, the signaling pathway mediating glucose-induced mRNA regulation is poorly understood. One hypothesis is that glucose-6-phosphate (G-6-P) is the primary metabolite responsible for glucose-induced mRNA regulation. This has prompted several investigators to focus on glucose-6-phosphate utilizing pathways (1). Products of the glycolysis pathway have been excluded as regulatory metabolites because pyruvate and lactate failed to up-regulate lipogenic enzymes (1,2). Intermediates of the pentose phosphate pathway have been proposed to mediate glucose regulation of gene expression based on the ability of xylitol to enter the pentose pathway and regulate pyruvate kinase and S14 genes in liver (3,4). Despite these studies, there is no clear mechanistic rationale for how G-6-P, or formation of glucose metabolites, could directly regulate mRNA levels.

The current studies were initiated to examine the hypothesis that the glucose-mediated up-regulation of lipogenic enzymes in isolated adipocytes is mediated by glucose flux through the hexosamine biosynthesis pathway (HBP) and the subsequent regulation of lipogenic enzyme mRNA levels. The rationale for exploring this hypothesis is based on the 1991 discovery that glucose-induced desensitization of the glucose transport system is linked to hexosamine biosynthesis (5,6). At that time, it was proposed that the HBP serves as a glucose sensor coupled to a metabolic transducer that regulates the insulin-responsive glucose transport system. Subsequent studies have expanded upon the idea of hexosamine-mediated regulation by implicating the HBP in the regulation of genes for pyruvate kinase, leptin, transforming growth factor α (TGFα), and transforming growth factor β (TGFβ) (7-14).

EXPERIMENTAL PROCEDURES

Materials - Sources of materials were as follows: Porcine insulin, Sigma, St. Louis MO.; collagenase, Worthington Biochemicals, Freehold, N.J.; bovine serum albumin (CRG-7), Armour Company, Kankakee, Ill.; Penicillin-Streptomycin and Dulbecco’s modified Eagle’s medium (DMEM) Gibco, Grand Island, NY, RNazol B, Cinna/Biotecx, Friendswood, TX. All other reagents were from Sigma, St. Louis, MO or Fisher, Santa Clara, CA, unless otherwise specified.
Preparation of Sterile Isolated Adipocytes - Isolated adipocytes were obtained from the epididymal fat pads of male Sprague-Dawley rats (180-225 g) by collagenase digestion (15) as previously described (16). Briefly, minced tissue (1-2 g) in 4 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing collagenase (1 mg/ml) and albumin (40 mg/ml) was shaken in 4-oz. sterile polypropylene containers at 37°C for 45 min. At the end of the digestion period, cells were filtered through nylon mesh (1000 µm) and then washed three times in Hepes-buffered balanced saline solution (HBSS). HBSS contains 25 mM Hepes, 120 mM NaCl, 0.8 mM MgSO₄, 2 mM CaCl₂, 5.4 mM KCl, 1 mM NaH₂PO₄, 1 mM sodium pyruvate, 100 Units/ml penicillin, 100 µg/ml streptomycin, and 1% BSA, pH 7.6. After washing, adipocytes were resuspended as a 10% (W/V) solution in HBSS (final concentration about 5 x 10⁵ cells/ml).

Primary Culture of Adipocytes and Extraction of Total RNA – Adipocytes were maintained in primary culture as previously described (16). Briefly, adipocytes were added to sterile 250 ml polypropylene bottles or 50 ml polypropylene tubes and diluted to a final volume of 20 or 60 ml (~2 x 10⁵ cells/ml) in sterile incubation medium consisting of glucose-free DMEM supplemented with 1 mM pyruvate and 1% BSA (SIM0) or HBSS. Cells were then incubated at 37°C for 18 hrs with various combinations of glucose (20 mM), insulin (25 ng/ml), glutamine (16 mM), or glucosamine (2 mM) unless otherwise indicated. After treatment, adipocytes were washed 3 times with HBSS. During the final wash, the infranatant was aspirated and total RNA was extracted from adipocytes by adding 500 µl of RNAzol B, 200 µl of chloroform, and 200 µl of 10 mM Tris pH 7.4. After centrifugation for 15 min at 4°C, the upper aqueous phase containing the RNA was precipitated at −20°C for 90 minutes with 0.3 M sodium acetate and isopropanol. The RNA pellets were washed with 75% ethanol and then resuspended in 10 mM Tris pH 7.4. RNA was quantified by measuring OD₂₆₀ and deemed pure (OD₂₆₀:OD₂₈₀ ~2). RNA was stored at −80°C prior to use in the ribonuclease protection assay.

Plasmids - S2 Ribosomal protein (S2) cDNA from bp 121 to 906 (GenBank accession # X57432) was amplified from rat adipocyte cDNA using GeneAmp PCR kit with amplitaq (Perkin Elmer, Foster City, CA) and primers (Keystone Biosource, Foster City, CA) designed to facilitate subsequent cloning into the AscI/PacI sites of pJMR1 (17). The sequences of the primers used were 5’-actggccgccttaggggccgctgtgg-3’ and 5’-aaattaattaattatgtgtagccactgtgagct-3’. Cytosolic glycerophosphate dehydrogenase (GPDH; EC 1.1.1.8) cDNA from bp 1 to 1050 (GenBank accession # AB002558) was similarly cloned. The sequences of the GPDH primers
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were 5’-caggccgcagttgcaaatggctgc-3’ and 5’-atttaatacatgttgtctc-3’.
The hormone sensitive lipase (HSL; EC 3.1.1.3) plasmid was a gift from Dr. Allan Green (Bassett Research Institute, Cooperstown, NY). The pCRII-FAS and pCRII-ACC plasmids were gifts from Sankyo Ltd. (Shinagawa, Japan).

**Generation of Nucleic Acid Probes** - Portions of the various cDNAs (above) were subcloned into either pT7/T3-18 vector (Ambion, Austin, TX) or pJMR1. The resulting plasmids were subsequently linearized, gel purified with gene clean (Bio 101, La Jolla, CA), and then used as a template for T7 RNA polymerase mediated *in vitro* transcription. The MAXIscript *in vitro* transcription kit (Ambion, Austin, TX) and [α-32P] UTP (New England Nuclear, Boston, MA) were used to prepare antisense RNA probes that protect S2 mRNA (bases 734-906 GenBank accession #X57432), FAS (bases 7340-7612 of GenBank accession #X14175), GPDH (bases 1-299 of GenBank accession #AB002558), ACC (bases 6827-7004 GenBank accession #J03808), and HSL (bases 1-337 GenBank accession #51415). RNA probes were purified over a NucTrap push column (Stratagene, La Jolla, CA) before use in the ribonuclease protection assay.

**Ribonuclease Protection Assay** - The ribonuclease protection assay (RPA) was performed using the Ambion RPA II kit (Ambion, Austin, TX) following the manufacturers instructions. Multiplexed antisense RNA probes (25-50,000 cpm/probe) were hybridized with 5-8 µg of total adipocyte RNA for 16 hrs at 42°C. After RNase digestion, the protected probes were resolved on a 6% polyacrylamide-Urea gel in Tris-Borate-EDTA buffer and then quantified using the Storm 840 phosphorimaging system (Molecular Dynamics, Piscataway, NJ).

**Northern Analysis** – 20 µg of total RNA was separated on a 1% (w/v) agarose-formaldehyde gel. RNA was transferred to nylon membranes (Schleicher & Scheull, Keene, NH) and then crosslinked using the UV stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed with radiolabeled cDNA probe for 5 hrs at 60 °C in ExpressHyb buffer (Clontech, Palo Alto, CA) containing 100 µg/ml salmon sperm DNA. After washing the blot, hybridization signals were visualized using the Storm 840 phosphorimaging system. Each blot was stripped and reprobed with a S2 cDNA probe to control for equal loading of RNA.

**RESULTS**
Glucose-induced up-regulation of FAS and GPDH mRNA levels. Primary cultured adipocytes were treated for 6-30 hrs in glucose-free DMEM containing 25 ng/ml insulin (control) or insulin plus 20 mM glucose. Total RNA was extracted and used for quantitation of mRNA levels using a ribonuclease protection assay.

Maximal changes in mRNA levels for FAS, GPDH, and ACC were observed between 12 and 24 hrs with little glucose regulation seen at 6 hrs (data not shown). Based on these preliminary studies, we decided to quantify mRNA levels at 18 hours in all subsequent experiments. As can be seen by Northern analysis in Fig. 1A, glucose treatment resulted in up-regulation of mRNA levels for both FAS and GPDH. Since S2 mRNA levels were unchanged with glucose treatment, it can serve as an internal control. The ribonuclease protection assay was used as a quantitative method to measure FAS, GPDH and S2 mRNA levels (Fig. 1B). Radiolabeled antisense probes for GPDH (299 bases), FAS (273 bases), and S2 (172 bases) were prepared using in vitro transcription and were then multiplexed in a hybridization reaction with RNA from control or glucose-treated adipocytes. After normalization to S2 mRNA levels, glucose treatment for 18 hrs resulted in a 241% increase in FAS mRNA levels and a 576% increase in GPDH mRNA levels. It is important to note that the addition of the transcription inhibitor actinomycin D (200nM) completely blocked glucose-induced up-regulation of mRNA levels (data not shown). All subsequent measurements of mRNA levels were performed using the RPA.

Figs. 1C&D depicts experiments in which adipocytes were treated for 18 hrs with 25 ng/ml insulin plus various sugars (all at a concentration of 20 mM). D-glucose effectively increased both FAS (Fig. 1C) and GPDH mRNA levels (Fig. 1D), whereas L-glucose had no effect. This indicates sugar specificity and eliminates osmolarity changes as a causative factor. Mannose was nearly as effective as glucose in elevating mRNA levels of FAS and GPDH, whereas fructose and galactose had much smaller effects. The differential effects of the various sugars on FAS and GPDH mRNA levels are most likely due to differences in uptake and metabolism of these sugars in isolated adipocytes.

To better quantify glucose-induced up-regulation of mRNA levels, we incubated adipocytes for 18 hrs in glucose-free DMEM containing insulin and various concentrations of glucose (Fig. 2). Glucose treatment resulted in a dose-dependent increase in mRNA levels for both FAS (ED$_{50}$ 15.5 mM) and GPDH (ED$_{50}$ 7.5 mM). Maximal concentrations of glucose (40 mM) increased FAS mRNA >3-fold and elevated GPDH mRNA about 9-fold. The fact that FAS and GPDH mRNA
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levels were relatively unchanged in the hypoglycemic to euglycemic range (1 to 5 mM glucose) indicates that the glucose regulation of mRNA levels is not a consequence of glucose deprivation but rather is a result of hyperglycemia. We also examined the effect of xylitol on FAS and GPDH mRNA levels (Fig. 2 insets) because glucose flux into the pentose phosphate shunt has been postulated to mediate up-regulation of various glucose-responsive genes (3,4). Xylitol is known to directly enter the pentose phosphate shunt through the intracellular formation of xylulose-5-phosphate. Because xylitol treatment had no effect on either FAS or GPDH mRNA levels in insulin-treated cells, we conclude that the pentose phosphate shunt does not contribute significantly to glucose up-regulation of FAS and GPDH mRNA in isolated adipocytes.

**Facilitative role of insulin in glucose-induced up-regulation of mRNA levels** – Glucose regulation of gene expression can be either insulin-dependent or insulin-independent. Examples of insulin-dependent regulation include glucose-mediated up-regulation of FAS mRNA in liver (through activation of glucokinase); insulin-independent regulation is typified by glucose regulation of stearoyl-CoA desaturase mRNA in 3T3-L1 adipocytes in the absence of insulin (1,18). To examine the role of insulin in glucose-induced up-regulation of GPDH and FAS mRNA in isolated adipocytes, we treated cells for 18 hrs in HBSS in the absence or presence of 25 ng/ml insulin (Fig. 3). Compared to controls (no additions) insulin alone down-regulated FAS and GPDH mRNA levels. In contrast, treatment with insulin and glucose resulted in a glucose dose-dependent, up-regulation of FAS and GPDH mRNA levels (Fig. 3). Since insulin is known to stimulate glucose transport in adipocytes by > 10 fold, we believe that insulin facilitates glucose action by enhancing glucose uptake into cells.

**Glutamine potentiates glucose-induced up-regulation of FAS and GPDH mRNA levels** – Glucose-induced insulin resistance in isolated adipocytes is mediated by glucose flux through the HBP (5). Evidence supporting this conclusion include the finding that desensitization requires the presence of three components in the medium – glutamine, glucose, and insulin (5,6). Glutamine was necessary because it serves as an essential amide donor for the conversion of glucose to hexosamine products. Specifically, glutamine-fructose-6-P amidotransferase (the first and rate-limiting enzyme of the hexosamine pathway) requires glutamine for conversion of fructose-6-P to glucosamine-6-P.

To investigate whether the HBP mediates glucose-induced up-regulation of FAS and GPDH mRNA, we incubated adipocytes with 25 ng/ml insulin and 20 mM glucose in the absence or
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presence of 16 mM glutamine. As shown in Fig. 3, inclusion of glutamine potentiated the glucose-induced increase of FAS and GPDH mRNA. This indicates that formation of hexosamine products underlies glucose regulation of FAS and GPDH mRNA levels.

**Ability of glucosamine to up-regulate FAS and GPDH mRNA levels** – To obtain additional evidence for hexosamine-mediated regulation of FAS and GPDH mRNA, we treated adipocytes for 18 hrs with 25 ng/ml insulin and various concentration of glucosamine. Glucosamine was used because it is readily transported into adipocytes through the glucose transport system where it directly enters the hexosamine pathway at the level of glucosamine-6-phosphate (5). The data depicted in Fig. 4 shows that glucosamine treatment resulted in a dose-dependent up-regulation of mRNA levels for FAS (ED$_{50}$ ~ 0.45 mM) and GPDH (ED$_{50}$ ~ 0.75 mM). Glucosamine was approximately 15-30 times more potent than glucose in inducing FAS and GPDH mRNA levels.

**Regulation of Acetyl-CoA Carboxylase mRNA levels by glucose and glucosamine** – To explore whether the HBP may control other mRNAs involved in lipid metabolism, we evaluated the regulation of mRNA for the lipogenic enzyme acetyl-CoA carboxylase (ACC) and the lipolytic enzyme hormone sensitive lipase (HSL). As depicted in Fig. 5A, ACC mRNA levels were up-regulated by co-treatment with insulin and glucose, but the extent of up-regulation was significantly enhanced by the inclusion of glutamine. Treatment with insulin and glucosamine up-regulated ACC mRNA levels to a greater extent than glucose, suggesting that ACC represents another lipogenic enzyme under the control of the HBP. Considered together, these data suggest that ACC mRNA levels are also regulated through the HBP. As shown in Fig 5B&C, HSL mRNA levels were not up-regulated by glucose, glutamine, or glucosamine treatment. In fact, HSL mRNA levels were actually down-regulated with insulin treatment. Since HSL is a lipolytic enzyme, these data highlight the specificity of hexosamine action on lipogenic enzymes in isolated rat adipocytes.

**DISCUSSION**

When food sources are readily available and consumed in excess, adipose tissue plays a major role in converting ingested metabolic fuel into lipids. Stored lipids then serve as an essential energy source during periods of famine. Facilitating fuel storage at the cellular level is a regulatory system in adipose and liver tissue that entails up-regulation of mRNA levels for lipogenic enzymes such as FAS and ACC. An important finding of the current study is that GPDH
mRNA is also up-regulated by glucose in isolated adipocytes. This fits well with the established role of GPDH in forming the glycerol backbone of triglycerides (by catalyzing biosynthesis of glycerol-3-phosphate within the glycolysis pathway). The finding that GPDH mRNA is regulated by glucose in adipocytes is consistent with earlier observations that GPDH activity and mRNA levels are up-regulated in the hyperinsulinemic obese Zucker rat (19) and agrees with studies showing that GPDH activity is nutritionally regulated in rat jejunum and liver (20). Although we found that glucose is more effective than fructose in elevating GPDH mRNA levels in adipocytes (Fig. 1), Stifel et al. reported that fructose was more potent than glucose in activating GPDH activity in other tissues (20). Whether these divergent findings represent differences in the regulation of GPDH among tissues, or tissue-specific differences in the uptake and metabolism of various sugars requires clarification.

Although previous studies have established that intracellular glucose metabolism results in up-regulation of lipogenic enzyme mRNA levels, the cascade of metabolic events leading to up-regulation remains obscure. Formation of glucose-6-phosphate has been postulated to regulate lipogenic mRNA levels (1-3); however, there is no mechanistic rationale for how G-6-P could modify mRNA levels (1). Another idea is that xyulose-5-phosphate functions as a metabolite regulator of mRNA levels (1-3) through activation of protein phosphatase 2a and dephosphorylation of transcription factors mediating the glucose response (3,21,22). To better understand the mechanism(s) underlying glucose-mediated regulation of lipogenic enzyme mRNA levels, we used isolated adipocytes maintained in primary culture as our model system so that we could investigate mRNA regulation under defined in vitro conditions.

When adipocytes were treated for 18 hrs with 25 ng/ml of insulin and various concentrations of D-glucose, we observed increases in mRNA levels for FAS (280%), ACC (93%), and GPDH (633%). The glucose ED$_{50}$ values were ~15 mM for FAS and ~7 mM for GPDH. Sugar specificity studies revealed that mannose elevated mRNA levels almost as effectively as glucose, whereas galactose and fructose were only partially effective. L-glucose, which is a non-metabolizable analog of D-glucose, had no effect on mRNA levels. Insulin was required for the expression of glucose-induced up-regulation of mRNA levels. However, insulin itself appears to play no direct role in mRNA up-regulation since treatment of adipocytes with insulin alone (in the absence of glucose) resulted in an actual decrease in FAS and GPDH mRNA levels (Fig. 3).
Since insulin enhances the rate of glucose uptake in isolated adipocytes by >10-fold, it is likely that insulin is required to facilitate glucose entry into cells.

Two lines of evidence support the hypothesis that mRNA regulation of lipogenic enzymes is coordinately regulated by enhanced glucose flux through the HBP. First, we found that glutamine significantly augmented the stimulatory effect of glucose on mRNA expression of FAS and GPDH (Fig. 3). This is consistent with the idea that intracellular formation of hexosamine products requires a supply of both glucose (in the form of F-6-P) and glutamine (as a cofactor for GFAT in the transfer of an amide group to F-6-P). The primary role of insulin in this scheme is to facilitate the uptake of glucose (about 10- to 20-fold). The second line of evidence for hexosamine involvement entailed the use of glucosamine, which has previously been shown to enter adipocytes through the glucose transport system (5) where it directly enters the hexosamine pathway distal to GFAT (through formation of GlcN-6-P). In insulin-treated adipocytes, glucosamine was 15-30 times more potent than glucose in up-regulating FAS and GPDH mRNA levels. Considered together, these studies lead to the conclusion that the HBP plays an integral role in lipid metabolism by up-regulating mRNA levels for various lipogenic enzymes. In contrast, mRNA levels of the lipolytic enzyme HSL were unaffected by treatment of isolated rat adipocytes with glucose or glucosamine for 18 hours. It should be mentioned that in human adipocytes and cultured 3T3-F442A adipocytes, prolonged glucose treatment for 48 hrs has been shown to culminate in up-regulation of HSL (23,24). The reason for the discrepancy between primary cultured rat adipocytes and other adipocyte model systems remains unclear.

Up-regulation of mRNA levels can result from transcriptional activation and/or changes in mRNA stability. Although the exact mechanism(s) by which glucose regulates lipogenic mRNAs in primary cultured adipocytes remains to be elucidated, changes in both transcription rates and mRNA stability may be involved (25,26). A glucose-inducible mRNA-binding protein has previously been shown to stabilize FAS mRNA levels in HepG2 cells (27). Whether the hexosamine pathway regulates mRNA-binding proteins in rat adipocytes is unclear. Since we found that the transcription inhibitor actinomycin D completely inhibits the glucose-induced up-regulation of lipogenic mRNA levels in primary cultured adipocytes, this suggests that transcriptional regulation plays a prominent role in regulating mRNA levels. This conclusion fits well with previous studies implicating the hexosamine pathway in the transcriptional regulation of pyruvate kinase (7), GFAT (13), leptin (8,9), TGFα (9,11,12), and TGFβ (13,14).
Hexosamine-mediated regulation of lipogenic enzymes at the level of transcription becomes even more compelling given that glucosamine was also found to be nearly six times more potent than glucose at inducing the glucose response element of TGFα (10).

The model depicted in Fig. 6 schematically integrates the current data on regulation of mRNA levels with previous studies on glucose-induced insulin resistance (28,29). Upon entering adipocytes, glucose is phosphorylated to G-6-P and rapidly converted to F-6-P. From these two metabolites, glucose fluxes into various pathways involved in glucose storage and utilization. These include the glycogen biosynthesis pathway, the pentose phosphate shunt, and the glycolysis / lipogenesis pathway. Although these three pathways represent the major routes traversed by the vast majority of glucose, a small percentage of incoming glucose (about 1-2%) is routed into the HBP. It was originally proposed that the HBP serves as glucose-sensor coupled to a biological transduction system that functions to reduce glucose uptake as the rate of glucose transport exceeds the capacity of the major glucose utilizing pathways (5). In other words, enhanced flux of glucose through the HBP culminates in insulin resistance of the glucose transport system. Based on the current study, it appears likely that the regulatory role of the HBP also encompasses control of lipid storage through gene regulation of lipogenesis enzyme mRNA levels. This coordinated response to hyperglycemia makes sense in that overall glucose uptake would be reduced (by development of insulin resistance) and excess incoming glucose would be stored as triglycerides (through enhancement of lipogenesis).

Under hyperglycemic conditions, as occurs after eating, the body mounts a normal, adaptive response to re-establish glucose homeostasis. However, under prolonged hyperglycemic conditions, as occurs in diabetes mellitus, cellular adaptation to excessive glucose uptake may lead to many of the pathophysiological consequences of diabetes, including insulin resistance, impaired glucose metabolism, and dyslipidemia. Accordingly, it can be hypothesized that prolonged hyperglycemia and excessive glucose flux through the HBP may play a role in the etiology and pathogenesis of diabetes. Therefore, pharmacological intervention targeting the HBP may ameliorate hyperglycemia and/or hyperlipidemia and possibly prevent or delay the onset of diabetic complications that accompany disease progression.

REFERENCES

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**FOOTNOTES**

The abbreviations used are; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase, GPDH, glycerol-3-phosphate dehydrogenase; HSL, hormone sensitive lipase; RPA, ribonuclease protection assay; HBP, hexosamine biosynthesis pathway; TGFα, transforming growth factor α; TGFβ, transforming growth factor β; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Heps-buffered balanced saline solution; GlcNAc, N-acetylglucosamine; UDP-GlcNAc, UDP-N-acetylglucosamine; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; GFAT, glutamine:fructose-6-phosphate amidotransferase; OGT, UDP-GlcNAc transferase, (uridine diphospho-N–acetylglucosamine:polypeptide β-N-acetylglucosaminyl transferase).
FIGURE LEGENDS

Fig. 1. Glucose up-regulates FAS and GPDH mRNA levels. Isolated adipocytes were incubated for 18 hrs with 25 ng/ml insulin (control) or with insulin and 20 mM glucose. Total RNA was isolated and mRNA levels of FAS and GPDH were quantified by either Northern analysis (A) or a ribonuclease protection assay (B). Levels of S2 mRNA were measured and used as an internal control. (C&D) Adipocytes were incubated for 18 hrs with 25 ng/ml insulin (control) or with insulin and 20 mM of d-glucose, L-glucose, mannose, galactose, or fructose. Total RNA was isolated and mRNA levels of FAS (C) and GPDH (D) were measured using an RPA and then normalized to the S2 mRNA control. RPA data from a representative experiment is shown in the upper panels. Black bars represent mRNA levels as a percent increase above control values (insulin treatment alone). Data is derived from three independent experiments and values are graphed as the mean +/- S.E.M.

Fig. 2. Dose-dependent ability of glucose to up-regulate FAS and GPDH mRNA levels. Adipocytes were incubated for 18 hrs with 25 ng/ml insulin (control) or with insulin and increasing doses of glucose. mRNA levels of FAS (panel A) and GPDH (panel B) were measured using an RPA. In related experiments, insulin-treated adipocytes were incubated with increasing concentrations of xylitol (insets). Each point represents mRNA level as a percent increase above control values (insulin-treatment alone). Data is derived from a representative experiment and each point represents the mean +/- SEM of two replicates.

Fig. 3. Effects of insulin, glucose and glutamine on FAS and GPDH mRNA levels. Adipocytes were incubated in HBSS for 18 hrs in the absence (no additions) or presence of 25 ng/ml insulin alone, insulin and 20 mM glucose, or insulin, glucose and 16 mM glutamine. FAS and GPDH mRNA levels were measured using an RPA. Data is derived from three independent experiments and values are graphed as the mean +/- S.E.M. Significance of glutamine potentiation was assessed using a pair-wise student t test.

Fig. 4. Glucosamine dose-response curves on up-regulation of FAS and GPDH mRNA levels. Adipocytes were incubated for 18 hrs with 25 ng/ml insulin alone (control) or with insulin and
increasing concentrations of glucosamine. FAS and GPDH mRNA levels were measured using an RPA. Data is derived from three independent experiments and values are graphed as the mean +/- S.E.M.

**Fig. 5. Effect of glucose and glucosamine on ACC or HSL mRNA levels.** (A) Adipocytes were incubated in HBSS for 18 hrs with 1) 25 ng/ml insulin, 2) insulin and 20 mM glucose, 3) insulin, glucose and 16 mM glutamine, or 4) insulin and 2 mM glucosamine. ACC mRNA levels were measured using an RPA. Black bars represent mRNA levels as a percent increase above control values (insulin treatment alone). Significance of glutamine potentiation was assessed using a pair-wise student t test. (B) Adipocytes were incubated in HBSS for 18 hrs with 1) no additions, 2) 25 ng/ml insulin, 3) insulin and 20 mM glucose, or 4) insulin, glucose and 16 mM glutamine. Hormone sensitive lipase (HSL) mRNA levels were measured using an RPA. (C) Adipocytes were incubated in glucose-free DMEM for 18 hrs with 25 ng/ml insulin or with insulin plus 2 mM glucosamine. HSL mRNA levels were measured using an RPA. Data is derived from three independent experiments and values are graphed as the mean +/- S.E.M.

**Fig. 6. Model depicting the postulated role of the hexosamine biosynthesis pathway in coordinating the regulation of the insulin-responsive glucose transport system and lipogenic enzyme gene expression.**
### A Northern Analysis

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### B Ribonuclease Protection Assay

![Ribonuclease Protection Assay](image)

### C FAS mRNA (% Increase)

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### D GPDH mRNA (% Increase)

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**A**

FAS mRNA (% Increase) vs. Glucose (mM)

ED$_{50} =$ 15.5 mM

**B**

GPDH mRNA (% Increase) vs. Glucose (mM)

ED$_{50} =$ 7.5 mM
**Figure A**

- **FAS mRNA (normalized)**
  - No Additions
  - Insulin
  - Insulin Glucose
  - Insulin Glucose Glutamine
  
  
  - P<0.05

**Figure B**

- **GPDH mRNA (normalized)**
  - No Additions
  - Insulin
  - Insulin Glucose
  - Insulin Glucose Glutamine
  
  
  - P<0.01

Glucose → G-6-P → F-6-P
Glutamine + Glutamate → Glucosamine-6-P → UDP-GlcNAc
OGT → O-Linked Glycoproteins

Insulin Action Cascade

Insulin Resistance of Glucose Transport System

Lipogenesis Pathway

Triglycerides

Fructose-1,6 bisP

Lipogenic Enzymes

FAS mRNA
GPDH mRNA
ACC mRNA

Hexosamine Signaling Pathway

UDP-GlcNAc
OGT

Glucosamine-6-P

Translocation

Glucosamine

Inhibition of Glucose Transport System

G-6-P F-6-P
Role of hexosamine biosynthesis in glucose-mediated up-regulation of lipogenic enzyme mRNA levels: Effects of glucose, glutamine and glucosamine on glycerophosphate dehydrogenase, fatty acid synthase, and acetyl-CoA carboxylase mRNA levels

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