GLUCOSAMINE REDUCES GLYCOGEN STORAGE IN L6 SKELETAL MUSCLE CELLS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of NICOLE MUNOZ find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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The impact of glucosamine supplementation on glycogen content in skeletal muscle fibers was examined *in vitro* using L6 myotubes. Serum starved L6 myotubes were exposed to either 5 mM or 25 mM glucose, with and without insulin (0 nM or 100 nM) and glucosamine (0 mM, 0.06 mM, 1 mM, 5 mM) for 1, 3 or 5 hr in culture. Glycogen content in L6 myotubes was quantified by image analysis following PAS staining. Insulin increased glycogen content in L6 myotubes across all treatments ($P = 0.023$). After exposure to a euglycemic condition, glycogen content was significantly increased at 3 and 5 hr as compared with glycogen content at 1 hr ($P = 0.00002$ and $P = 0.006$ respectively), independent of glucosamine treatment. Glucosamine supplementation in a hyperglycemic condition (25 mM glucose) reduced glycogen content in L6 myotubes. After 1 hr. incubation with 0.06 mM, 1 mM or 5 mM glucosamine supplementation, glycogen content was significantly less than that present in the absence of glucosamine (all $P = 0.000$ at each comparison, respectively). Additionally, glycogen content in L6 cells decreased over time in the 25 mM glucose treatment groups. Glycogen content was significantly reduced following exposure to 1 mM glucosamine versus results obtained at 3 and 5 hr ($P = 0.000$ and $P = 0.001$, respectively). These data indicate that

glucosamine supplementation can reduce glycogen content in L6 myotubes, and this effect is dependent on glucose concentration in the media and the duration of exposure in culture.

LIST OF FIGURES (Appendix A)

Dedication

This thesis is dedicated to my father, Richard Munoz, who has guided me from up above every step of the way.

It is also dedicated to my loving grandparents, Frank and Marge Stevens, who have supported me in every form possible, and have been instrumental in my success.

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INTRODUCTION

During the last twenty years, the study of the insulin-signaling pathway in skeletal muscle has lead to several breakthroughs in understanding the regulation of glucose uptake. Skeletal muscle accounts for the largest percentage of glucose uptake in a post-prandial state (6). Skeletal muscle is well adapted to ensure circulating glucose homeostasis, and its energetic demands regulate glucose uptake. Under basal conditions, glucose transporter 1 (GLUT1), located in the cell membrane, mediates glucose uptake (40). Upon insulin stimulation, glucose uptake into the cell is dramatically increased as glucose transporter 4 (GLUT4) migrates to the cell membrane and facilitates glucose uptake (79). After entering the cell, intracellular glucose has a variety of different fates. It can be metabolized to support cellular energy demands through glycolysis and aerobic metabolism, or stored in the cell in the form of glycogen (27). Alternatively, glucose can enter the hexosamine synthetic pathway (14). In addition to exercise, insulin is the most potent stimulator of glucose uptake in skeletal muscle. Failure to increase intracellular glucose concentrations upon insulin stimulation is known as insulin resistance (77) and has a series of negative outcomes for the energy status of the cell (37).

A number of different factors may cause inhibition of glucose entry into the cell. Insulin-resistance in skeletal muscle has been proposed to occur at various stages of the insulin-signaling cascade. While the specific mechanisms distinctively responsible for insulin resistance are not delineated, dysregulation of the hexosamine synthesis pathway may play a key role in this process. Although

this pathway is only responsible for a small percentage of intracellular glucose, it is thought to play a large role in the development or progression of insulin resistance (14, 59). The initial step of the hexosamine synthesis pathway and its relationship to insulin resistance has been extensively studied (9, 14, 17, 33, 47, 59, 65, 68, 76). However, the hexosamine synthetic pathway is not the only mechanism identified in relationship to insulin resistance. An inhibition of insulin to induce the translocation of GLUT4 to the plasma membrane has been implicated in insulin resistance as well (76, 77, 87). Impaired translocation of GLUT4 results in decreased glucose uptake into the cell and a decrease in downstream glucose storage in the form of glycogen (7).

Glycogen is a polysaccharide of glucose and serves as short term energy storage in skeletal muscle (1). Accumulation of intramuscular glycogen indicates the cell is in positive energy balance (29). Changes in skeletal muscle glycogen content influences energy demands within the cell (41). Regulation of intramuscular glycogen content has long been studied in conjunction with endurance performance (42). Therefore, insulin-resistance leading to impaired glucose uptake into the cell and decreased accumulation of glycogen would presumably have a negative impact on the regulation of the energy demands within the cell and forseeably, endurance performance.

Skeletal muscle has had a significant role in the research focused on the mechanisms explaining insulin-resistance. A plethora of different intracellular environments have been implicated in the development of insulin-resistance in skeletal muscle (25, 30, 34, 35, 38, 50, 66, 70).

Type 2 diabetes mellitus and hyperglycemia accounts for the predominant focus of research studies examining insulin-resistance (77). A number of different supplements have also been shown to dysregulate glucose uptake. Glucosamine, an over the counter supplement used in the treatment of osteoarthritis (5), possesses a chemical structure similar to glucose, and has been studied for it's ability to induce insulin resistance in various tissues (7). The effect of glucosamine supplementation on glucose uptake is somewhat controversial; however, evidence suggests that glucosamine may contribute to the development of insulin resistance (2, 4, 7, 30, 33, 34, 36, 39, 46, 69, 75, 90). Interestingly, the effects of biological concentrations of glucosamine supplementation on insulin-stimulated glucose uptake have yet to be determined in skeletal muscle. This effect will therefore be examined indirectly in this study as reflected by changes in glycogen content with glucosamine supplementation *in vitro*.

ENERGY REGULATION

Muscle cells are often the focus of cellular research regarding energy regulation. Muscle cells are studied for their impact on all functions of life as cardiac muscle, smooth muscle and skeletal muscle serve as the foundation machinery for all bodily functions. The two most noted substrates for fuel in skeletal muscle are carbohydrates and fat (19). Carbohydrates are most noted for their “quick energy” capabilities. Carbohydrates are transported into skeletal muscle cells by glucose transporters. After glucose is transported into the cell it is phosphorylated by hexokinase and then further catabolized via glycolytic and oxidative pathways (7). Downstream of the phosphorylation of glucose, carbohydrate is stored in muscle in the form of glycogen through activation of glycogen synthase (18). The regulation of energy within the cell is very important in maintaining the function of the cell.

Skeletal muscle cell lines constitute a useful system to study skeletal muscle biology and compliment *in vivo* experimental approaches for assessing glucose uptake. Cell culture models allow for careful analyses of cellular processes that are difficult to document within *in vivo* models. Specifically, rat L6 skeletal muscle cells are frequently used as a model for translating *in vitro* results to the *in vivo* condition. Rat L6 myoblasts have the capability to differentiate into multi-nucleated myotubes possessing functional GLUT4 and therefore mimic insulin-stimulated glucose uptake similar to human skeletal muscle fibers (80). Properties of the L6 cell line mimic isolated skeletal muscle fibers and their signaling events (79). Insulin signaling that occurs in a cell culture model allows

for careful analyses of each of the proteins and enzymes involved in insulin-stimulated glucose uptake.

GLUCOSE UPTAKE

Skeletal muscle is responsible for roughly 70-90% of insulin-stimulated glucose uptake in a postprandial state (6, 83). Physiologically, the two most important regulators of glucose uptake are insulin and exercise (79). Skeletal muscle responds to an acute increase in insulin concentration by increasing glucose uptake two-to-eight fold as compared with no insulin stimulation (31). The rate of glucose uptake and glycogen accumulation in skeletal muscle is dependent on the presence of insulin, but generally a threshold exists between 3 and 6 hr (24) after which glycogen accumulation rate declines.

Activation of an insulin receptor through the binding of insulin triggers a series of signaling cascades that are responsible for the translocation of GLUT4 (43). Although many forms of glucose transporters exist, GLUT1 and GLUT4 remain the two most predominant glucose transporters in skeletal muscle. Only 5-10% of glucose uptake is accounted for by GLUT1 which is responsible for basal glucose uptake (95). Of the 13 known glucose transporters, GLUT4 remains the only isoform that is insulin responsive in skeletal muscle (43).

In a cell specific manner, insulin evokes metabolic, mitogenic and anti-apoptotic actions depending solely on the intrinsic tyrosine kinase activity of its receptor (Fig 1.) (81). Insulin binding to the extracellular α-subunits of the insulin receptor induces a conformational change that serves to activate the two

transmembrane β-subunits. This results in a series of intermolecular transautophophorylation reactions (55) allowing for appropriate recognition of insulin receptor substrates (IRSs) (56). Tyrosine phosphorylated insulin receptor substrate-1 (IRS-1) binds with the SH2 domain of the p85 subunit on phosphoinositol 3 kinase (PI3K) converting membrane bound phosphatidylinositol (4,5)-bisphosphate (PIP2) via phosphorylation, into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (3). This step is pertinent to the downstream signaling that facilitates increased glucose uptake into the skeletal muscle cell. Formation of PIP3 is required for activation of phosphoinositide dependent kinase 1 (PDK1). PDK1 relays the signaling message to phosphorylate the Akt protein, protein kinase B (PKB) and protein kinase C (PKC) (43, 48). These proteins each serve important and unique roles in glucose uptake into a skeletal muscle cell.

Each of the proteins Akt, PKB and PKC signal for the redistribution of the GLUT4 protein via vesicle exocytosis and GLUT4 translocation to the plasma membrane (43). Translocation of GLUT4 to the plasma membrane facilitates an increase in glucose uptake into the cell. In conjunction, Akt and PKB phosphorylate glycogen synthase kinase 3 (GSK3) contributing to the increased activity of glycogen synthase via it's dephosphorylation (41).

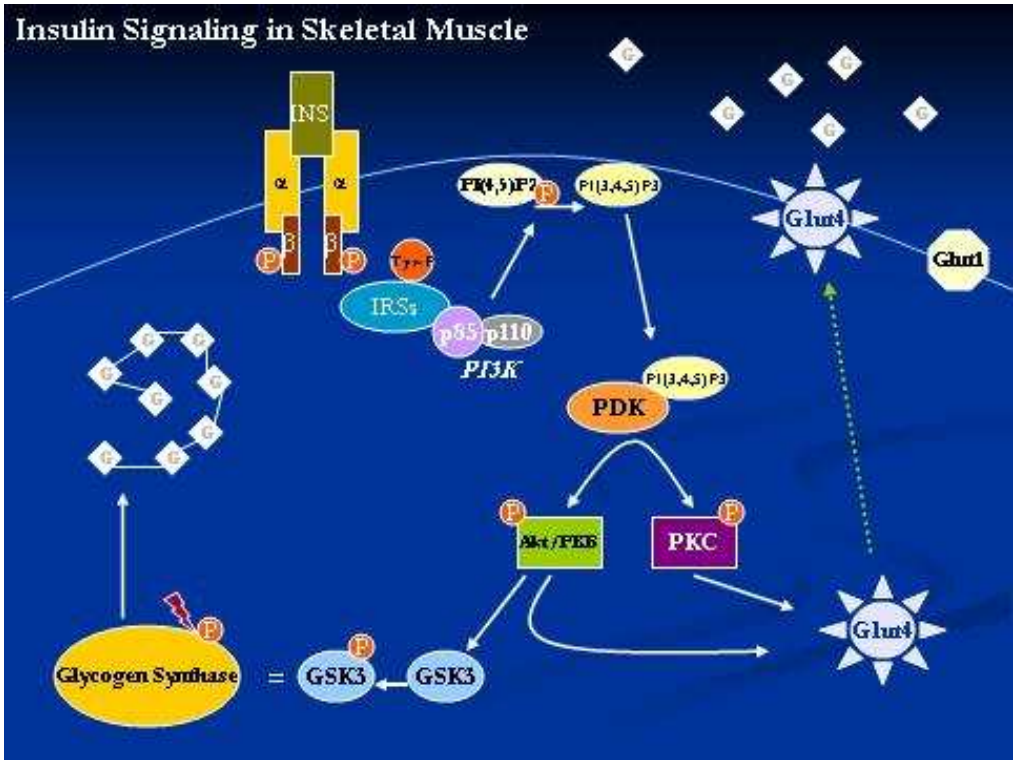

Fig. 1. Schematic representation of insulin-stimulated glucose uptake. Insulin-stimulated glucose uptake results in downstream signaling proteins of the insulin receptor, translocation of glucose transporter isoform 4 (GLUT4), and glycogen synthase activation. Insulin binding with the insulin receptor stimulates increased glucose uptake via GLUT4 translocation and increased glycogen storage via the activation of glycogen synthase (10, 23, 27, 29, 32, 37, 40, 41, 47, 50, 56, 68, 73, 81, 95).

Upon termination of insulin stimulation, GLUT4 protein is internalized through calithrin-dependent endocytosis and is subjected to an intracellular sorting process as it is returned to GLUT4 storage compartments within the cytoplasm of the skeletal muscle cell (43). The dynamic processes of GLUT4 trafficking is directly orchestrated through the binding of insulin to the insulin receptor and the intracellular signaling cascade that occurs as a result. A reduction in glucose uptake into the skeletal muscle cell could be the result of an interference of any of the outlined signaling steps of insulin-stimulated glucose uptake.

INTRACELLULAR FATE OF GLUCOSE

The fate of glucose after it enters the cell determines how it will be utilized as a source of energy (Fig. 2). Upon entry into the cell, glucose is phosphorylated, becoming glucose-6-phosphate (G-6-P) and can be converted further into fructose-6-phosphate (50). Signaling that occurs beyond the phosphorylation of fructose-6-phosphate is determinant upon the cell's energy status.

Glucose may be utilized as an immediate energy source where, once it is converted to fructose-6-phosphate, it leads to lactate formation or pyruvate oxidation in the Krebs cycle which is metabolically linked to the process of glycolysis and supported by oxidative phosphorylation (50, 78).

If the cell is in positive energy balance, as depicted by the balance of glycogen synthase and glycogen phosphorylase (29), glucose will be phosphorylated to glucose-1-phosphate from glucose-6-phosphate. Furthermore, glucose-1-phosphate is converted into UDP-glucose and stored as glycogen through the dephosphorylation of glycogen synthase with the subsequent phosphorylation of glycogen synthase kinase 3 (GSK3) (28, 43, 50).

Additionally, glucose may also enter the hexosamine synthesis pathway, whereby glutamine:fructose-6-phosphate amidotransferase (GFAT) catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate (14, 17, 75). Although this pathway only accounts for ~ 3% of total glucose utilized (57), it is ultimately responsible for the production of UDP-N-acetylglucosamine (UDP-

GlcNAc) and UDP-N-acetylgalactosamine that are incorporated into higher order macromolecules such as glycoproteins and proteoglycans (14, 75).

The first and rate-limiting enzyme of the hexosamine pathway is GFAT, and current evidence is controversial regarding its role in the development of insulin resistance and impaired glucose uptake (14, 57, 58). An increased flux through the hexosamine pathway via the increased activity of GFAT may function as a cellular nutrient sensor and play a role in the development of insulin resistance (11, 12, 59, 76).

GLYCOGEN STORAGE

Glycogen is stored and used to provide substrate for cellular energy balance. Glycogen is stored in the liver and the muscle. If intracellular glucose concentration exceeds the need for energy, glucose is stored as glycogen under the control of glycogen synthase (18). Glycogen can be utilized during the onset of exercise when glycogen phosphorylase is activated and this activity increases exponentially with exercise intensity (67). Diets high in carbohydrate composition have been shown to increase glycogen storage intramuscularly (20) and are therefore frequently used by endurance athletes.

Glycogen reservoirs are essential in ensuring energetic demands of skeletal muscle. Storage of glycogen occurs via intracellular signaling and is dependent on the energy demands of the cell. Enhanced glucose uptake into the skeletal muscle cell promotes the storage of glycogen within the cell. However, the energy requirements of the cell dictate the signaling pathways that occur with

glycogen storage and the rate of glycogen accumulation in the cell. Changes in G-6-P concentration within physiological range also modulate the flux through hexokinase and glucose transport systems (25, 78) and therefore have an effect on glycogen accumulation. Increased UDP-glucose is directly linked to activation of glycogen synthase via it's dephosphorylation (41). Controversy exists on whether basal glycogen concentration determines the rate of glycogen deposition or the concentration of UDP-glucose within the cell (28). Regardless, glycogen accumulation in skeletal muscle ultimately relies on the presence of insulin, amount of extracellular glucose present, and the intracellular signaling for glucose uptake related to the energy status of the cell.

INSULIN RESISTANCE

The detrimental effects of exposing cells to high glucose concentrations include impaired glucose metabolism and insulin resistance (77). Insulin resistance is associated with diseases such as type 2 diabetes and obesity (47). As a condition that exists prior to the onset of disease, insulin resistance can be attributed to genetic factors and other factors such as hyperglycemia (22). Clinically, insulin-resistance is defined as the reduced ability of insulin to lower plasma glucose in tissues expressing GLUT4 (skeletal and heart muscle and adipocytes) (14). Sustained hyperglycemia is one of the most common risk factors leading to insulin resistance (94). Activation of the hexosamine synthesis pathway is attributed to an excess glucose flux into the cell (11, 12). Activation of the hexosamine signaling pathway outlined in an earlier section (see

INTRACELLULAR FATE OF GLUCOSE), results in an accumulation of UDP-GlcNAc in the cell. As the predominant end-product of the hexosamine pathway, UDP-GlcNAc is an allosteric feedback inhibitor of GFAT, the rate-limiting enzyme of the hexosamine pathway (14). A number of different hypotheses have described the potential mechanisms explaining insulin resistance.

GLUCOSAMINE INDUCED INSULIN RESISTANCE

Glucosamine is a supplement taken prophylatically and used to alleviate joint discomfort associated with osteoarthritis (86). As a building block of proteoglycans which act to lubricate and nourish collagen found in cartilage, glucosamine has beneficial effects on repairing and maintaining cartilage found in the body (53). Glucosamine has been successful in clinical research for preserving cartilage associated with overuse from aging and/or excessive activity (89).

Glucosamine as a substrate has a similar chemical structure as glucose. However, it is also a metabolic product of glucose that occurs through an irreversible enzymatic pathway (86). Glucosamine may interfere with the hexosamine biosynthesis pathway and contribute to insulin resistance or an impairment in glucose uptake (59, 90) (Fig. 2).

Under conditions in which intracellular energy needs are met, glucose metabolism shifts to the hexosamine pathway resulting in accumulation of endogenous glucosamine (86). Accumulation of endogenous glucosamine from the conversion of endogenous glucose results in diminished glucose uptake as

the cell recognizes glucosamine as a substrate (90). Exogenous glucosamine also interferes with glucose uptake into skeletal muscle. Uptake of extracellular glucosamine contributes to an increase in endogenous glucosamine concentration and leads to a down-regulation of glucose uptake (86).

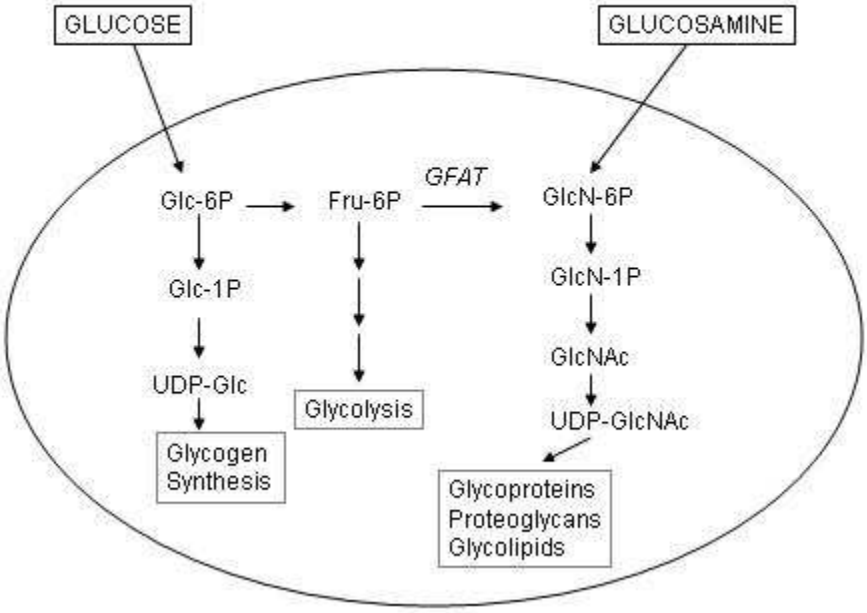

Fig. 2. Simplified schematic representation of hexosamine biosynthesis pathway. Following entry via glucose transport system into the cell, glucose is primarily utilized by pathways for glycogen synthesis and glycolyis. The hexosamine pathway accounts for ~3% of intracellular glucose via the conversion of fructose-6-phosphate (Fru-6P) to glucosamine-6-phosphate (GlcN-6P) by the rate limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Glucosamine transport into the cell bypasses the GFAT enzyme an results in accumulation of the hexosamine synthesis pathway end-products (59, 65, 91).

There is considerable evidence from *in vitro* and animal studies suggesting that glucosamine negatively impacts insulin sensitivity and glucose metabolism (2), although *in vivo* studies with humans subjects dispute these findings (72). According to Pouwels et al. (72) the discrepancy between the results can be explained by differences in species, the presence or absence of

diabetes, and the experimental design, including the duration of hyperglycemia. Results from i*n vitro* experiments in animal models demonstrate that increasing concentrations of glucosamine interfere with glucose transport, glycogen synthesis and responsiveness of insulin secretion, in conjunction with high glucose concentrations (30).

One such example demonstrated the ability of 5 mM glucosamine supplementation to induce insulin resistance in 3T3-L1 adipocytes (39). Although mechanisms of glucosamine induced insulin resistance seem were not elucidated in this study, prolonged incubation with glucosamine has been shown to impair GLUT4 translocation in skeletal muscle (74), resulting in decreased glucose uptake into the cell and decreased glycogen storage. Other researchers have also noted an inhibition of GLUT4 translocation in the presence of glucosamine. For example, 3T3-L1 adipocyte cells exhibit signs of insulin resistance, by inhibition of GLUT4 translocation, when treated with 2 mM glucosamine (75).

Translocation of GLUT4 in skeletal muscle is also inhibited by glucosamine. Baron et al. (7) demonstrated that glucosamine had an inhibitory effect on GLUT4 translocation in skeletal muscle *in vivo*. In this study, a constant infusion of glucosamine at various concentrations was associated with skeletal muscle insulin-resistance in Sprague Dawley rats (75). Furthermore, subfractionation of the sarcolemma from various skeletal muscles of the rats in the study indicated that GLUT4 expression in plasma membrane subfractions

was reduced after glucosamine treatment (75). In parallel, reduced GLUT4 expression was associated with insulin resistance (49).

Glucosamine seems to interfere with GLUT4 translocation in a similar fashion to non-insulin dependent diabetes mellitus (NIDDM) (33). This is hypothesized to occur in part due to an increase in endogenous glucosamine leading to the negative feedback on glucose uptake (7, 74). However, it is not completely clear which upstream protein(s) may be responsible for this dysregulation.

One mechanism by which glucosamine interferes with GLUT4 translocation may involve GFAT, an enzyme that is widely expressed in human tissues (65). Glucosamine, taken into the cell via GLUT1 and GLUT4 in skeletal muscle, activates the hexosamine biosynthetic pathway (63). The hexosamine pathway may serve as the fuel sensor for insulin-sensitive cells, indicating that when the cell is inundated with substrates (i.e., glucose, and/or glucosamine) insulin resistance develops (34, 57, 59). Glucosamine is utilized to increase the flux through the hexosamine-phosphate pathway (7, 38, 46), because it is metabolized to glucosamine-6-phosphate by the coupled glucose transporter/hexokinase and therefore bypasses GFAT leading to a glucose-independent increase in the hexosamine pathway (Fig 3.) (63).

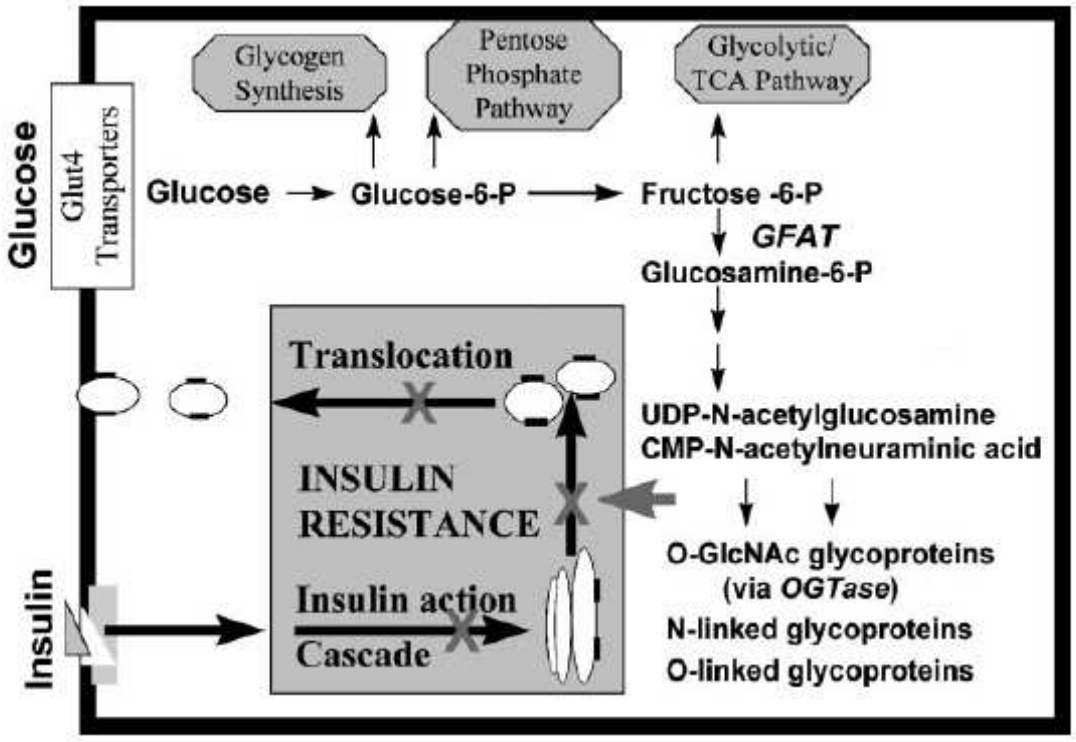

Fig. 3. Schematic representation of insulin resistance elicited by flux through hexosamine synthesis pathway. As an end-product of the hexosamine pathway, glucosamine induces insulin resistance by interfering with the insulin signaling cascade and translocation of GLUT4. Reduced translocation of GLUT4 leads to impaired glucose uptake into the cell. Adapted from ref 46.

Interestingly, increases in the products resulting from the increase in hexosamine biosynthetic pathway, such as glucosamine, correlate with a depletion in intracellular ATP (26). This does not explain the mechanism for hyperglycemia and GFAT overexpression induced insulin-resistance, because glucose does not cause a depletion in intracellular ATP (39). Activation of 5'-AMP-activated protein kinase (AMPK) that is attributed to an increased AMP:ATP ratio (43) has also been associated with GLUT4 translocation (35, 52). Thus, the energy status of the cell reflected by the AMP:ATP ratio and activation of AMPK may serve as an individual mechanism regulating the translocation of GLUT4 as

well. On the other hand, a reduction in intracellular ATP occurs in conjunction with the inhibition of insulin receptor autophosphorylation and IRS-1 tyrosine phosphorylation (39) and may therefore be independent of the activation of AMPK.

It is apparent that glucosamine has been shown to induce insulin resistance, even though it seems that the mechanisms underlying glucosamine induced insulin-resistance and glucose induced insulin-resistance are the not same (61). These findings suggest that increased endogenous glucosamine inhibits the insulin-signaling cascade starting at the insulin receptor in a separate fashion than that which is observed during hyperglycemic conditions.

FUTURE RESEARCH

While glucosamine has been reported to reduce glucose uptake (19, 36, 39) and glycogen storage (74), the exact mechanisms regulating these events remain elusive. Glucosamine concentrations that are linked to insulin-resistance are greater than those obtained through oral supplementation, e.g., plasma concentration = 0.06 mM glucosamine (2). Over-the-counter glucosamine supplements are ingested in quantities equal to or slightly less than 5 mM dosage. However, the maximum intracellular concentration is believed to equal 0.1 to 0.2 mM glucosamine (64). Research on the impact of biological or pharmacological doses of glucosamine on muscle glycogen accumulation is scarce. It is foreseeable that if biological and/or pharmacological concentrations

of glucosamine have the ability to reduce glucose uptake into the cell, then glucose homeostasis and glycogen storage may be impaired.

It is important to consider how glucosamine is marketed as a supplement to treat osteoarthritis since glucosamine supplementation may contribute to human disease such as insulin-resistance, especially in patient populations such as the elderly and/or obese, who are most often utilizing the supplement (5). Although glucosamine supplementation may have a detrimental role in glucose homeostasis, it could hypothetically be considered an “ergogenic aid” for athletes. If in fact, glucosamine does induce insulin resistance in individuals, it might be a useful supplement for athletes seeking glycogen depletion prior to performance. Athletes who use glycogen depletion and super-compensation methods to improve endurance performance (42) may be able to utilize glucosamine as a supplement to reduce glycogen storage as opposed to caloric restriction methods. To date, it is unknown if glucosamine can be used as a viable ergogenic aid for endurance performance. Therefore, the purpose of this study was to examine the effects of 0.06 mM, 1 mM, and 5 mM glucosamine on glycogen storage in L6 myotubes.

STATEMENT OF THE PROBLEM

Glucosamine is a common over-the-counter oral supplement utilized by a variety of individuals for the treatment of osteoarthritis and related symptoms. As a supplement that is arguably detrimental to glucose uptake and glycogen storage in a variety of different species and tissues, the effects of glucosamine supplementation in skeletal muscle have not been elucidated. Additionally, the effects of the complex extracellular environment, in conjunction with glucosamine supplementation, warrant further examination. Inhibition of glucose uptake and/or glycogen accumulation in skeletal muscle in competitive endurance athletes may prove to be detrimental to endurance performance and training. To date, the effects of glucosamine supplementation during euglycemic and hyperglycemic conditions, with and without the presence of insulin on glycogen content in skeletal muscle are unknown. The objectives of this study are: 1) to examine the effect of varying concentrations of glucosamine supplementation on glycogen content in L6 myotubes; 2) to examine the effect of glucosamine supplementation on glycogen content in L6 myotubes during incubation periods of 1, 3 or 5 hrs; 3) to examine in L6 myotubes the effect of glucosamine supplementation on glycogen content under euglycemic and hyperglycemic conditions with and without the presence of insulin.

HYPOTHESES

The hypotheses of the study are: 1) insulin will increase glycogen content independent of glucosamine concentration; 2) a time threshold exists after which glucosamine has no effect on glycogen content in L6 myotubes; and 3) in a hyperglycemic/hyperinsulinemic environment, glycogen content will be reduced by 0.06 mM, 1 mM, and 5 mM glucosamine in a dose-dependent manner.

RESEARCH DESIGN AND METHODOLOGY

Materials

Rat L6 myoblasts were purchased from ATCC (product #CRL-1458, Manassas, VA). Fetal calf serum (FCS, product #SFBU-100) and donor equine sera (product # SE-0100) were purchased from Equitech Industries (Kerrville, TX). Minimum Essential Eagle Medium (αMEM, product #M0894), penicillin/streptomycin (product # P4333), porcine insulin (product #57595), Periodic-Acid Schiff staining kits (product# 395B), and glucosamine hydrochloride (product #G1514) were all purchase from Sigma-Aldrich (St. Louis, MO). Thermanox coverslips (product #26030) were purchased from Ted Pella, Inc., (Redding, CA).

Cell Culture

Myoblasts were seeded into 100mm petri dishes containing four 22mm thermanox coverslips at a density of ~7500 cells/cm^2 in αMEM (5mM glucose) supplemented with 10% (vol/vol) FCS, 100U/mL penicillin/100µg/ml streptomycin, and 2.2 g/L sodium bicarbonate at 37^0C in the presence of 5% CO_2. Myoblasts were grown in monolayers and allowed to reach confluence (~ seven days). After confluence, the media was changed to αMEM (5mM glucose) supplemented with 2% (vol/vol) horse serum, 100U/mL penicillin/100µg/ml streptomycin, and 2.2 g/L sodium bicarbonate to induce differentiation. Cells were maintained in culture and allowed to fully differentiate from days 7-14. The

media was changed every 48 hours and cells were used from passage numbers 3-4. Experiments were performed at day 14.

Experimental Treatments

Cells were serum starved in αMEM (5 mM glucose) for five hours prior to experimental treatments. Cells were treated with αMEM for 1-, 3-, or 5-hr time periods. Treatment media contained either 5 mM or 25 mM glucose concentrations to mimic a euglycemic or hyperglycemic state. Media was supplemented with 100 nM insulin to emulate a post-prandial state. Concentrations of glucosamine varying from 0.06 mM, 1 mM or 5 mM were used in treatment groups.

Periodic-Acid Schiff (PAS) Staining

Thermanox coverslips were rinsed with phosphate buffered saline and fixed with 4% formaldehyde for one minute at room temperature. Slides were rinsed for one minute under tap water. Coverslips were then placed in columbia jars and immersed in 9 mL of Periodic Acid Solution (Sigma-Aldrich, product #395-1) for five minutes at room temperature. Following this, slides were rinsed in five changes of distilled water. Next, coverslips were immersed in 9 mL of Schiff's reagent (Sigma-Aldrich, product #395-2) for 15 minutes at room temperature and washed under running tap water for five minutes. Coverslips were allowed to dry in columbia jars for 24 hours and mounted on glass slides with low viscosity Cytoseal-60 (Richard-Allan Scientifc, product #8310-16).

Image analysis

Digital photographs were randomly taken of each coverslip with Nikon CoolPix 995 Digital Camera attached to a Nikon Eclipse E200 microscope. Standardized manual camera settings were used for each image obtained (HI setting, 1/125 exposure time). Within each coverslip, four images were taken at random with a 4x objective.

Image J Pro software was used to analyze staining intensities. Color Deconvolution, an additional plug-in for the Image J software, was used to separate out red, green and blue images specific to the PAS staining solution.

Images uploaded into Image J were separated into red, green, and blue images using the H PAS option for the Color Deconvolution plug-in. Red images, representing the wavelength depicting PAS-positive staining (600-650 nm), were used for the quantitative analysis of glycogen content. White images taken prior to each sequence of digital photographs were used to quantify absorbance of PAS-positive staining based on the following equation:

$$A_\lambda = \log_{10}(I_0/I)$$

8-bit red images were divided by 8-bit white images and converted to a 32-bit resulting image. The log of the resulting image was used for analyses. Integrated optical density units were measured after subtraction of the modal value.

Statistical Analysis

Data were tested for normality and were analyzed by two or three way ANOVA and appropriate post hoc analyses (i.e., Pairwise Multiple Comparison Procedures by the Holm-Sidak method or Dunn's method for unequal sample sizes). Data that were not normally distributed with equal variance were analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks. All analyses were conducted using Sigma-Stat (SPSS Inc., Chicago, IL). Significance was accepted at $P < 0.05$.

RESULTS

Effect of insulin on glycogen content in L6 myotubes

After fully differentiating, L6 myotubes were serum starved for five hr and subjected to experimental treatments. Treatments included varying concentrations of glucose (5 mM or 25 mM), insulin (0 nM or 100 nM) and glucosamine (0 mM, 0.06 mM, 1 mM or 5 mM) for incubation times of 1, 3 or 5 hr. A significant main effect for insulin treatment was observed. Overall, insulin increased glycogen content in L6 cells by approximately 118% as compared with cells given no insulin treatment ($P = 0.023$, Fig. 4).

Effect of glucosamine supplementation on glycogen content during a euglycemic condition

L6 myotubes were subjected to a euglycemic condition (5 mM glucose) for 1, 3 or 5 hr with varying concentrations of glucosamine (0.06 mM or 5 mM) (Fig. 7). As the duration of incubation was prolonged, glycogen content increased in L6 myotubes. Glycogen content in L6 myotubes was significantly greater after 5 hr. than after 1 and 3 hr incubation, across all concentrations of glucosamine used ($P < 0.00002$ and $P = 0.006$ respectively, Fig 5.). Glycogen content was significantly less in myotubes incubated with 5 mM glucosamine than with 0.06 mM glucosamine, independent of insulin treatment at 3 and 5 hr ($P = 0.0469$, Fig 5.).

Effect of glucosamine supplementation on glycogen content during hyperglycemic conditions

L6 myotubes were subjected to a hyperglycemic condition (25 mM glucose) for 1, 3, or 5 hr with either 0 mM, 0.06 mM, 1 mM or 5 mM glucosamine (Fig. 8 & 9). A significant interaction was observed between the effects of glucosamine concentration and incubation time on glycogen content, independent of insulin ($P < 0.001$, Fig 6.). Glycogen content in L6 myotubes was greater (119%) following 5 hr incubation with 1 mM glucosamine than after 0.06 mM glucosamine supplementation (56063$\pm$475 vs. 47237$\pm$227 IODU, respectively, $P = 0.004$, Fig 6.).

After 1 hr incubation, 0.06 mM glucosamine treatment corresponded with greater glycogen content (137%) than observed after no glucosamine exposure (56825$\pm$275 vs. 41520$\pm$435 IODU, respectively, $P < 0.001$, Fig 6.). Glycogen content was also greater (139%) after 1 hr incubation with 1 mM glucosamine supplementation (57733$\pm$653 IODU) than the content visualized for the no glucosamine condition (41520$\pm$435 IODU, $P < 0.001$, Fig 6.). Alternatively, 5 mM glucosamine treatment resulted in significantly less glycogen observed after 1 hr (8539$\pm$218 IODU, $P = 0.011$, Fig 5.) and 5 hr (33744$\pm$249 IODU, $P = 0.009$, Fig 5.) than identified with 1 mM glucosamine treatment (57733$\pm$653 IODU, 1 hr, 56063$\pm$282 IODU, 5 hr. respectively).

The time of incubation influenced L6 myotube glycogen content. In the absence of glucosamine (0 mM), significantly more glycogen was visualized in the cells after 3 and 5 hr as compared with 1 hr incubation ($P = 0.000158$, for

differences between 1 and 3 hr, and $P = 0.000718$, for differences between 3 and 5 hr. respectively, Fig 6.). Supplementation with 1 mM glucosamine for 3 or 5 hr resulted in significantly less glycogen content versus 1 hr incubation with a similar glucosamine concentration ($P < 0.001$, for differences between 1 and 5 hr, and $P = 0.001$, for differences between 3 and 5 hr respectively, Fig 6.).

DISCUSSION

The results of this study demonstrated that accumulation of glycogen content in L6 myotubes, as determined by PAS staining and image analysis, was influenced by glucose and glucosamine concentrations and the duration of the incubation period. As expected, 100 nM insulin supplementation increased glycogen content independent of time and concentrations of glucose and glucosamine in the culture media. These findings are consistent with previous studies (6, 9, 10, 13, 23, 27, 29, 32, 47, 56, 60, 79, 81, 82, 87, 91, 92). Glycogen accumulation increased with incubation time in the 5 mM glucose treatment groups. These results support the work of Elsner et al. (24) indicating that the rate of glycogen accumulation in serum-starved L6 myotubes that are subsequently cultured with 5 mM glucose reaches a threshold at approximately five hr. Glycogen accumulation in the 5 mM glucose treatment groups reflects glucose uptake through GLUT1, as non-insulin dependent glucose uptake (49, 62, 71, 93). It is assumed that the increase in glycogen content observed in the present study resulted from activation of increased glycogen synthase activity as the availability of intracellular glucose improved upon exposure to media containing 5 mM glucose (18, 29, 47).

Glycogen content in L6 cells was reduced after incubation with 5 mM glucosamine as compared to that of 0.06 mM glucosamine concentration. Glycogen content in the cell represents the dynamic balance of glycogen utilization and synthesis. Glycogen synthesis can be inhibited by accumulation of intracellular glucosamine metabolites which induce GFAT activity and lead to

desensitization of the glucose transport system in adipocytes (33, 57, 58). If such conditions existed in myotubes used in the present study, it is logical to assume that dysregulation of glucose transport occurred to a greater extent in the 5 mM versus the 0.06 mM glucosamine treatment. This assumption is based on evidence of similar glucose signaling pathways in adipocytes and skeletal muscle (15, 16).

The results of this study indicated that glucosamine can impair short-term glycogen accumulation in L6 myotubes cultured in a hyperglycemic environment. These findings are consistent with previous reports of the inhibitory effect of glucosamine on glycogen synthesis and the signaling mechanisms regulating glycogen deposition (2, 4, 19). Glycogen content was reduced after 1 hr incubation with significant differences in glycogen content observed between 0.06 mM and 5 mM glucosamine treatments. Moreover, the results indicate that the inhibitory effect on glycogen content was overcome with time as no differences in glycogen straining were observed after 3 and 5 hr incubation.

A limitation of this portion of the study is that no data were collected with 0 mM glucosamine in the euglycemic environment. This information would be useful for clarifying the overall effect of glucosamine on glycogen content after exposure to 5 mM glucose and for examining the predominant reduction in glycogen content observed after 5 mM glucosamine treatment as compared with the 0.06 mM glucosamine concentration.

The physiological relevance of these findings is unknown. Hyperglycemia *in vivo* would occur in tangent with hyperinsulinemia (12) and thus, it is

interesting to note that a significant reduction in glycogen content occurred only in the 25 mM glucose plus 100 nM insulin treatments. Glycogen content decreased over time with 0.06 mM and 1 mM glucosamine concentrations and was particularly evident within the insulin-stimulated treatments. Extracellular concentration of glucosamine between 0.06 and 1 mM is achievable *in vivo*, representing biological and pharmacological doses, respectively (54).

Insulin signaling is the primary mechanism for increased glucose uptake into skeletal muscle *via* the recruitment of GLUT4 by a variety of signaling proteins including PI3K, PDK, Akt, PKC (37, 60, 88). Although glucosamine has been shown to interfere with the translocation of GLUT4 in rodent skeletal muscle (7), one mechanism by which glucosamine may act on GLUT4 signaling occurs through the insulin receptor (8, 14, 84), as insulin receptor phosphorylation is impaired with glucosamine supplementation (84). This is the first step in initiating the insulin-signaling cascade. Impairment in this step would prevent the translocation of GLUT4, decrease glucose uptake and decrease activation of glycogen synthase, thus explaining a decrease in cellular glycogen accumulation. Other mechanisms that may contribute to glucosamine-induced inhibition of glycogen storage in skeletal muscle include increased GFAT expression (4, 14-17, 21, 36, 65, 76) and a decrease in GLUT4 translocation (4, 21, 26, 34, 36). These mechanisms may be intricately involved in the decreased glucose uptake observed in L6 cells with glucosamine treatment. Overexpression of GFAT correlates with interference in the insulin-signaling cascade even though the exact mechanisms have not yet been delineated (69).

It is also possible the glucosamine supplementation *in vivo* has no negative effects on glycogen content during a euglycemic condition in skeletal muscle (85). Glycogen content was not significantly reduced after incubation with 5 mM glucosamine, 25 mM glucose and 100 nM insulin. Steiler et al. (85) observed a similar effect of euglycemia and glucosamine treatment on muscle glycogen content in the soleus muscle of Goto Kakizaki (diabetic) rats. These authors and others (44, 45, 85) reported that hyperglycemia but not euglycemia correlated with an increase in protein kinase C (PKC)ζ activity/phosphorylation, a predominant protein responsible for the translocation of GLUT4 to the plasma membrane (27). These findings were observed with and without the presence of insulin (85). Increased PKCζ activity/phosphorylation without the presence of insulin suggest that the extracellular milieu can directly alter the kinetic properties of insulin-signaling intermediates and intracellular enzymes (85). Although it may seem paradoxical that increased PKC activity/phosphorylation may inhibit glucose uptake, long-term activation of PKC is observed with diabetes mellitus (85). Together with other proteins, such as PI3K and Akt (51), PKC is involved in the inhibition of the insulin signaling cascade and presumably glucose uptake (85).

Glucosamine has similar effects on the inhibition of the insulin-signaling cascade observed in rodent skeletal muscle (69). Implications of the effects of hyperglycemia on the insulin-signaling cascade may explain the results of the present study. Furthermore, these results indicate that glucosamine may

interfere with glycogen accumulation in skeletal muscle under hyperglycemic conditions.

It is reasonable to speculate that if high levels of extracellular glucosamine interfere with insulin signaling proteins, then prolonged exposure to such concentrations may also induce insulin resistance in skeletal muscle, independent of glucose concentration. Further research that focuses on prolonged exposure of skeletal muscle cells to glucosamine, independent of glucose concentration may be useful in determining if glucosamine directly affects the proteins involved in the insulin-signaling cascade and may elucidate mechanisms responsible for glucosamine induced insulin-resistance.

In conclusion, it may be plausible that in the presence of a hyperinsulinemic and hyperglycemic environment, glucosamine has the ability to interfere with glycogen accumulation and presumably glucose uptake *in vivo*. If this were the case, a target population impacted by these phenomena would include endurance athletes who use glucosamine supplements. During prolonged endurance exercise, athletes consume simple carbohydrates approximately every 45 minutes in an effort to preserve intramuscular glycogen stores (20, 42). Prior supplementation with doses eliciting 0.06 - 1 mM glucosamine in the extracellular milieu may limit glucose uptake into skeletal muscle and potentially compromise endurance performance. Further research examining the interactions among glucosamine supplementation, carbohydrate intake, and glycogen metabolism will determine whether glucosamine supplementation is detrimental to athletic performance.

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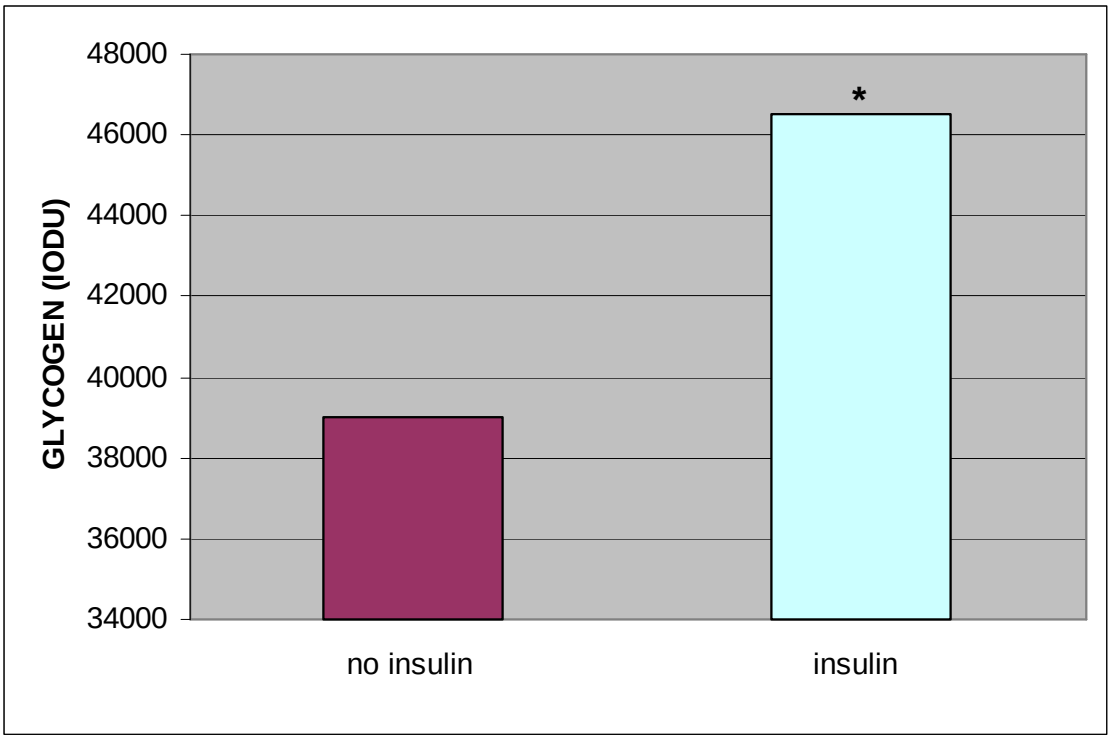

Fig. 4. **Main effect of insulin on glycogen content in L6 myotubes.** * P = 0.023 vs no insulin. Data are mean±SEM of three experiments. (IODU- Integrated Optical Density Units).

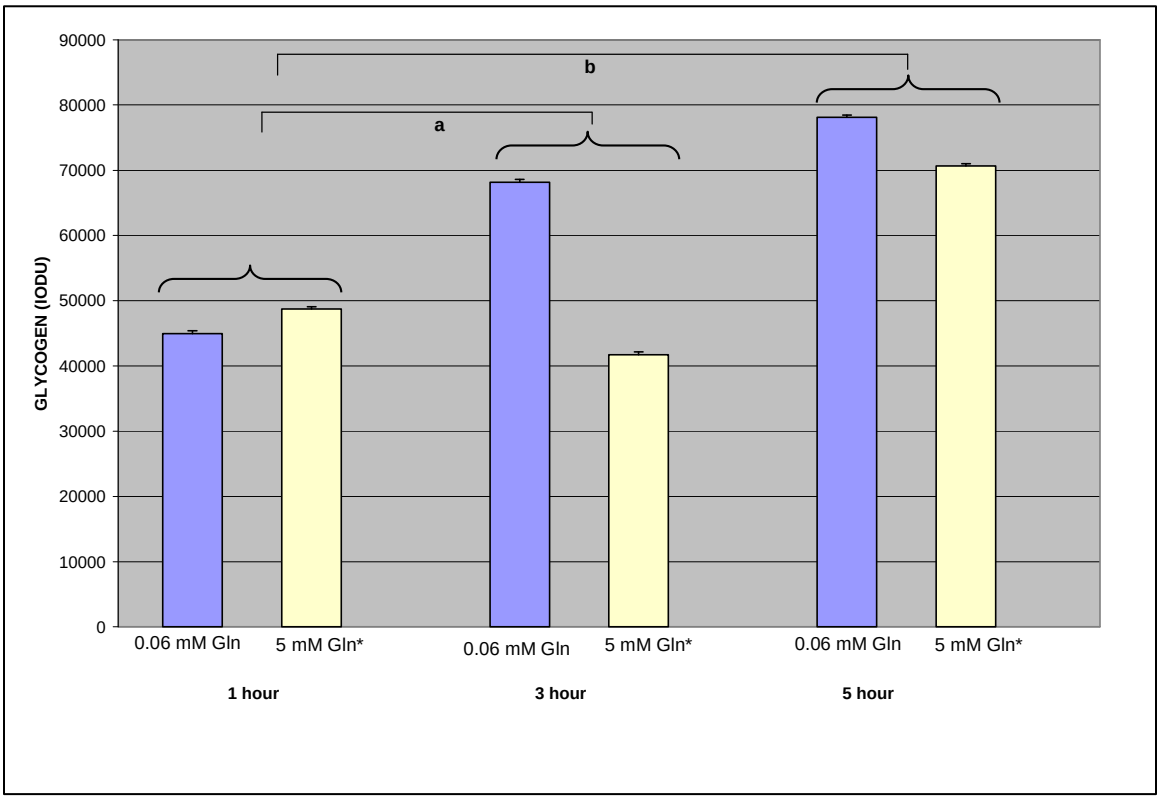

Fig. 5. **Effect of glucosamine treatment in euglycemic condition (5 mM glucose) on glycogen content in L6 myotubes at 1, 3, 5 hr.** *, P = 0.0469 vs. 0.06 mM Gln. [a], P = 0.00002 for 1 hr treatment vs 3 hr treatment. [b], P = 0.006 for 1 hr treatment vs. 5 hr treatment. Data are mean±SEM of three experiments. (IODU- Integrated Optical Density Units).

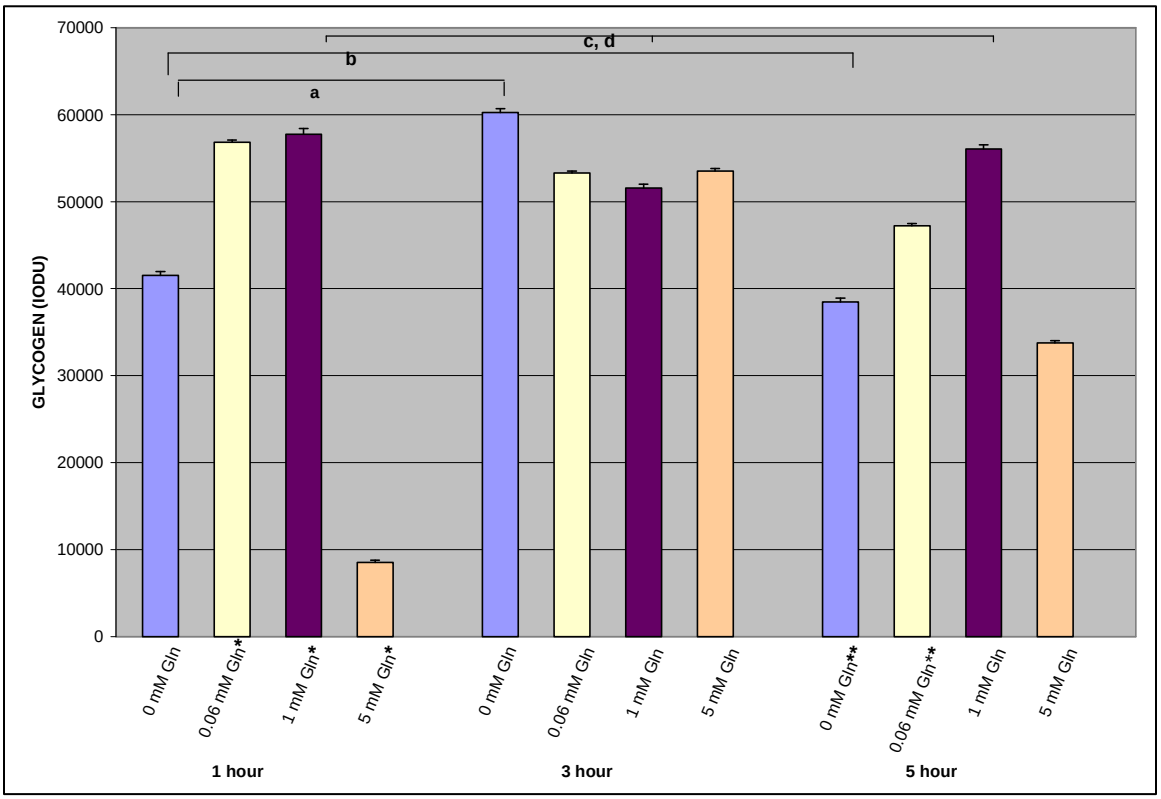

Fig. 6. **Effect of glucosamine treatment in hyperglycemic conditions (25 mM glucose) on glycogen content in L6 myotubes.** * $P < 0.001$ for 0.06 mM, 1 mM and 5 mM Gln at 1 hr vs. 0 mM Gln at 1 hr. **, $P = 0.004$ for 0 mM Gln at 5 hr vs. 1 mM Gln at 5 hr and $P = 0.009$ for 0.06 mM Gln at 5 hr vs. 1 mM Gln at 5 hr. [a], $P = 0.000158$ for 0 mM Gln at 1 hr vs. 0 mM Gln at 3 hr. [b], $P = 0.000718$ for 0 mM Gln at 1 hour vs. 0 mM Gln at 5 hour. [c], $P < 0.001$ for 1 mM Gln at 1 hour vs. 1 mM Gln at 5 hr. [d], $P = 0.001$ for 1 mM Gln at 3 hr vs. 1 mM Gln at 5 hr. Data are mean±SEM of three experiments. (IODU- Integrated Optical Density Units).

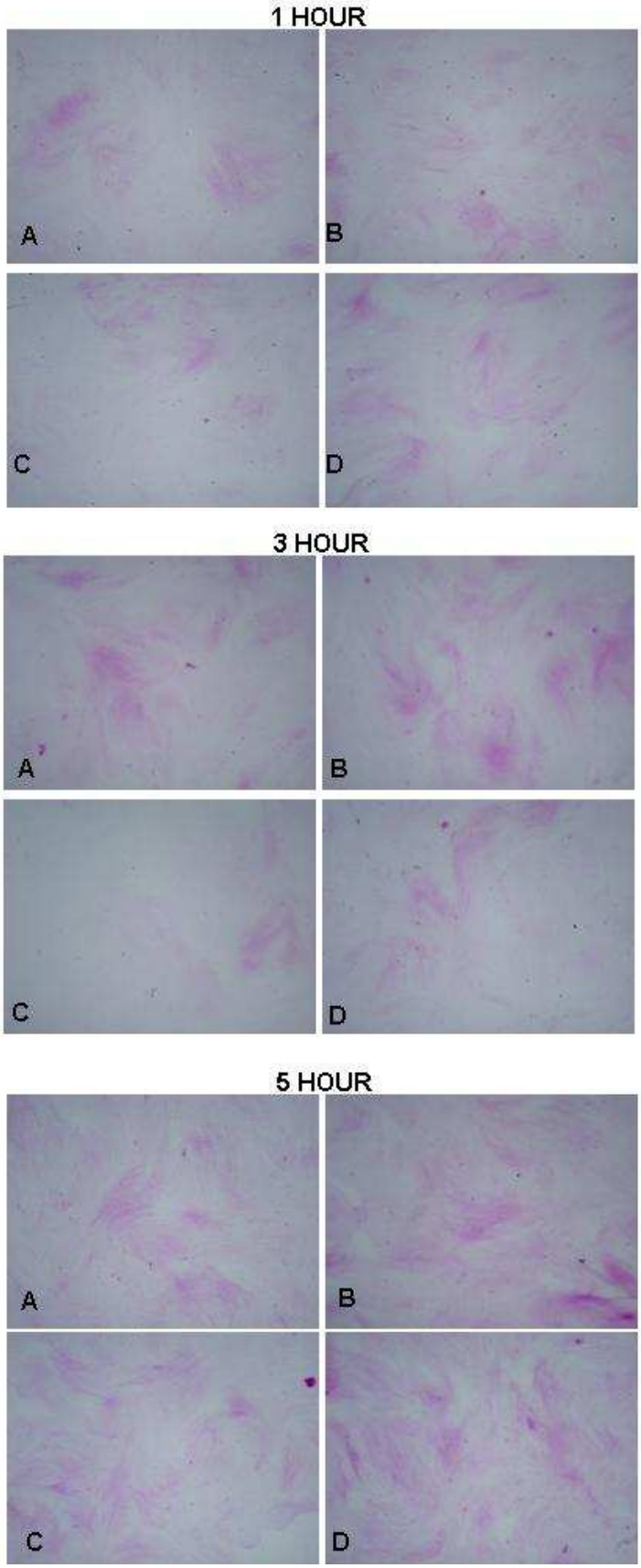

Fig 7. Glycogen content visualized by PAS staining in L6 myotubes following experimental treatments in a euglycemic enviroment (5 mM glucose) with glucosamine supplementation (0.06 mM or 5 mM) and with or without insulin (100 nM). ***A*** *= 0.06 mM glucosamine,* ***B*** *= 0.06 mM glucosamine, 100 nM insulin* ***C*** *= 5 mM glucosamine,* ***D*** *= 5 mM glucosamine, 100 nM insulin.* Images represent one of twelve images for each experimental group.

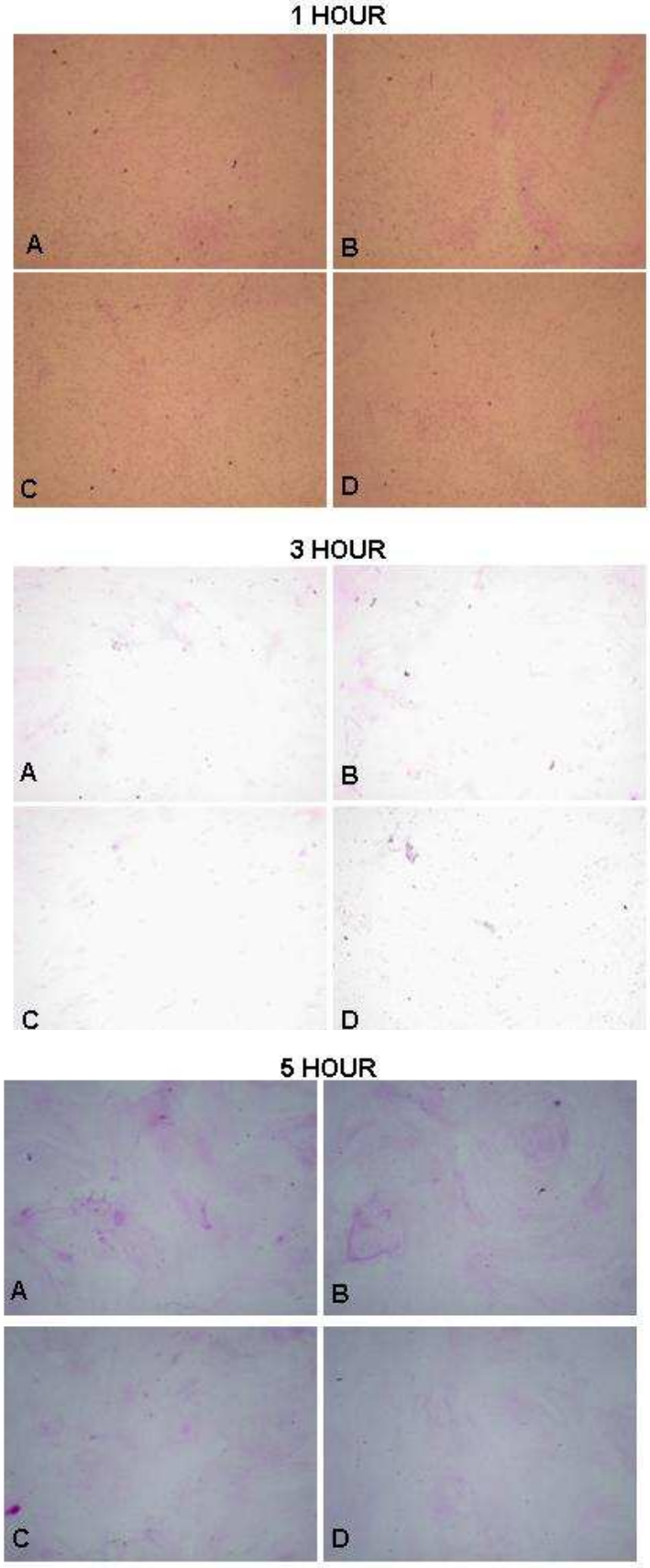

Fig 8. Glycogen content visualized by PAS staining in L6 myotubes following experimental treatments in a hyperglycemic enviroment (25 mM glucose) with and without glucosamine (0 mM and 5 mM) at 1, 3, or 5 hr. ***A*** *= 25 mM glucose,* ***B*** *= 25 mM glucose, 100 nM insulin,* ***C*** *= 25 mM glucose, 5 mM glucosamine,* ***D*** *= 25 mM glucose, 5 mM glucosamine, 100 nM insulin. Images represent one of twelve images for each experimental group.*

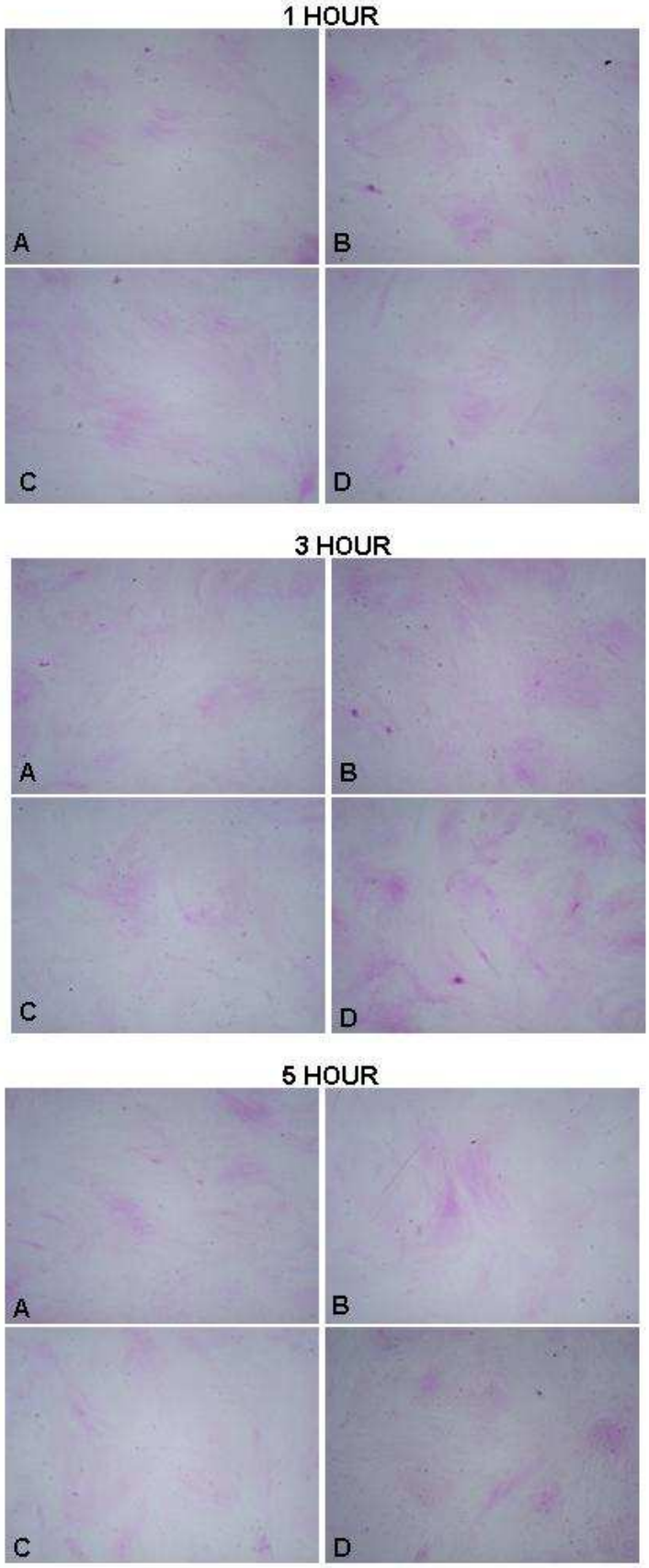

Fig 9. Glycogen content visualized by PAS staining in L6 myotubes following experimental treatments in a hyperglycemic enviroment (25 mM glucose) with glucosamine (0.06 mM and 1 mM) at 1, 3, or 5 hr. ***A*** *= 25 mM glucose, 0.06 mM glucosamine* ***B*** *= 25 mM glucose, 0.06 mM glucosamine, 100 nM insulin,* ***C*** *= 25 mM glucose, 1 mM glucosamine,* ***D*** *= 25 mM glucose, 1 mM glucosamine, 100 nM insulin. Images represent one of twelve images for each experimental group.*