THE EFFECT OF LITHIUM CHLORIDE ON THE DISTAL INSULIN SIGNALING CASCADE AND ON P38 MAPK IN THE SOLEUS MUSCLE OF FEMALE LEAN ZUCKER RATS

by

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STATEMENT BY AUTHOR

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DEDICATION

This thesis is dedicated to my loving family. To my wonderful husband, John: you are truly the wind beneath my wings. To my precious children Jess, Zach, Missy, Jake, and Sierra: I could not have completed this thesis without your patience and support. To my incredible parents, Wendell and Lucy Smith: your role model to me of a strong work ethic and all of your encouragement helped me to complete this thesis.

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ABSTRACT

This project focused on determining the effect of lithium on glucose uptake, glycogen synthesis, and insulin signaling proteins, protein kinase B (Akt1) and GSK-3, in isolated soleus muscle from female lean Zucker rats. We also investigated the role of the stress-activated p38 MAPK in the action of lithium to activate skeletal muscle glucose transport. In the absence of insulin, lithium (10 mM LiCl) increased basal glucose transport by 62% ($p<0.05$) and glycogen synthesis by 112%. Lithium did not alter phosphorylation of Akt ser473, but enhanced GSK-3β ser9 phosphorylation by 41%. Lithium further enhanced the effect of insulin on glucose transport (42%), glycogen synthesis (44%), and GSK-3ß phosphorylation (13%). Lithium increased phosphorylated p38 MAPK 31% without and 19% with insulin. Moreover, a selective p38 MAPK inhibitor, A304000, completely prevented the lithium-induced enhancement of glucose transport revealing the critical involvement of p38 MAPK phosphorylation in lithium-induced glucose transport in isolated skeletal muscle.

PART I: INTRODUCTION

A. Cause and symptoms of Type 2 Diabetes.

Type 2 diabetes has become one of the major health issues both in the United States and abroad, affecting 200 million people worldwide (www.idf.org). Unlike Type 1 diabetes, which is caused by auto-immune destruction of insulin-secreting pancreatic β cells, Type 2 is often caused by physiological changes associated with obesity. Reaven (1993) classifies the primary defect as insulin resistance, and has described a clustering of atherogenic risk factors that he named "Syndrome X". Symptoms of this syndrome include glucose intolerance, hyperinsulinemia, and dyslipidemia. To better understand those factors that contribute to this insulin resistance, one must first consider the normal action of insulin on glucose transport.

B. Regulation of skeletal muscle glucose transport.

Glucose transport into muscle and fat cells has been a primary focus of diabetes research, with particular interest in the insulin signaling cascade (Figure 1). Pancreatic β cells secrete insulin when plasma glucose concentrations are elevated. Insulin then binds to the membrane bound insulin receptor (IR) of muscle cells. This binding initiates a sequence of events beginning with tyrosine phosphorylation of the receptor substrate-1 (IRS-1) by tyrosine protein kinase. The phosphorylated IRS-1 then causes activation of phosphatidylinositol 3-kinase (PI-3K), which catalyzes the production of phosphatidylinositol lipid moieties (PIP_3). These lipid moieties activate a protein kinase, phosphoinositide-dependent kinase (PDK-1) that phosphorylates protein kinase B (also

known as Akt1) (Chang, et al, 2004; Holman & Kassuga, 1997). GLUT-4 glucose transporter proteins, activated by downstream signals originating at Akt1, then migrate to the cell membrane to facilitate glucose transport into the cell (Guma, et al, 1995). Furthermore, Akt1 phosphorylates and by this means inactivates glycogen synthase kinase-3 (GSK-3). The non-phosphorylated GSK-3 can phosphorylate and activate glycogen synthase, thereby mediating glucose incorporation into glycogen (Le Marchand-Brustel, 1999).

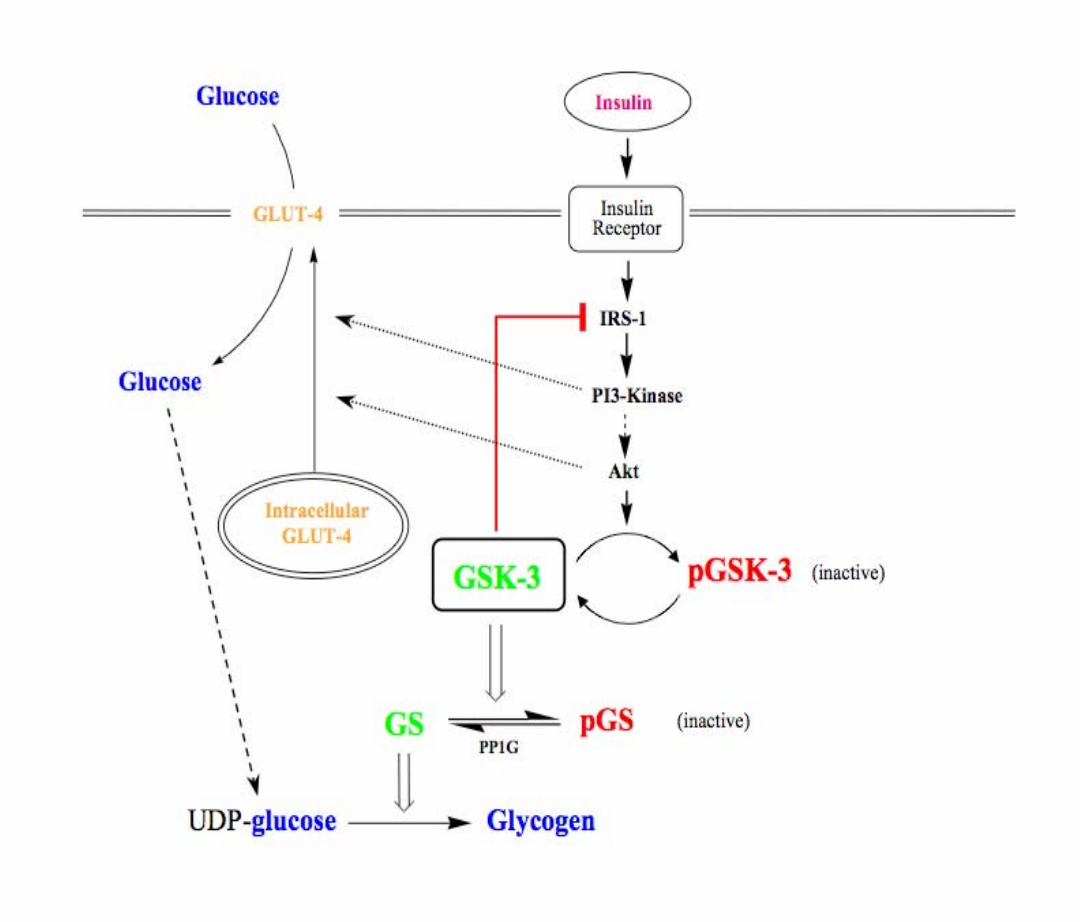

Figure 1: Diagram of the insulin signaling cascade (Henriksen & Dokken, 2006).

Glucose transport also may be affected by the actions of the p38 mitogen-activated protein kinase (p38 MAPK), which Somwar et al (2000) suggest may stimulate glucose uptake by muscle cells by interacting with the insulin signaling cascade and/or boosting GLUT-4 functionality. Furtado et al (2003) reported that insulin rapidly activates both α and β isoforms of p38 MAPK in mature skeletal muscle and that these isoenzymes are involved in the GLUT-4 activation pathway. The mechanism remains to be elucidated, but some evidence implies that p38 MAPK may itself be recruited to the plasma membrane, the site of the insertion of translocated GLUT-4 proteins.

C. Insulin resistance of muscle glucose transport

According to Zierath et al (2000), the predominant characteristic of Type 2 diabetes is insulin resistance in somatic cells (e.g., skeletal muscle cells). This resistance may be due to decreased insulin receptor (IR) and/or insulin receptor substrate (IRS-1) protein concentration or reduced phosphorylation of these proteins (Zierath et al., 2000) or from defective GLUT-4 trafficking and translocation due to defective insulin activation of PI-3 kinase (Zierath et al, 1997). Dresner et al (1999) found that elevated fatty acid concentration can prevent translocation of GLUT-4 and its fusion with the cell membrane. Grundleger & Thenen (1982) showed that a high fat diet decreases insulin binding by 35% due to reduced insulin receptor number, but not receptor affinity. According to Paolisso et al (1996), high free fatty acid concentrations enhance oxidative stress, created by redox reactions resulting in the formation of reactive oxygen species (ROS), which may in turn interfere with insulin-stimulated glucose uptake and further

contribute to insulin resistance. Henriksen (2006) and Evans et al (2002) suggest that oxidative stress may lead to the activation of p38 MAPK, which can interfere with insulin signaling. This interference may result from the phosphorylation of serine, rather than tyrosine, residues on IRS-1.

Insulin resistance also can be attributed to an overexpression and overactivity of GSK-3 (Henriksen and Dokken, 2006). Nikoulina et al (2000) found that muscle tissue taken from type 2 diabetes subjects contained twice the normal amount of both α and β isoforms of GSK-3 and this increase "may contribute to the impaired glycogen synthase activity and skeletal muscle insulin resistance present in type 2 diabetes." Since GSK-3 inhibits glycogen synthase, which is responsible for glycogen synthesis from glucose, the plasma concentration of glucose would remain elevated with increased GSK-3 activity. Furthermore, Dokken et al (2005) provided evidence that GSK-3β inactivates IRS-1 by phosphorylation, causing downregulation of the insulin signaling cascade. All of these factors could contribute to the body's inability to transport glucose into the cell and thus reduce its conversion into glycogen for storage.

D. Effects of lithium on the glucose transport system.

Recently, much attention has been given to lithium as an effective GSK-3 inhibitor. Dokken et al. (2005) showed that by selectively inhibiting GSK-3β, upregulation of the insulin signaling cascade occurs, thus enhancing glucose uptake and glycogen synthesis. Strunecka et al (2005) note that because lithium's structure is similar to that of sodium and potassium, due to the single electron in its outer shell that can react

readily with other compounds, lithium may replace such ions in certain chemical reactions. Choi & Sung (2000) determined that lithium inhibits GSK-3β without affecting GSK-3β phosphorylation. Haugaard, et al. (1974) and Tabata, et al (1994) showed that lithium upregulates glycogen synthesis in the presence of insulin. However, it remains unclear whether the LiCl has an effect on other parts of the insulin signaling cascade or if it operates by another mechanism in enhancing glucose transport.

E. Purpose of my research

The purpose of this research project is to characterize the effect of lithium on the glucose transport system in mammalian skeletal muscle. Specifically this will be determined by investigating the effect of lithium chloride on 1) the functionality of Akt1 and GSK-3β within the insulin signaling cascade, and 2) the stimulation of p38-MAPK.

PART II: MATERIALS AND METHODS

Animals

Lean (150-170 g) female Zucker rats (Fa/-) (age: 6-7 weeks old) (Gulve et al, 1993) were obtained from Harlan (Indianapolis, IN).

In vitro treatment of skeletal muscle

Rats were fasted overnight and injected intraperitoneally with 50 mg/kg body weight of Nembutal (pentobarbital sodium). The soleus muscle was extracted from each hindleg and split into two strips (~25-35 mg each). Each soleus strip was then incubated in vitro for 1 hour in a solution containing 3 ml of pH 7.4 Krebs-Henseleit buffer (KHB) and 8mM glucose, 32mM mannitol, 0.1% BSA (RIA grade from Sigma Chemical) and gassed with 95% oxygen/5% CO_2. Also added to the KHB was 5 mU/ml insulin in the absence or presence of 10 mM LiCl. At the end of the 1-hour incubation, the muscle strips were frozen with an aluminum clamp chilled in liquid nitrogen, trimmed of fat and connective tissue, and immediately weighed on an analytical balance. The tissue was then stored at -80° F until use.

Assessment of insulin signaling

Each soleus muscle strip was homogenized in 8 volumes of ice-cold homogenization buffer solution (50 mM HEPES pH 7.5, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM ß-glycerophosphate, 10 mM NaF, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM $CaCl_2$, 10 ug/ml aprotinin, 10 µg/ml leupeptin, 0.5 µg/ml pepstatin, 2 mM phenylmethylsulfinyl flouride) using a rotating glass rod grinder. The amount of homogenization buffer for each sample was determined by performing a total

protein assay of each sample (BCA method, Pierce, Rockford, IL). The appropriate amount of buffer was then added to each sample to yield a final protein concentration of 250µg/35µl. Samples were kept on ice for 20 minutes, centrifuged at 13,000g for 20 minutes at 4°C and aliquoted to the appropriate protein concentration. 3X Laemmli sample buffer (LSB) with dye was added (12 µl) and the samples were frozen at -80°C until use.

Each sample was loaded on a 7.5% or 12% polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) and run on an electrophoresis apparatus for 1.5 hours at 220mV in a freshly made electrode buffer mixture. Each gel was then transferred onto a pre-chilled Towbin solution transfer sponge/filter paper and a chilled nitrocellulose paper was placed on top of the gel. The nitrocellulose paper was then covered with another filter paper and sponge, placed in a cartridge, and submerged in chilled Towbin transfer solution adjacent to an ice container and run in an electrophoresis apparatus for 40 minutes. This process was repeated so each cartridge received 40 minutes adjacent to the ice pack. The nitrocellulose paper was then removed and placed immediately in a 5% dry milk solution and agitated for one hour at room temperature. After three 5-minute washes in TBS-T solution, the paper was placed in a small sealed plastic pouch containing a 5% primary antibody (against phospho-Akt ser^{473}, phospho-GSK-3α/ß $ser^{21/9}$, or phospho-p38 MAPK thr^{180}/tyr^{182}) (Cell Signaling Technologies, Berkley, MA). This was chilled overnight at 4° C with rotation. The nitrocellulose paper was then washed 3 X 5 minutes again in TBS-T solution, placed in a sealed plastic pouch with the secondary goat anti-rabbit antibody coupled with horseradish peroxidase (HRP; Chemicon, Temecula, CA)

and anti-biotin for 1 hour with rotation. After three 5 min washes in TBS-T, the nitrocellulose paper was placed in ECL solution for 1 minute, the paper was exposed on Kodak X-Omat Ar film (Kodak, Rochester, NY) and developed. The films were then scanned on a Bio-Rad imaging densitometer (model GS-800) to determine concentrations of p-Akt1, p-GSK-3, and p-p38 MAPK.

Using the same protocol as above, another experiment tested 10 uM A304000, a selective inhibitor of p38 MAPK (Abbott Labs, Abbott Park, IL) to distinguish between lithium's and p38 MAPK's effect on glucose transport.

Muscle glucose transport activity

Muscles were incubated for 20 minutes in 2 ml KHB containing 39 mM (U-^{14}C) mannitol (0.8 μCi/mmol; ICN Radiochemical, Irvine, CA) and 1 mM 2-deoxy-(1,2-^{3}H) glucose (2-DG) (300μCi/mmol; Sigma Chemical). Thereafter, muscles were frozen, trimmed, and weighed as stated above, and then dissolved in 0.5 ml of 0.5 N NaOH. Scintillation cocktail (5 ml) was added and the samples were analyzed in a scintillation counter to determine the intracellular accumulation of 2-DG (Henriksen et al, 1990; Hansen, et al, 1994).

Glycogen synthesis

Net glycogen synthesis was measured as ^{14}C-glucose incorporation into glycogen. Muscles were incubated for 60 minutes in 3 ml KHB containing 8 mM (U-^{14}C) glucose (0.1 μC/ml; Sigma Chemical) and 0.1% BSA. Thereafter, muscles were frozen, trimmed, and weighed as above. Glycogen was isolated as described by Hassid and Abraham (1957) using ethanol precipitation. The glycogen pellet was dissolved in 0.5 ml

of ddH_20 and 5 ml of scintillation cocktail was added. Thereafter, the samples were counted for activity in the ^{14}C-channel.

Statistical analysis

A paired Student's t test between samples from the same muscle was performed. A level of $p<0.05$ was set for statistical significance.

PART III: RESULTS

Effects of Lithium on Glucose Transport

Lithium chloride has significant effects on a number of physiological processes in the body, one of which is glucose transport. For our experiment, the overall effect of lithium on glucose transport needed to be determined before targeting its action at specific metabolic step(s). The effect of lithium on glucose transport in the rat soleus muscle was measured in the absence or presence of insulin (Figure 2).

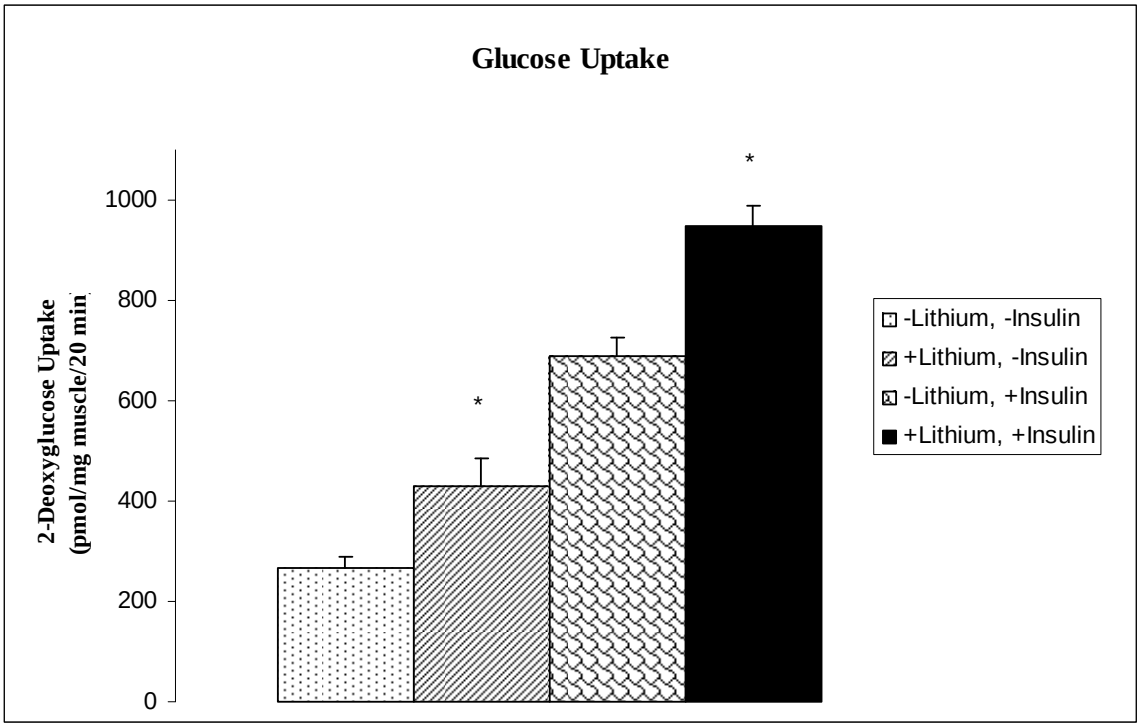

Figure 2: Effect of 10 mM lithium chloride on glucose transport in rat soleus muscle in the absence or presence of insulin (5 mU/ml). Units are pmoles of intracellular 2-deoxyglucose per mg of tissue per 20 minutes. Values are means ± SE for 4 muscles per group. (* $p<0.05$, plus lithium vs. minus lithium).

In the absence of insulin, lithium enhanced glucose transport by 62% ($p<0.05$). Insulin alone caused a 157% ($p<0.05$) increase in the rate of glucose transport. In the

presence of insulin, lithium induced a further increase (42%, $p<0.05$) in glucose transport activity.

Glucose Incorporation into Glycogen

Glucose incorporation into glycogen is an index of the rate of glycogen synthesis in a tissue. Hence the response of glucose incorporated into glycogen in rat soleus muscle in the presence and absence of lithium chloride was determined both with and without insulin stimulation.

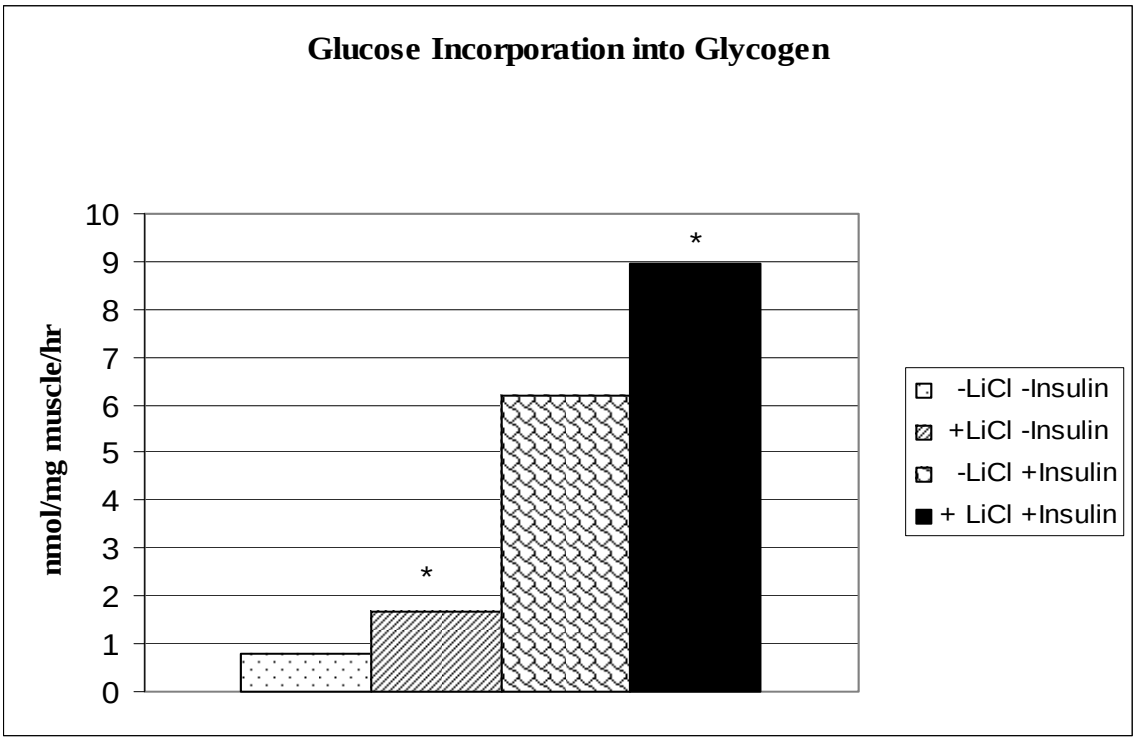

Figure 3: Effects of 10 mM lithium chloride on 14C glucose incorporation into glycogen in both non-insulin stimulated and insulin-stimulated (5mU/ml) rat soleus muscle. Units are nmoles of glycogen isolated per mg of tissue per hour. Values are means ± SE for 4 muscles per group. (* $p<0.05$, plus lithium vs. minus lithium).

Lithium increased glucose incorporation into glycogen in the absence of insulin by 212% ($p<0.05$). Insulin alone stimulated this process by 790% ($p<0.05$). Lithium appeared to have a greater than additive effect with insulin on glycogen synthesis by increasing the rate of insulin-stimulated glycogen synthesis by 44% ($p<0.05$), to 11.4 times the basal rate.

Phosphorylation of Protein Kinase Bα (Akt1)

Akt1, also known as protein kinase Bα, becomes activated upon phosphorylation of the serine 473 residues. Activated Akt1 initiates migration of GLUT-4 proteins to the plasma membrane for glucose uptake (Guma, et al, 1995) as well as phosphorylation, and thus inactivation, of GSK-3 (Le Marchand-Brustel, 1999). Therefore we compared the amount of phosphorylated Akt1 produced in the rat soleus muscle in the presence and absence of lithium, both with and without insulin stimulation (Figure 4).

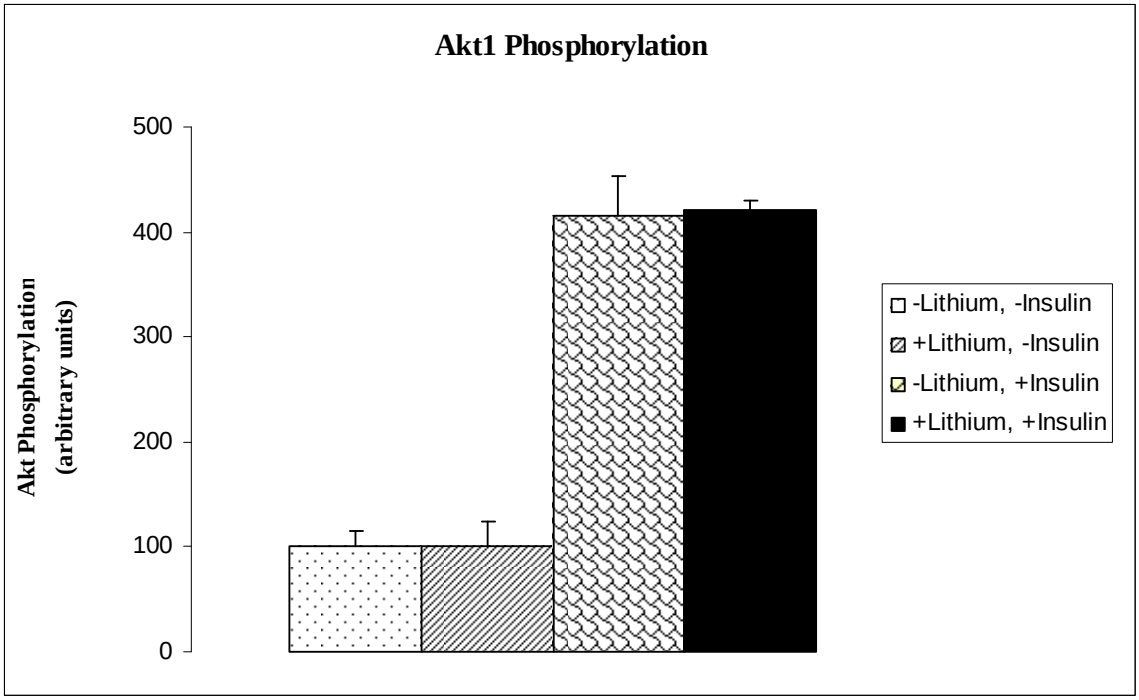

Figure 4: Effects of 10 mM lithium chloride in the absence or presence of 5 mU/ml insulin on serine 473 phosphorylation of Akt1. Units are based on the average densitometry optical density readings obtained from autoradiography film, with basal value was arbitrarily set at 100%. Values are means ± SE for 4-7 muscles per group.

Insulin caused a 314% increase ($p<0.05$) in ser473 phosphorylation of Akt1. However, lithium had no effect on phosphorylation of Akt1 either in the presence or absence of insulin stimulation.

Phosphorylation of Glycogen Synthase Kinase-3

Phosphorylated GSK-3 is responsible for activating glycogen synthase, which initiates production of glycogen from glucose. The extent of GSK-3ß serine-9 phosphorylation generated in the rat soleus muscle in the presence and absence of lithium was measured, both with and without insulin stimulation (Figure 5).

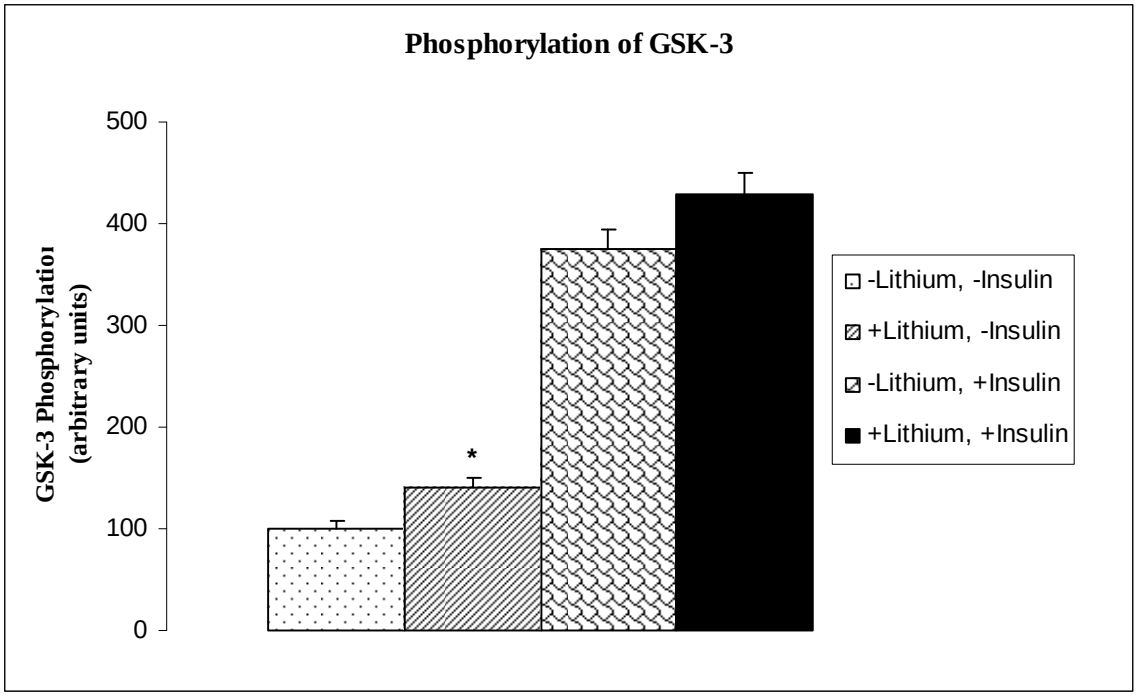

Figure 5: Effects of 10 mM lithium chloride in the absence or presence of 5 mU/ml insulin on serine 9 phosphorylation of GSK-3ß. Units are based on the average densitometry optical density readings obtained from autoradiography film, with basal value was arbitrarily set at 100%. Values are means ± SE for 4-8 muscles per group. (* $p<0.05$, plus lithium vs. minus lithium).

With the addition of lithium, phosphorylation of GSK-3β was enhanced by 41% ($p<0.05$) in the non-insulin stimulated soleus muscle tissue. Insulin alone increased this variable by 330% ($p<0.05$). There was a trend (13%; $p>0.05$) for an enhancement of insulin-stimulated GSK-3β phosphorylation by lithium.

Phosphorylation of p38 mitogen activated protein kinase

Phosphorylated p38 MAPK activates transcription factors which initiate cell replication enhancing glucose uptake simply by the increase in cell number. Furthermore, Furtado et al (2003) suggests in the presence of insulin p38 MAPK stimulates GLUT-4 activity, which also would enhance glucose uptake. The amount of

phosphorylated p38 MAPK generated in the rat soleus muscle in the presence and absence of lithium chloride also was analyzed, either with or without insulin stimulation (Figure 6).

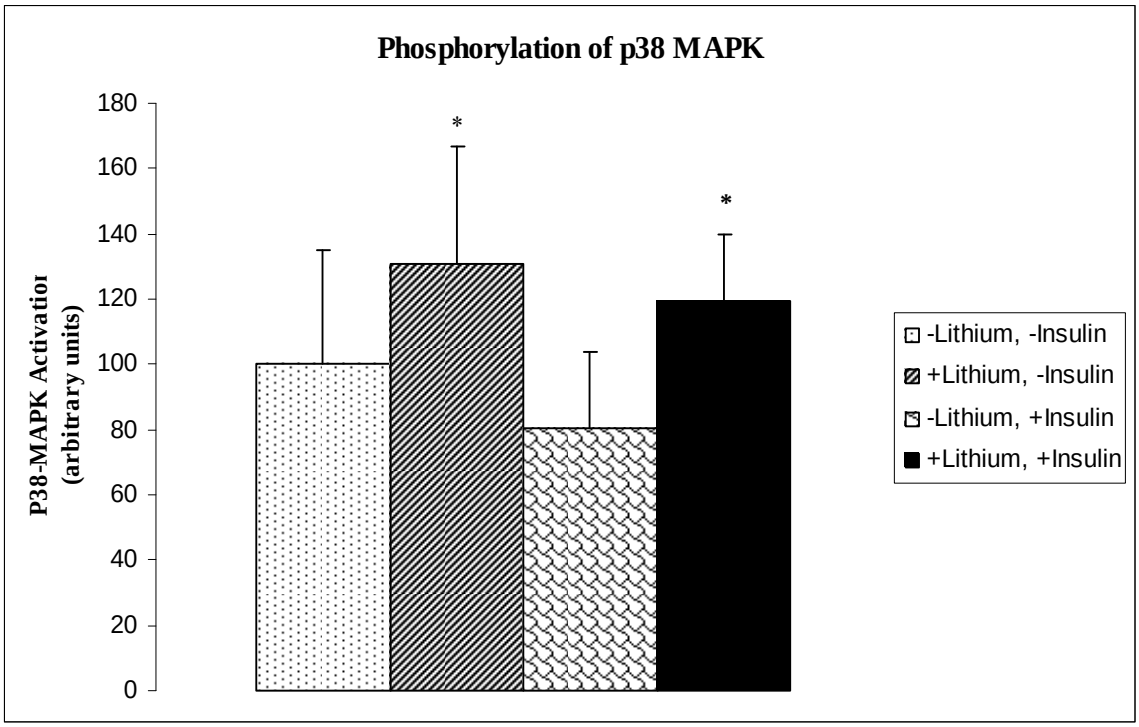

Figure 6: Effects of 10 mM lithium chloride on phosphorylation of p38 MAPK in both non-insulin-simulated and insulin-stimulated (5 mU/ml) rat soleus muscle tissue. Units are based on the average densitometry optical density readings obtained from autoradiography film, with basal value was arbitrarily set at 100%. Values are means ± SE for 4 muscles per group. (* $p<0.05$, plus lithium vs. minus lithium).

In the presence of lithium without insulin stimulation, phosphorylation of p38 MAPK was enhanced by 31% ($p<0.05$). Insulin alone had no significant effect on this variable. However, in the presence of insulin, the addition of lithium increased p38 MAPK phosphorylation by 19% ($p<0.05$).

Effect of selective p38 MAPK inhibitor A304000 on lithium-stimulated glucose transport

To determine if p38 MAPK was a contributor to the increased glucose transport induced by lithium (as shown in Figure 1), the selective p38 MAPK inhibitor A304000 was used (Figure 6). By preventing action initiated by p38 MAPK, it was possible to distinguish if this enzyme was directly affecting lithium-stimulated glucose uptake into the rat soleus muscle.

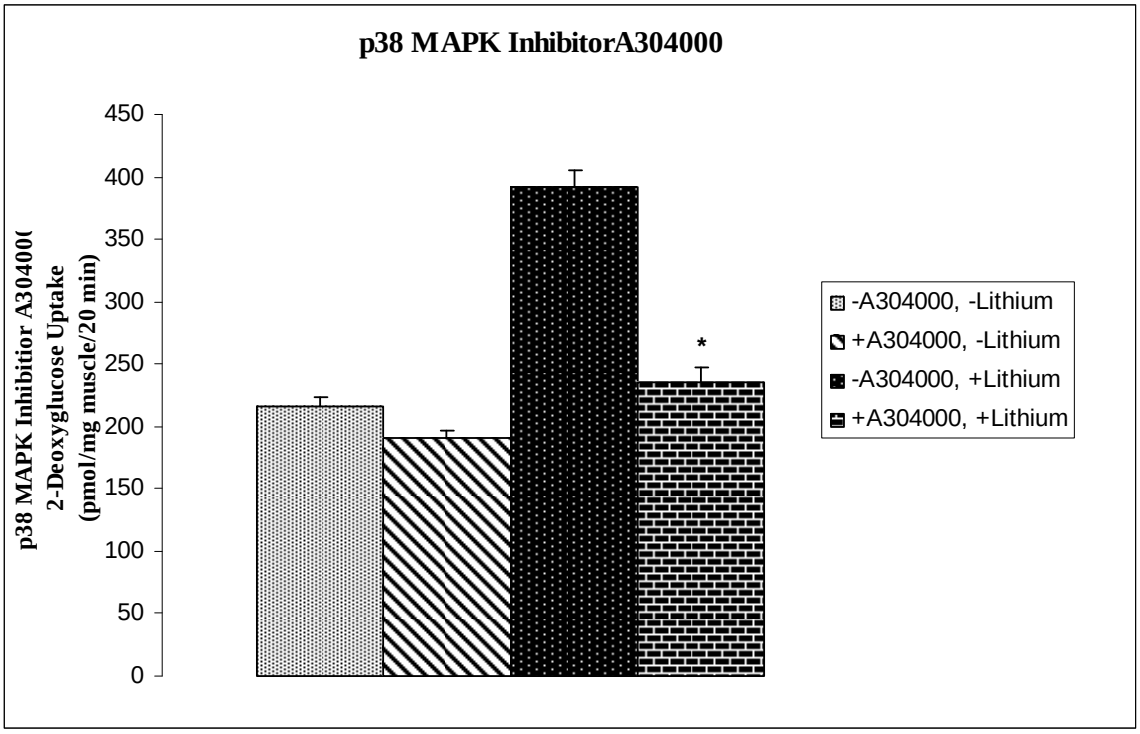

Figure 7: Effects of selective p38 MAPK inhibitor A304000 on lithium-stimulated glucose transport activity in lean rat soleus muscle tissue. Soleus strips were incubated both with and without lithium (10 mM) and 10 μM p38 MAPK inhibitor A304000 for 1 hour. 2-Deoxyglucose uptake was then assessed as described in the Methods section. Values are means ± SE for 4 muscles per group. (* $p<0.05$, plus lithium vs. plus lithium plus A304000).

In the absence of lithium stimulation, the p38 MAPK inhibition did not affect glucose uptake ($p>0.05$). However, the A304000 inhibitor nearly completely abolished ($p<0.05$) the increase in glucose transport induced by lithium.

PART IV: DISCUSSION

Our current findings of in vitro lithium-induced increases in both glucose transport (Fig. 2) and glycogen synthesis (Fig. 3) in insulin-sensitive lean Zucker rats confirm results observed by MacAulay et al (2003) in cultured L6 myotubes and 3T3-L1 adipocytes. Although lithium is a simple cation, it induces significant physiological changes, particularly with respect to enzyme inhibition. Lithium is highly effective in suppressing the activity of the serine/threonine kinase glycogen synthase kinase-3 in L6 myocytes (Choi and Sung, 2000) and inhibits GSK-3β in 3T3-L1 adipocytes (Orena et al, 2000). Henriksen et al (2003) found similar results in rat epitrochlearis and soleus muscles. Upon serine phosphorylation, the GSK-3β becomes inactivated, causing upregulation of glycogen synthase, which is partially responsible, coupled with increases in glucose transport, for initiating conversion of glucose into glycogen (Azpiazu et al, 2000). Zhang et al (2003) found that lithium directly inhibits GSK-3 by phosphorylating its inhibitory N-terminal. Phiel and Klein (2001) suggest that lithium may non-competitively displace the divalent cations in GSK-3 and similar enzymes that are catalyzed by metal ions. Our findings confirm that lithium increases the ser9 phosphorylation of GSK-3 in skeletal muscles (Fig. 4). In contrast, Choi and Sung (2000) found that lithium does not affect phosphorylation of GSK-3 in L6 myotubes, which have characteristics common to both skeletal and smooth muscle. Since elevated levels of GSK-3 are common in insulin-resistant subjects (Nikoulina et al, 2000), the

development of a modified lithium therapy to reduce these levels may be a fruitful area of diabetes research.

Though there appeared to be an activation of the insulin distal signaling cascade by lithium, as seen by the increase in glycogen synthesis, lithium failed to affect upstream Akt phosphorylation (and thus activation) both in the presence and absence of insulin stimulation (Fig.4). Choi & Sung (2000) and MacAulay et al (2003) observed similar results in L6 myocytes and 3T3-L1 adipocytes. Since phosphorylated Akt1 initiates translocation of GLUT4 glucose transporters to the plasma membrane, which may also be enhanced by contractions (Gao et al, 1994), the increase in glucose transport must be the result of activation, rather than translocation, of GLUT4. This activation may be initiated by other mechanisms in addition to Akt1, as seen by several studies from the Klip laboratory (Sweeney et al, 1999; Somwar et al, 2000; Somwar et al, 2002). These studies showed that insulin can increase glucose transport in muscle cell lines and isolated skeletal muscle by a p38 MAPK-dependent activation of GLUT4 glucose transporters.

In this context, one novel and extremely important finding in the present study was the essential role of p38 MAPK in glucose transport activation by lithium, both with and without insulin stimulation (Fig. 6). Kim et al (2006) also demonstrated the importance of p38 MAPK in glucose transport activation in mammalian skeletal muscle by an oxidant stress. Geiger et al (2005) found that both the α and β isoforms of p38 MAPK, stimulated by anisomycin, enhance sensitivity of muscle glucose transport to insulin, but this effect was independent of increased insulin sensitivity induced by contractile activity. Our results imply that insulin stimulation of p38 phosphorylation is

not direct, but may be caused by insulin signaling proteins such as IRS-1 or PI3-kinase. Further investigation is warranted on this issue.

Our findings also showed that addition of the selective p38 MAPK inhibitor, A304000, almost completely reversed the lithium-induced increases in glucose transport. MacAuley, et al (2003) reported similar glucose transport suppression with the p38 MAPK inhibitor SB203580 in L6 myocytes and 3T3- L1 adipocytes. In addition, Geiger et al (2005) observed reversal in insulin sensitivity to glucose transport caused by the p38 MAPK inhibitor, SB-202190. These observations support an essential role of p38 MAPK in modulating glucose transport. We were not able to conclusively determine if p38 MAPK activation is a crucial aspect of insulin stimulation of glucose transport in mammalian skeletal muscle, and other studies (Kayali et al, 2000; Fujishiro et al, 2001; Ho et al, 2004; O'Keefe et al, 2004) do not support the insulin-dependent role of p38 MAPK in glucose transport. The effect of insulin on p38 MAPK is certainly an important avenue of research yet to be pursued.

PART V: SUMMARY

In summary, lithium induced significant increases in glucose transport, glycogen synthesis, phosphorylation of GSK-3β, and p38 MAPK phosphorylation in both insulin and non-insulin stimulated mammalian type 1 muscle tissue. Akt phosphorylation was unaffected, demonstrating that the action of lithium occurs upstream in the insulin signaling cascade, perhaps at PI3-kinase or IRS-1. These findings show there are potential solutions to the problem of insulin resistance so common in type 2 diabetic subjects. By activating mechanisms involved in glucose uptake, such as p38 MAPK, it may be possible to override the defective insulin signaling cascade and lower blood glucose levels by other means. Development of a pharmaceutical other than LiCl (which also affects the nervous system), capable of activating p38 MAPK and/or suppressing GSK-3, may be a future treatment for type 2 diabetes

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