

Endoplasmic Reticulum Stress Increases Glucose-6-Phosphatase and Glucose
Cycling in Liver Cells

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Running Title: ER stress in hepatocytes

Key Words: Unfolded protein response, diabetes, insulin action, glucose production

Supported by grants from the National Institutes of Health DK47416 and DK072017 (MJP). D.W., Y.W., K.N.M. and M.J.P. have nothing to declare. D.S. is employed by Sanofi-Aventis.

Abstract

Impaired regulation of hepatic glucose production is a characteristic feature of the metabolic syndrome, a cluster of diseases that includes obesity, insulin resistance, type 2 diabetes and cardiovascular disease. It has been proposed that sustained endoplasmic reticulum stress, which appears to occur in obesity and diabetes, modulates insulin action in the liver. In this study, we show that experimental induction of endoplasmic reticulum stress increases expression and activity of glucose-6-phosphatase, and the capacity for glucose release and glucose cycling in primary rat hepatocytes and H4IIE liver cells. Increased expression of the catalytic subunit of glucose-6-phosphatase was largely due to increased transcription. Deletion analysis of the glucose-6-phosphatase promoter identified an endoplasmic reticulum stress responsive region located between -233 and -187 with respect to the transcriptional start site. Experimental induction of endoplasmic reticulum stress increased the activity of c-jun N-terminal kinase. Prevention of endoplasmic reticulum stress-mediated activation of c-jun N-terminal kinase reduced the expression of the catalytic subunit of glucose-6-phosphatase, glucose-6-phosphatase activity, glucose release and glucose cycling. These data demonstrate that sustained endoplasmic reticulum stress in the hepatocyte provokes adaptations, mediated in part via activation of c-jun N-terminal kinase, that act to increase hepatocellular capacity for glucose release and glucose cycling.

Introduction

An essential function of the endoplasmic reticulum (ER) is the synthesis and processing of secretory and membrane proteins (1). Several pathologic stresses disrupt ER homeostasis and lead to the accumulation of unfolded proteins and protein aggregates in the ER lumen, which can be detrimental to cell survival (2-4). Disruption of ER homeostasis, collectively termed “ER stress”, activates the unfolded protein response (UPR), a signaling pathway that links the ER lumen with the cytoplasm and nucleus (2, 4, 5).

The PKR-like endoplasmic reticulum eukaryotic initiation factor-2 α kinase (PERK) couples protein folding in the ER to polypeptide biosynthesis through phosphorylation of the eukaryotic initiation factor-2 α (eIF2 α) on serine 51, which attenuates translation initiation in response to ER stress (3, 6, 7). PERK $-/-$ mice develop diabetes due to a rapid and progressive decline in endocrine and exocrine pancreatic function (7). Conversely, mice with a homozygous mutation of serine 51 on eIF2 α (Ser⁵¹, serine to alanine), died within 18 hr of birth due to hypoglycemia and impaired induction of liver phosphoenolpyruvate carboxykinase (PEPCK), a key gluconeogenic enzyme (8). Thus, PERK-deficient and Ser⁵¹ mutant mice exhibited severe but opposing defects in glucose homeostasis. Programmed cell death in response to ER stress is mediated, in part, through transcriptional activation of C/EBP homologous protein (CHOP; also known as Growth Arrest and DNA Damage-Inducible Gene 153 or GADD153) (9, 10). Targeted disruption of the CHOP gene in Akita mice, a mouse line that spontaneously develops hyperglycemia with reduced β -cell

mass, delayed the onset of diabetes (9, 11). Oxygen-regulated protein 150 (ORP150), a molecular chaperone found in the ER, protects cells from ER stress (12). ORP150 overexpression in the liver of obese diabetic mice significantly improved insulin resistance and glucose tolerance (13). In addition, systemic overexpression of ORP150 delayed, whereas heterozygous disruption of the ORP150 gene facilitated progression into diabetes in Akita mice (14). Thus, it has been proposed that chronic ER stress may contribute to the attrition of β -cell function and to impaired regulation of glucose homeostasis in diabetes (7, 15).

Although significant progress has been made in identifying pathophysiologic stimuli that induce ER stress (2, 16, 17), components of the UPR (18), and putative physiologic roles for ER stress and the UPR (9, 13, 19, 20), very little is known about the direct effects of ER stress in hepatocytes. Understanding the role and function of ER stress and the UPR in hepatocytes is particularly relevant given recent evidence demonstrating the presence of ER stress in the liver from murine models of obesity (19). The present study examined ER stress-mediated regulation of hepatocyte glucose metabolism using primary rat hepatocytes and a rat hepatoma liver cell line, H4IIE. The data demonstrate that sustained ER stress increases expression of the catalytic subunit of glucose-6-phosphatase, and acts to increase both glucose release and glucose:glucose-6-phosphate cycling, in part, via activation of c-jun N-terminal kinase.

Materials and Methods

Primary Cell Culture. Hepatocytes were isolated from male, Wistar rats (Charles River Laboratories, Wilmington, MA) by collagenase perfusion (21). All procedures involving rats were reviewed and approved by the Colorado State University institutional animal care committee. Cells were first incubated in RPMI 1640 (HyClone, Logan, UT) containing 11 mM glucose, 10^{-7} M dexamethasone, 10^{-7} M insulin on matrigel coated plates (for RNA) or on collagen coated plates containing 5% FBS (for protein) for 4h (attachment period). The media was then changed to one containing RPMI, 8 mM glucose, 10^{-7} M dexamethasone, and 10^{-8} M insulin. The following morning experimental treatments were performed using RPMI that contained 8 mM glucose and 10^{-7} M dexamethasone (22). Results shown represent 5-9 independent rat preparations performed in triplicate.

Experimental Agents. Tunicamycin (Tu), an inhibitor of N-linked protein glycosylation (23) and thapsigargin (Th), a tumour-promoting sesquiterpene lactone that discharges calcium from the ER (24) were used to induce ER stress. SP600125 (20 μ M, Calbiochem, San Diego, CA), an anthrapyrazolone (25), was used to inhibit c-jun N-terminal kinase (JNK). SB203580 (20 μ M, Calbiochem) was used to inhibit p38 MAP kinase.

RNA Isolation. Total RNA was extracted using TRIzol reagent using the manufacturer's protocol (Invitrogen, Carlsbad, CA).

PCR and Northern Blot Analysis. A two-step protocol was used for reverse transcription PCR using SuperScript II reverse transcriptase and Taq polymerase (26). Northern blot analysis was performed as described previously (22).

Real-Time PCR. Purified RNA was treated with DNase (RQ1, Promega, Madison, WI) and reverse transcription was performed using 0.5 µg of DNase-treated RNA using Superscript II RnaseH and random hexamers. PCR reactions were performed in 96 well plates using transcribed cDNA and IQ-SYBR green master mix (Bio Rad, Hercules, CA) using the following primer sets designed by the Beacon designer program version 3.1:

Glucose-6-phosphate catalytic subunit (G6Pase; GenBank NM_013098):

5'- GTGGGTCCTGGACACTGACT

3' – AATGCCTGACAAGACTCCA

Glucose-6-phosphate translocase (G6PT; GenBank NM_031589):

5' – GCCTACGCCATCAGCAAGTT

3' – AGCTCCACGAGAAGACTACGT

Glucose regulated protein 78 (GRP78; GenBank S63521):

5' – AACCCAGATGAGGCTGTAGCA

3' –ACATCAAGCAGAACCAGGTCAC

β2-microglobulin (used as a control; GenBank NM_012512):

5' – GGTGACCGTGATCTTTCTGGTG

3' – GGATGGCGAGAGTACACTTGAATT

PCR efficiency was between 90-105% for all primer and probe sets and linear over 5 orders of magnitude. The specificity of products generated for each set of primers was examined for each fragment using a melting curve analysis and gel electrophoresis. Reactions were run in triplicate and data calculated as the delta

cycle threshold (CT) for the target gene relative to the delta CT for β 2-microglobulin according to the procedures described by Muller et al (27).

G6Pase mRNA decay. Primary rat hepatocytes were provided Tu (2 μ g/ml) or Th (150 nM) for 4 h and transcription was halted by the administration of actinomycin D (10 μ g/ml). Incubations were continued either in the absence or presence of Tu or Th. RNA was isolated and G6Pase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) mRNA were analyzed by Northern blot analysis.

Nuclear run-on assay. The procedure used was as described by Massillon with the exception that assays were performed on freshly isolated nuclei (28).

G6Pase promoter reporter constructs. In order to investigate regulation of the G6Pase promoter we used the pGL3-3.9 plasmid (29, 30). This construct contains the 5'-flanking region of the human G6Pase gene spanning nucleotides -3919 to +61 relative to the transcription start site (29, 30). This -3919/+61 fragment was sub-cloned into the SacI/XhoI sites of the promoter reporter plasmid pGL3 upstream of the firefly luciferase gene (30). A series of truncated G6Pase promoter constructs with progressive 5'-end deletions and a conserved 3' end was generated by either restriction enzyme or PCR using the -3919/+61 as template. The sequence integrity of all constructs was verified by DNA sequencing (Macromolecular Resource Facility, Colorado State University).

Transient transfection assays. H4IIE cells (rat liver hepatoma cell line, American Type Culture Collection) were cultured in Dulbecco's modified Eagle's Medium (DMEM) and 10% fetal bovine serum (FBS). Cells were transfected at 75%

confluence using Lipofectamine Plus (Invitrogen), 1.2 µg/well of reporter-gene construct (firefly luciferase), and 0.02 µg/well of thymidine kinase (TK) control (renilla luciferase) in serum free media. Treatments were performed 18h post-transfection, in DMEM plus 10% FBS for time periods ranging from 1-8 h. Following treatment periods, cells were harvested by direct lysis in situ and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) according to the manufacturer's protocol.

Western Blot Analysis. Cells were harvested following three washings with phosphate-buffered saline in a lysis buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM sodium vanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 mM β-glycerophosphate, 3 mM benzamide, 10 µM leupeptin, 5 µM pepstatin, and 10 µg/ml aprotinin. Equivalent amounts of protein (50-100 µg) were subjected to SDS-PAGE, and subsequently transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and membranes incubated with antibodies against the p36 catalytic subunit of G6Pase (a generous gift from G. Mithieux), the p46 glucose-6-phosphate translocase (a generous gift from G. van de Werve), total and phosphorylated (Ser⁵¹) eIF2α (Cell Signaling), and Pan-actin (Cell Signaling), the latter used as a loading control. Total protein was determined according to the methods Lowry et al (31). Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and an enhanced

chemiluminescence reagent (Pierce, Rockford, IL). Density was quantified using a UVP Bioimaging system (Upland, CA).

In vitro glucose release, glucose phosphorylation and glucose cycling. Following treatments, cells were washed and incubated in RPMI containing no glucose, 5 mM lactate, and 0.5 mM pyruvate for 2 h. Media was sampled at 30 min intervals and glucose concentrations were measured enzymatically using reagents from Sigma Chemical Co. (St. Louis, MO). Glucose phosphorylation and cycling were estimated using 2-³H-glucose and 6-³H-glucose in the presence of 30 mM glucose as previously described (32). Glycogen was isolated according to the procedures of Chan and Exton (33). Ion-exchange chromatography was used to isolate labeled metabolites (32, 34).

Enzyme activity assays. PEPCCK and G6Pase activities were determined on whole cell extracts (35-38). JNK activity was determined using the N-terminal c-jun fusion protein bound to glutathione sepharose beads (Cell Signaling). P38 MAPK activity was determined using an immobilized phospho-p38 MAPK monoclonal antibody and ATF2 as substrate (Cell Signaling).

Calculations. Glucose phosphorylation was calculated from the sum of accumulated ³H₂O and tritiated glycogen from 2-³H-glucose. Estimation of tritiated glycogen served to decrease the error resulting from incomplete equilibration between glucose-6-phosphate and fructose-6-phosphate (39). Glycolysis was calculated as the sum of tritiated pyruvate, lactate, amino acids and tritiated water from 6-³H-glucose. Glucose cycling was calculated as the difference between glucose phosphorylation and total glucose metabolism

(glycolysis + $6\text{-}^3\text{H}$ -glucose incorporation into glycogen) (40). Thus, glucose cycling represents phosphorylated glucose not further metabolized via glycolysis or glycogen synthesis (40).

Data analysis and statistics. Statistical comparisons were calculated using a paired Student's t test, an unpaired Student's t test or analysis of variance. The level of significance was $p < 0.05$.

Results and Discussion

Induction of the UPR by tunicamycin and thapsigargin in primary rat hepatocytes.

ER stress and activation of the UPR involves a complex set of responses that include translational attenuation, via phosphorylation of Ser⁵¹ on eIF2 α , and upregulation of genes encoding ER chaperone proteins, such as GRP78 (3, 10). We first examined the time course of changes in eIF2 α phosphorylation and GRP78 mRNA in response to the UPR activating agents Tu and Th in primary rat hepatocytes (n=5). Phosphorylation of eIF2 α (Fig. 1A) was observed after 20 min of Tu treatment (2 $\mu\text{g/ml}$), whereas GRP78 mRNA (Fig. 1B, RT-PCR) was increased by 3 h. Th treatment (150 nM) increased phosphorylation of eIF2 α (Fig. 1A) after 20 min and GRP78 mRNA (Fig. 1B, RT-PCR) after 3h. These data demonstrate that agents used to induce ER stress and activate the UPR in other cell types (2, 41, 42), also activate specific components of the UPR in primary rat hepatocytes.

Tunicamycin and thapsigargin increase G6Pase gene expression. We next examined whether experimental induction of ER stress regulated the expression of genes involved in glucose production in primary rat hepatocytes.

Tu (2 $\mu\text{g/ml}$) and Th (150 nM) treatment increased G6Pase mRNA (Northern blot) after 4 h (Fig. 2A, n=9). In contrast, these treatments had no effect on glucose-6-phosphate translocase (G6PT) or phosphoenolpyruvate carboxykinase (PEPCK) mRNA (data not shown). These data demonstrate that Tu, an agent that induces ER stress by inhibition of protein glycosylation (23), and Th, an agent that induces ER stress through inhibition of the ER calcium-ATPase (24), both act to increase the expression of the catalytic subunit of G6Pase but had no effect on G6PT, PEPCK or GAPDH mRNA.

Insulin prevents induction of G6Pase by tunicamycin and thapsigargin. Insulin inhibits cAMP- and glucocorticoid-stimulated PEPCK gene expression and both basal G6Pase expression and the stimulatory effect of glucocorticoids on G6Pase (43, 44). We next examined the effects of insulin on Tu- and Th-mediated upregulation of G6Pase gene expression. The presence of 10 nM insulin prevented the induction of G6Pase mRNA by Tu (2 $\mu\text{g/ml}$) and Th (150 nM) (Fig. 2A, n=9). These data demonstrate that the suppressive effects of 10 nM insulin on G6Pase gene expression are dominant over the inductive effects of Tu (2 $\mu\text{g/ml}$) and Th (150 nM).

Dose-response relationship between ER stress and G6Pase. We next examined the effects of Tu and Th concentration on G6Pase gene expression in primary rat hepatocytes using Real Time PCR. There was a dose-dependent relationship between Tu and G6Pase mRNA levels (Fig. 2B, n=5) and between Th and G6Pase mRNA levels (Fig. 2B, n=5). Tu and Th also increased GRP78 mRNA

levels (marker of ER stress) at all concentrations studied (Fig. 2B). In contrast, Tu and Th had no effect on G6PT mRNA (Fig. 2B).

Tunicamycin and thapsigargin increase G6Pase p36 catalytic subunit protein expression, G6Pase activity, glucose release, and glucose cycling. We next examined whether Tu and Th treatment lead to changes in G6Pase protein expression and glucose metabolism in primary rat hepatocytes (n=5).

Hepatocytes were incubated in control media, or control media containing 2 µg/ml of Tu or 150 nM Th for 4 or 8 h. Tu and Th treatment increased G6Pase catalytic subunit protein (p36), G6Pase activity, glucose concentration in the media and total glucose release (Fig. 3A). In addition, Tu and Th treatment increased glucose cycling (Table 1). In contrast, the p46 G6PT protein (Fig. 3A) and PEPC activity (Fig. 3A and 3B) were not increased by either of these treatments. These data demonstrate that Tu- and Th-mediated upregulation of G6Pase leads to an increase in both the functional capacity of the hepatocyte to release glucose and glucose:glucose-6-phosphate cycling.

Tunicamycin and thapsigargin increase G6Pase transcription. We next examined whether Tu- and Th-mediated induction of G6Pase gene expression involved changes in transcription (n=6) and/or mRNA stability (n=4) in primary rat hepatocytes. Tu (2 µg/ml) and Th (150 nM) treatment increased G6Pase transcription by 2-4-fold (Fig. 4A). To examine mRNA decay, the transcription inhibitor actinomycin D (10 µg/ml) was added to hepatocytes after 4h of incubation in the presence of Tu (2 µg/ml) or Th (150 nM) and the incubation was continued in the presence or absence of these agents. The decrease in G6Pase

mRNA (Northern blot) over the course of 4h was not different in the presence or absence of Tu or Th (Fig. 4B). These data demonstrate that the observed upregulation of G6Pase gene expression by Tu and Th occurs primarily through activation of gene transcription in primary rat hepatocytes.

Regulation of the G6Pase gene promoter in H4IIE cells. To further elucidate the mechanism of ER stress-mediated upregulation of G6Pase, G6Pase-luciferase fusion genes were transiently expressed in H4IIE liver cells. To determine whether this cell line was appropriate, we first examined the effects of Tu and Th on G6Pase and GRP78 mRNA (n=4). Tu (2 µg/ml) and Th (150 nM) increased G6Pase mRNA (Real Time PCR) 2.5±0.3- and 2.1±0.2-fold, respectively, after 2h and 4.6±0.4- and 5.3±0.4-fold, respectively, after 4h. Tu and Th increased GRP78 mRNA 8.6±0.6-fold and 7.4±0.5-fold, respectively, after 4h. Tu and Th did not increase G6PT mRNA. These data demonstrate that H4IIE liver cells respond to Tu and Th in a manner similar to that observed in primary rat hepatocytes.

In contrast to our previous mRNA and transcription data, we were unable to detect any significant induction of G6Pase promoter activity in response to Tu treatment (Fig. 5A, 4h at 2 µg/ml, n=4). Treatment of H4IIE cells with a higher concentration of Tu (10 µg/ml, n=3) provided for either 1, 2, 4, or 8h had no discernible effect upon G6Pase promoter activity (data not shown). We then investigated the effect of Th treatment upon G6Pase promoter activity using the G6Pase promoter fragments -3919/+61, -1226/+61, -665/+61, -496/+61, and -233/+61 in independent transient transfections and luciferase assays (Fig. 5A).

Treatment of H4IIE cells with 150 nM Th for 4 h (n=7) increased relative luciferase activity between 2.9-3.7 fold (Fig. 5A). The scale of this induction was essentially identical for all of the different constructs used in this experiment.

When these experiments were repeated using G6Pase promoter reporter constructs containing fragments -141/+61 and -44/+61, we were unable to detect any induction of G6Pase promoter activity by Th (Fig. 5A). These results indicate that cis-acting sequence elements located between -233 and -141 are indispensable for Th-mediated activation of the G6Pase promoter.

Additional constructs were created to further characterize the region responsible for Th-mediated activation of the G6Pase promoter. Th-mediated activation of the -233/+61 promoter fragment was reduced by ~60% in the -217/+61 promoter fragment and 100% in the -187/+61 promoter fragment (Fig. 5B, n=3). These data demonstrate that the region located between -233 and -187 contains regulatory elements that are required for Th-mediated regulation of the G6Pase promoter.

Insulin repression is dominant over Th-mediated activation of the G6Pase promoter. In experiments described above, insulin suppressed Tu- and Th-mediated induction of G6Pase gene expression in primary rat hepatocytes. In order to characterize this regulatory interaction further in H4IIE liver cells, we examined whether insulin treatment diminished the effects of Th on the G6Pase promoter. We found that the scale of Th-mediated induction of G6Pase promoter activity from both the -1226/+61 and -233/+61 fragments was reduced by insulin treatment (Fig. 5C). These data demonstrate that, similar to the effects of insulin

on G6Pase gene expression in primary rat hepatocytes, the suppressive effects of 10 nM insulin are dominant over Th-mediated (150 nM) activation of the G6Pase promoter activity in H4IIE liver cells.

An inhibitor of JNK reduces thapsigargin-mediated activation of the G6Pase promoter and G6Pase gene expression. IRE1 (inositol requiring, ER-to-nucleus signaling protein) is an ER membrane-anchored endonuclease that directs the splicing of XBP1 mRNA and activation of transcriptional programs in response to ER stress (18). IRE1 also interacts with stress signaling proteins, such as JNK (45). In order to elucidate the possible mechanisms by which ER stress might upregulate G6Pase gene and protein expression, we next examined whether Th-induced activation of the G6Pase promoter involved signaling via JNK. H4IIE cells (n=3) were incubated with a control media, or a control media containing either Th (150 nM) or Tu (2 µg/ml) in the absence or presence of SP600125 (20 µM), an inhibitor of JNK, or SB203580 (20 µM), an inhibitor of p38 MAP kinase. Both Th and Tu increased JNK activity, whereas p38 activity was not increased by either agent (Fig. 6A). The presence of the JNK inhibitor prevented Tu- and Th-mediated activation of JNK (Fig. 6A). Transient transfection studies in H4IIE cells revealed that treatment with the SP600125 JNK inhibitor reduced Th-mediated activation of the G6Pase promoter fragments -1226/+61 and -233/+61 by ~50% (Fig. 6B). The presence of SB203580, a p38 MAP kinase inhibitor, had no effect on Th-mediated activation of the G6Pase promoter fragments -1226/+61 and -233/+61 (data not shown).

We next examined the effects of the JNK inhibitor (SP600125) on gene expression, protein activity, and glucose metabolism in response to Tu and Th in primary rat hepatocytes. Similar to H4IIE cells, Tu (2 μ g/ml) and Th (150 nM) increased (2.6-fold by Tu; 2.1-fold by Th) JNK activity, but not p38 MAP kinase activity (data not shown), in primary rat hepatocytes. The presence of the JNK inhibitor reduced Tu- and Th-mediated induction of G6Pase mRNA, but had no effect on PEPCK or G6PT mRNA (Fig. 7A, n=4). In addition, the presence of the JNK inhibitor reduced G6Pase activity, glucose release, and glucose cycling in primary rat hepatocytes (Fig. 7B, n=4). These data demonstrate that activation of JNK contributes to Th-induced activation of the G6Pase promoter in H4IIE cells and Tu- and Th-mediated upregulation of G6Pase gene expression, G6Pase activity, glucose release and glucose cycling in primary rat hepatocytes.

UPR signaling is essential for the maintenance of glucose homeostasis and ER stress appears to play an important role in the development of and/or complications associated with obesity and diabetes (7, 8, 13, 14, 19, 45, 46). However, very little is known regarding the role and downstream effects of ER stress and UPR signaling within the hepatocyte. Results from the present study demonstrate that experimental induction of ER stress using tunicamycin or thapsigargin acts to selectively increase expression of the catalytic subunit of glucose-6-phosphatase, glucose-6-phosphatase activity and the capacity for glucose release and glucose cycling in primary rat hepatocytes. ER stress-mediated induction of G6Pase gene expression occurred via transcriptional mechanisms that involved cis-elements located between -233 and -187. Finally,

experimental induction of ER stress activated JNK activity and inhibition of this activation reduced the effects of ER stress on G6Pase gene expression, G6Pase activity, glucose release and glucose cycling.

In the present study, tunicamycin and thapsigargin increased the expression of GRP78, an ER chaperone whose expression is increased in response to ER stress, and phosphorylation of eIF2 α on Ser⁵¹, a translation initiation factor that is phosphorylated by PERK in response to ER stress (2). Therefore, these agents appear to activate both the transcriptional and translational components of the UPR in primary rat hepatocytes and H4IIE liver cells. Experimental induction of ER stress also increased the capacity for glucose release and glucose-glucose-6-phosphate cycling. Overproduction of glucose is a classic metabolic sequela of diabetes, and glucose cycling between glucose and glucose-6-phosphate has been shown to be increased in some forms of diabetes, although the magnitude and physiologic significance of glucose cycling in diabetes is presently unclear (40, 47, 48). Thus, these data suggest that sustained ER stress within the hepatocyte may be linked to diabetes both via effects on insulin signaling (13, 19) and direct effects on hepatocyte glucose metabolism.

Tunicamycin and thapsigargin increased the expression of the catalytic subunit of G6Pase and increased G6Pase activity in primary rat hepatocytes. The G6Pase protein complex is located in the ER and catalyzes the hydrolysis of glucose-6-phosphate derived from glycogenolysis and gluconeogenesis in the liver (49, 50). The enzyme appears to exist as a multi-subunit complex that

minimally includes the catalytic subunit oriented to the ER lumen and a glucose-6-phosphate translocase (50). Notably, experimental induction of ER stress had no effect on the expression of the glucose-6-phosphate translocase or on the expression and activity of PEPCK. Thus, under these experimental conditions, the effects of sustained ER stress appear to be relatively specific to the catalytic subunit of G6Pase, although the present study has not provided a comprehensive analysis of all genes involved in glucose production.

Unfolded proteins in the ER induce cellular stress and activate JNK. Activation of JNK appears to be coupled to ER stress via interactions with IRE1 (45). Mouse models of obesity are associated with ER stress, activation of the UPR and JNK, and reduced insulin signaling in the liver (19). In the present study, experimental induction of ER stress activated JNK in both primary rat hepatocytes and H4IIE liver cells. Inhibition of this activation reduced Tu- and Th-mediated increases in G6Pase expression, G6Pase activity, glucose release and glucose cycling in primary hepatocytes. In addition, inhibition of Th-mediated activation of JNK in H4IIE cells reduced the activation of the G6Pase promoter in response to thapsigargin. These data suggest that sustained ER stress can provoke increased hepatocyte glucose release and cycling, in part, via JNK signaling. It could be postulated that activation of JNK may influence the expression of G6Pase and glucose metabolism via effects on insulin signaling, since activation of JNK can impair insulin action (51). However, induction of G6Pase and glucose release by Tu and Th occurred in the absence of insulin, the presence of insulin (10 nM) effectively suppressed this induction, and any

JNK-mediated effects on insulin signaling would be expected to also affect PEPCK gene expression, which did not occur in the present study (43). Therefore, it is hypothesized that JNK activation is linked to G6Pase gene expression and alterations in hepatocyte glucose metabolism via phosphorylation-mediated activation of a transcription factor(s).

Although Tu and Th increased G6Pase transcription in primary rat hepatocytes, only Th increased the activity of G6Pase-luciferase promoter constructs in H4IIE liver cells. Since both Tu and Th increased G6Pase mRNA in H4IIE cells, this result indicates that the induction of G6Pase gene expression by Tu and Th is mediated by different transcription factors and that the sequence elements involved in Tu-mediated induction lie outside of the G6Pase promoter reporter constructs used in this study. Deletion analysis of the G6Pase promoter revealed a thapsigargin-responsive region between -233 and -187. Whether this region is regulated by calcium specifically, can confer a stimulatory effect of thapsigargin on a heterologous promoter and identification of the transcription factors binding to this region in response to thapsigargin is under investigation.

In total, our data support the notion that sustained ER stress may represent an integrating mechanism underlying dysregulation of glucose homeostasis in diabetes (13, 15, 18, 19, 52). Greater understanding of the connection between ER stress, UPR signaling, and glucose homeostasis may be instrumental in the design of novel therapeutic strategies in diabetes and other metabolic diseases associated with ER stress and impaired regulation of hepatic glucose production.

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Figure Legends

Figure 1. *A*, The effects of tunicamycin (2 $\mu\text{g/ml}$) and thapsigargin (150 nM) on phosphorylation of eIF2 α (phospho-eIF2 α) as a function of time in primary rat hepatocytes. Insert is a representative western blot showing phosphorylated (p-eIF2 α) and total (eIF2 α) eIF2-alpha. *B*, The effects of tunicamycin (Tu, 2 $\mu\text{g/ml}$) and thapsigargin (Th, 150 nM) on GRP78 mRNA as a function of time in primary rat hepatocytes. Insert represents RT-PCR analysis of GRP78 mRNA and 18S rRNA (loading control) from a representative experiment. Data are normalized to 0 min which was set to 1. Graphs are means \pm SEM for n=5. *, significantly different from 0 time point ($p<0.05$).

Figure 2. *A*, The effects of tunicamycin (Tu, 2 $\mu\text{g/ml}$) and thapsigargin (Th, 150 nM) on G6Pase mRNA (northern blot) after 2 and 4 hours of incubation in the absence (-) or presence (+) of 10 nM insulin in primary rat hepatocytes. G6Pase mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) mRNA and data in the graph are expressed relative to LG (low glucose control incubation) at 2 hours which was set to 1. The insert is a blot from a representative experiment. Graph is the mean \pm SEM for n=9. *, significantly different from LG ($p<0.05$). *B*, The effects of tunicamycin and thapsigargin concentration on glucose-6-phosphatase (G6Pase), glucose-6-phosphate translocase (G6PT), and glucose-regulated protein 78 (GRP78) mRNA (Real Time PCR) in primary rat hepatocytes. In the graph, 0 concentration has been set to 1. Graph is the mean \pm SEM for n=5. *, significantly different from 0 concentration ($p<0.05$).

Figure 3. *A*, The effects of tunicamycin (Tu, 2 $\mu\text{g/ml}$) and thapsigargin (Th, 150 nM) on the p36 catalytic subunit of G6Pase protein (G6Pase p36), the p46 glucose-6-phosphate translocase protein (G6PT p46), glucose-6-phosphatase (G6Pase) activity, and phosphoenolpyruvate carboxykinase (PEPCK) activity after 4 or 8 h of incubation in primary rat hepatocytes. Enzyme activity data were normalized to total protein and data provided in the graph are expressed relative to untreated (LG) cells at 4 hours, which were set to 1. The insert is a western blot from a representative experiment and includes Pan-actin (actin) which was used as a loading control. *B*, the effects of tunicamycin (Tu, 2 $\mu\text{g/ml}$) and thapsigargin (Th, 150 nM) on glucose concentration in the media (left panel) and total glucose release (right panel) in primary rat hepatocytes. Media at time 0 was devoid of glucose and contained 0.5 mM pyruvate and 5 mM lactate as gluconeogenic substrates. Glucose concentration in the media was measured every 30 min over a 2 h period, following 4 or 8 h of incubation in the absence (LG) or presence of tunicamycin or thapsigargin. Glucose release, calculated as the rate of change in glucose concentration, was normalized to total protein and expressed relative to untreated (LG) cells at 4 hours, which were set to 1. Graphs are means \pm SEM for n=5. *, significantly different from LG (p<0.05).

Figure 4. *A*, The effects of tunicamycin (Tu, 2 $\mu\text{g/ml}$) and thapsigargin (Th, 150 nM) on G6Pase transcription following 0-4 h of incubation. Run-on assays were performed on freshly isolated nuclei from primary rat hepatocytes. Data are expressed relative to untreated (LG) cells. Graph is mean \pm SEM for n=6. *B*, The effects of tunicamycin (Tu, 2 $\mu\text{g/ml}$) and thapsigargin (Th, 150 nM) on the decay

of G6Pase mRNA in primary rat hepatocytes. Primary rat hepatocytes were cultured in the presence of Tu or Th. Transcription was then halted with addition of actinomycin D (10 µg/ml) and incubations were continued in the absence (-) or presence (+) of Tu or Th. G6Pase and GAPDH mRNA were measured at each time point by Northern blot analysis. G6Pase mRNA was normalized to GAPDH and data are expressed relative to the 0 time point (immediately prior to addition of actinomycin D), which was set to 100%. Graph is the mean±SEM for n=4. *, significantly different from 0 time point (p<0.05).

Figure 5. *A*, The effects of tunicamycin (Tu, 2 µg/ml) and thapsigargin (Th, 150 nM) on the G6Pase promoter in H4IIE liver cells. The indicated fragments of the G6Pase 5'-flanking region were cloned into the pGL3 basic vector. H4IIE cells were co-transfected with these G6Pase reporter constructs and thymidine kinase renilla luciferase control. Graph is mean±SEM for n=5-7. *B*, The effects of tunicamycin (Tu, 2 µg/ml) and thapsigargin (Th, 150 nM) on the G6Pase promoter in H4IIE liver cells. The indicated fragments of the G6Pase 5'-flanking region were cloned into the pGL3 basic vector. H4IIE cells were co-transfected with these G6Pase reporter constructs and thymidine kinase renilla luciferase control. Graph is mean±SEM for n=3. *C*, The effects of thapsigargin (Th, 150 nM) on the G6Pase promoter in the absence (- insulin) and presence (+ insulin) of 10 nM insulin in H4IIE liver cells. The indicated fragments of the G6Pase 5'-flanking region were cloned into the pGL3 basic vector. H4IIE cells were co-transfected with these G6Pase reporter constructs and thymidine kinase renilla luciferase

control. Graph is mean±SEM for n=3. All data are expressed relative to untreated fragments which were set to 1. *, significantly different from untreated fragment.

Figure 6. *A*, The effects of tunicamycin (Tu, 2 µg/ml) and thapsigargin (Th, 150 nM) on c-jun terminal kinase (JNK) and p38 MAPK (p38) activity in the absence (-) or presence (+) of the JNK inhibitor, SP600125, in H4IIE liver cells. Data are expressed relative to untreated (LG) controls in the absence of SP600125. Graph is mean±SEM for n=5. *B*, The effects of thapsigargin (150 nM) on the -1226/+61 and -233/+61 G6Pase promoter fragments in the absence (-) or presence (+) of the JNK inhibitor, SP600125, in H4IIE liver cells. Data are expressed relative to untreated fragments which were set to 1. Graph is mean±SEM for n=3. *, significantly different from LG- (6A) or –JNK inhibitor (6B) (p<0.05).

Figure 7. *A*, The effects of tunicamycin (Tu, 2 µg/ml) and thapsigargin (Th, 150 nM) on glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphate translocase (G6PT) mRNA (G6Pase and G6PT analyzed by Real Time PCR, PEPCK analyzed by northern blot) in the absence (-) or presence (+) of the c-jun terminal kinase (JNK) inhibitor, SP600125, in primary rat hepatocytes. Data are expressed relative to untreated (LG) controls in the absence of SP600125. Graph is mean±SEM for n=4. *B*, The effects of tunicamycin (Tu, 2 µg/ml) and thapsigargin (Th, 150 nM) on G6Pase activity, PEPCK activity, glucose release, and glucose cycling in the absence (-) or presence (+) of the c-jun terminal kinase (JNK) inhibitor, SP600125, in primary rat hepatocytes. Data are expressed relative to untreated (LG) controls in the absence of SP600125. Graph is mean±SEM for n=4. *, significantly different from

LG- ($p < 0.05$). +, significantly different from corresponding treatment in the presence of the JNK inhibitor ($p < 0.05$).

Table 1. Glucose phosphorylation and cycling in primary rat hepatocytes

	<u>Phosphorylation</u>	<u>Cycling</u>	<u>Phosphorylation</u>	<u>Cycling</u>
	<u>4 hours</u>		<u>8 hours</u>	
	(nmol · mg protein ⁻¹ · hr ⁻¹)			
LG	1.4 ± 0.16	0.2 ± 0.04	1.5 ± 0.12	0.1 ± 0.01
Tu	1.6 ± 0.2	0.9 ± 0.06*	1.7 ± 0.14	0.7 ± 0.05*
Th	1.7 ± 0.25	0.9 ± 0.07*	1.6 ± 0.11	0.8 ± 0.07*

Data are means ± SEM for n=5. LG, low glucose controls; Tu, tunicamycin; Th, thapsigargin. Glucose phosphorylation and glucose:glucose-6-phosphate cycling were determined as described in the methods section. *, significantly different from LG (p<0.05).

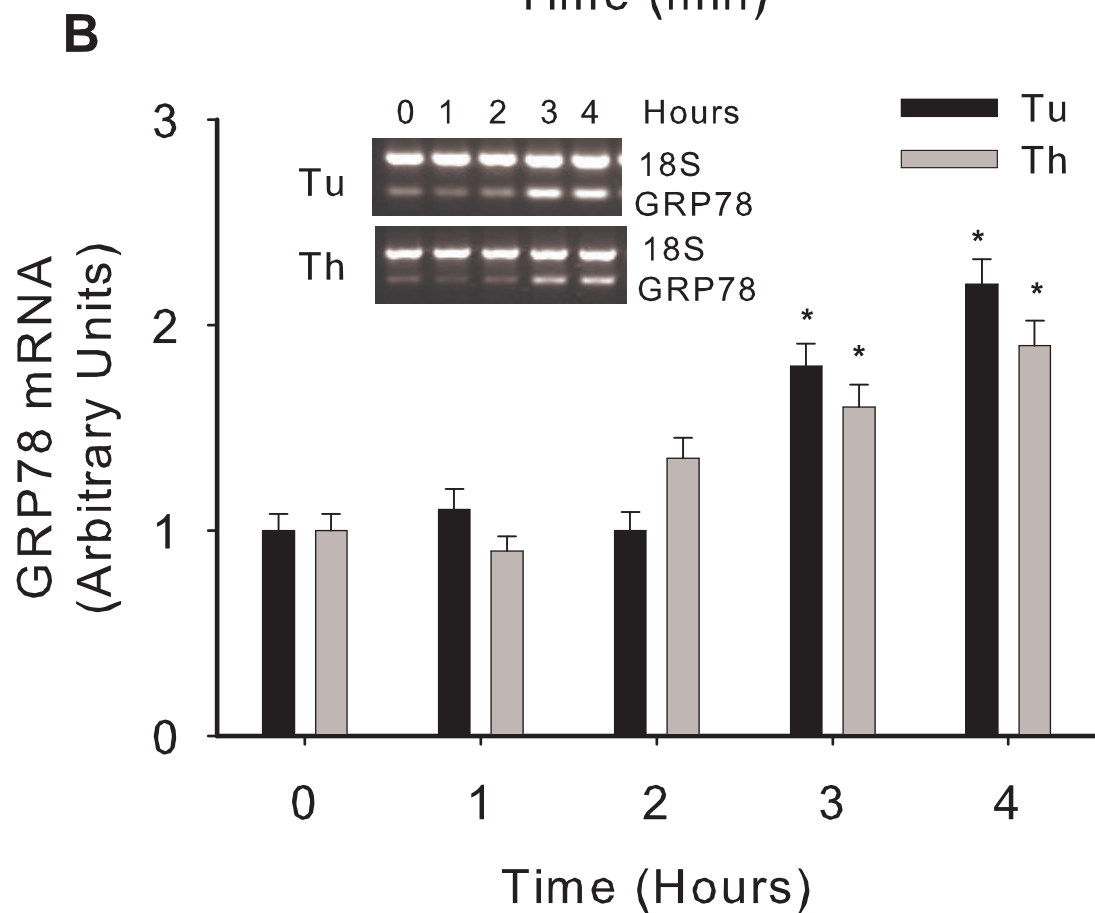
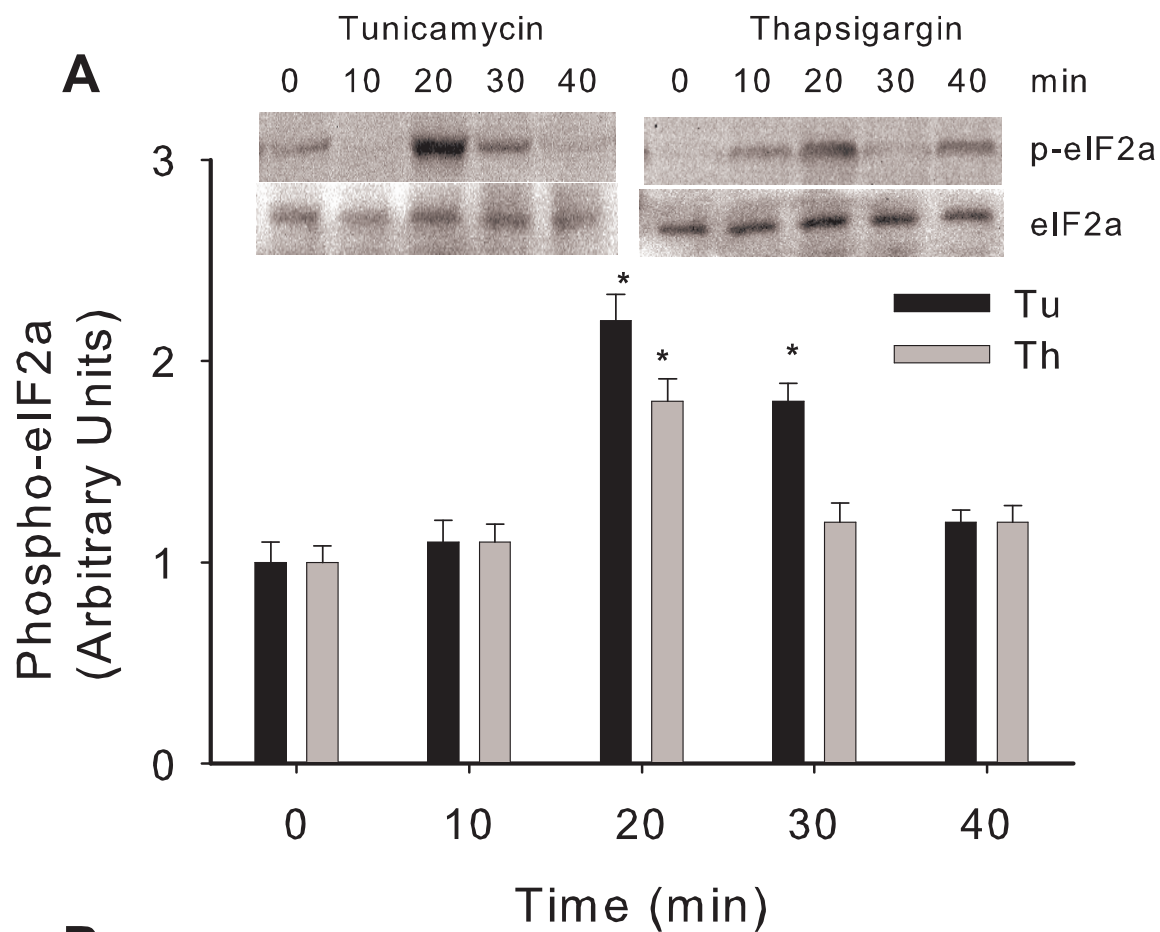


Figure 1

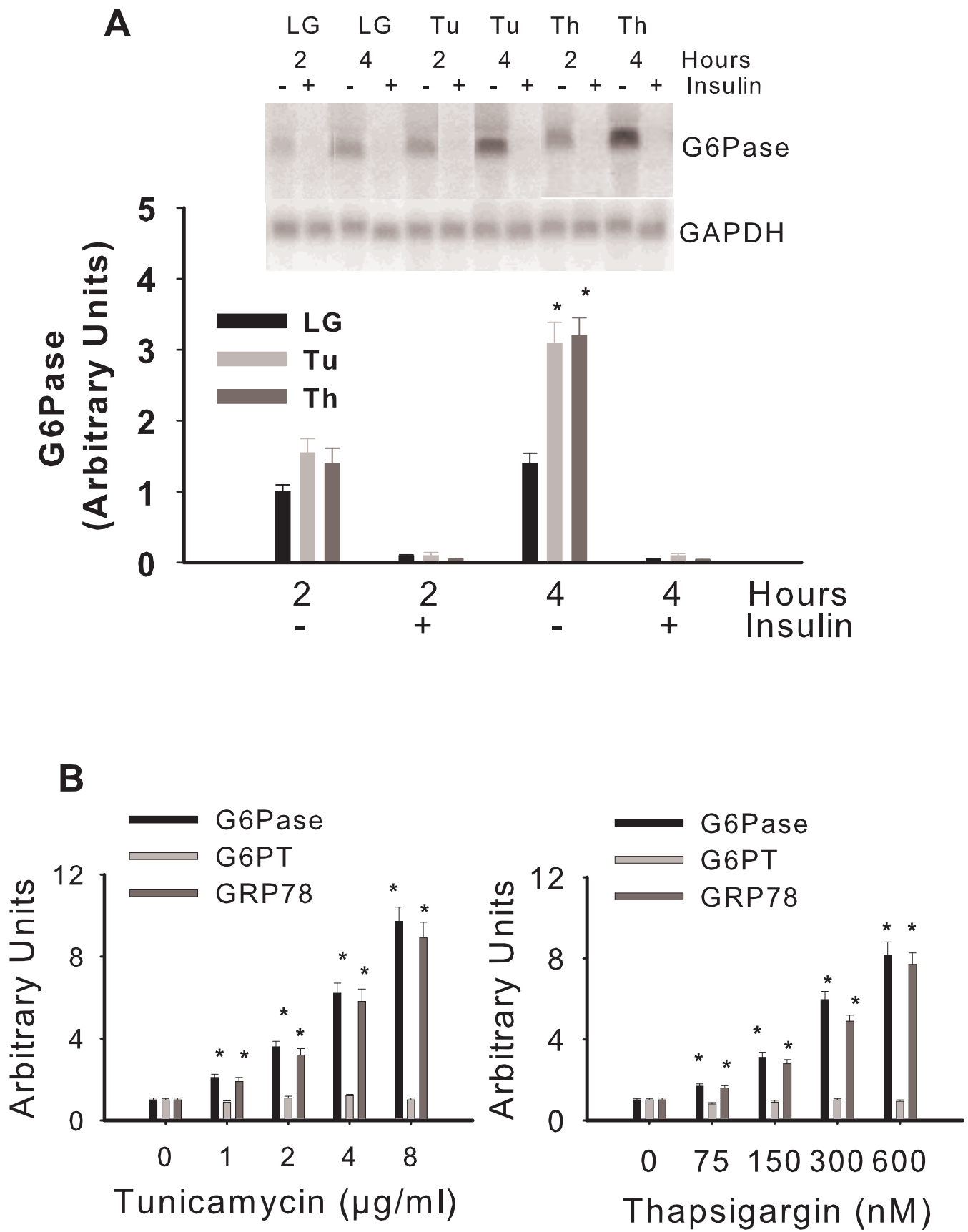


Figure 2

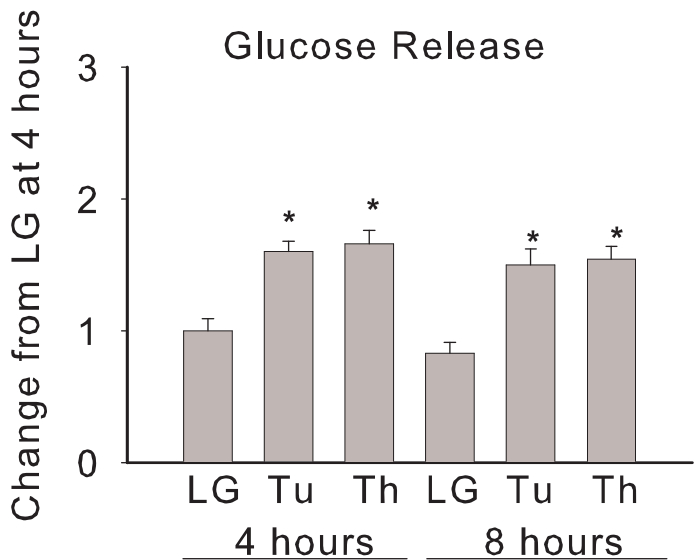
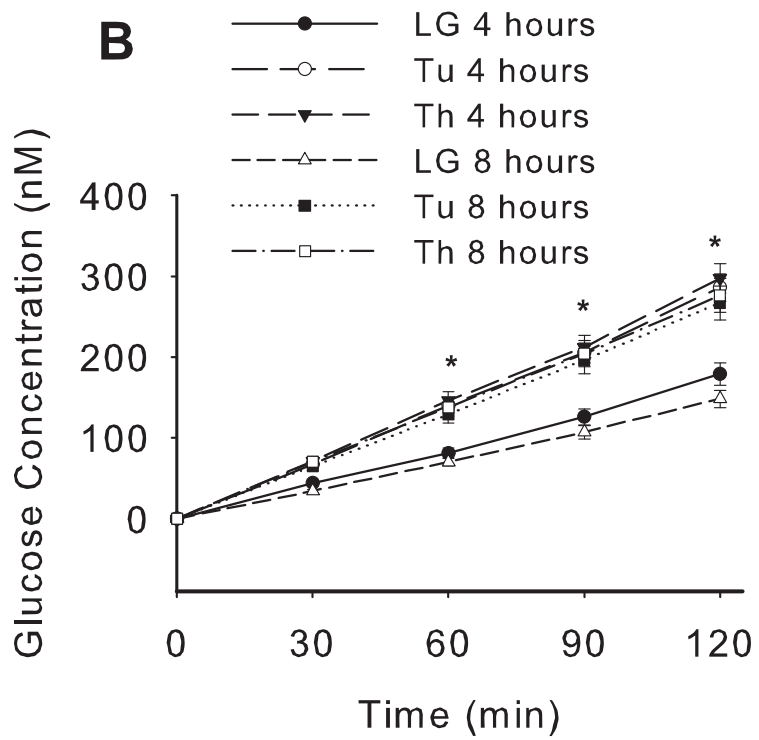
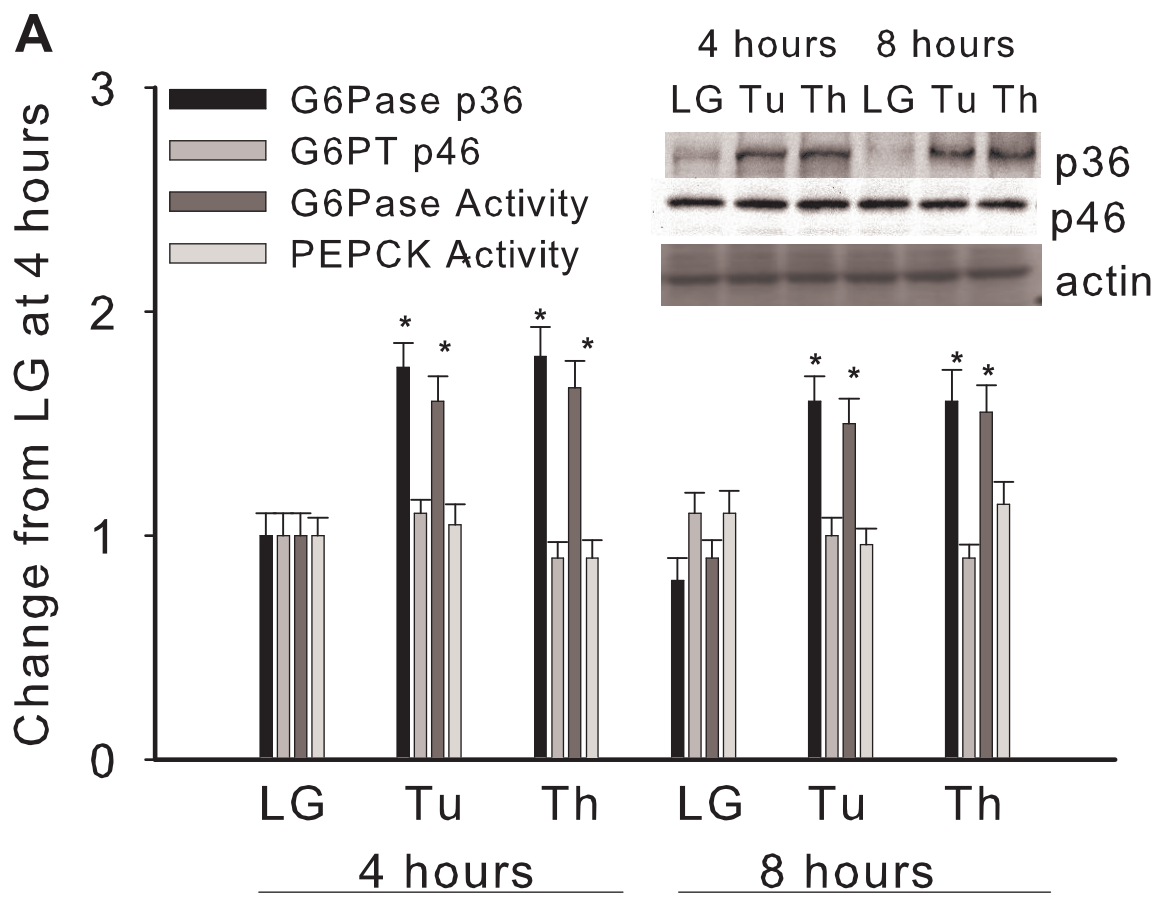


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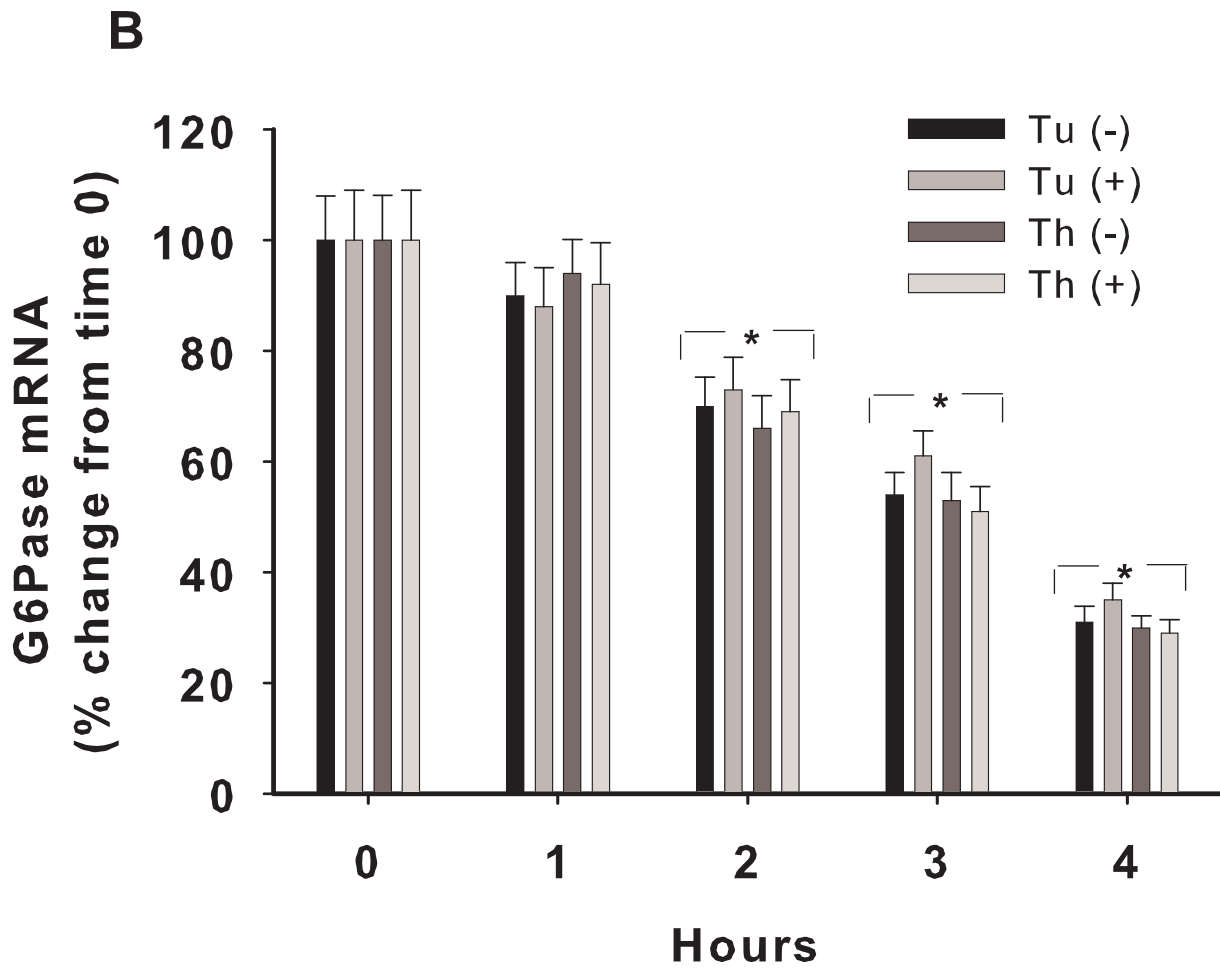
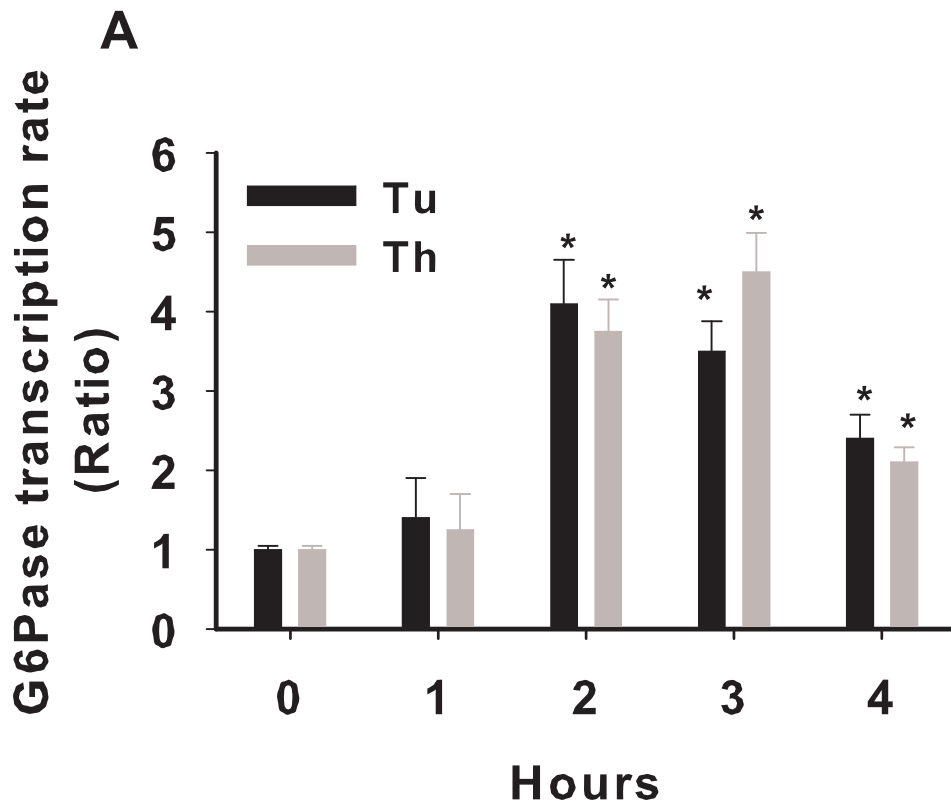


Figure 4

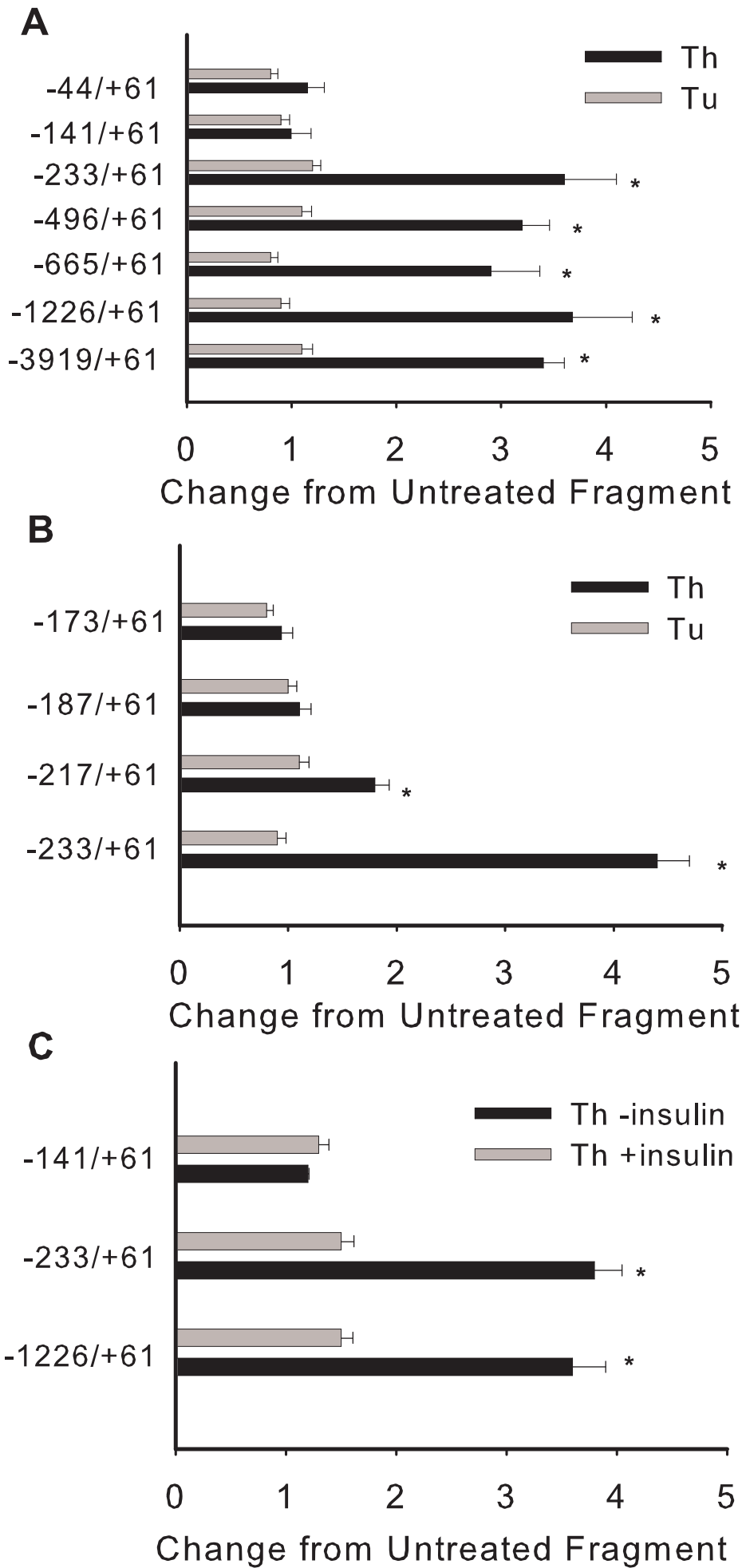


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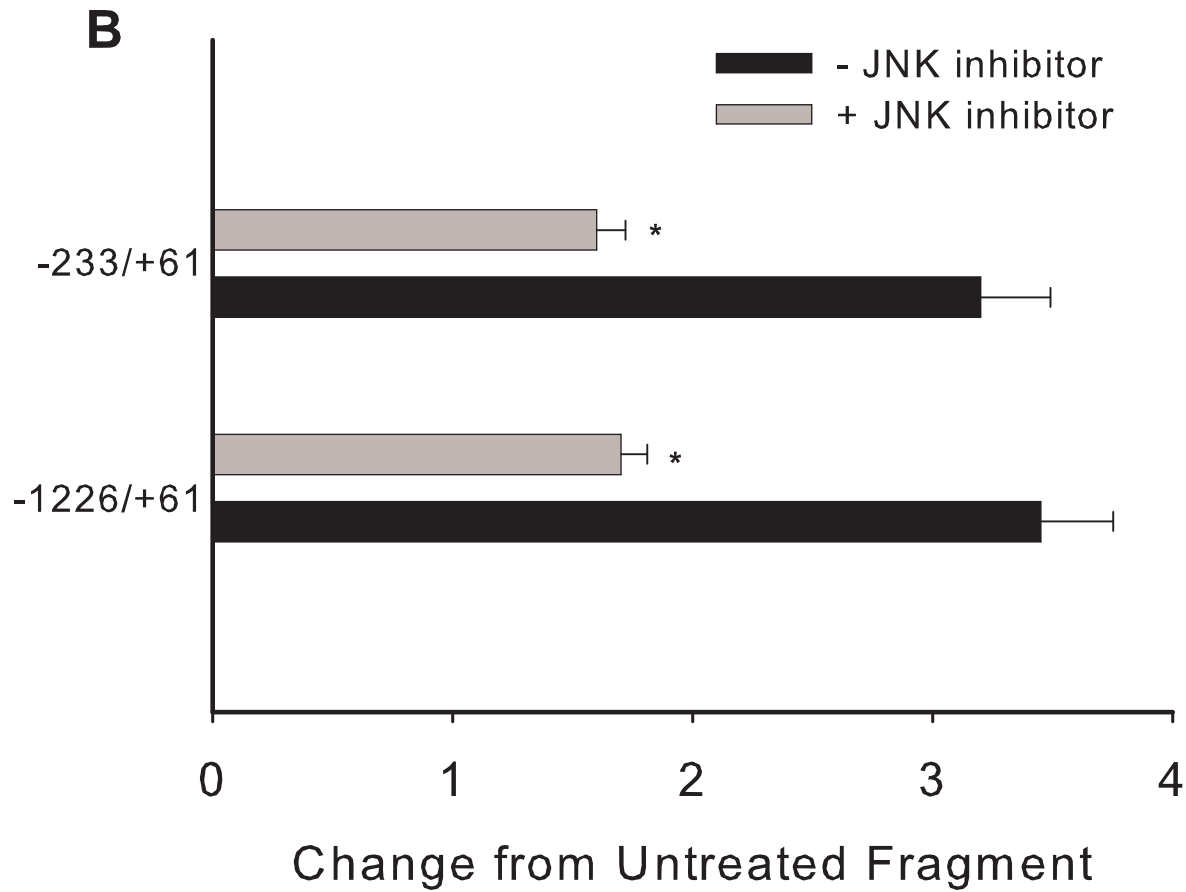
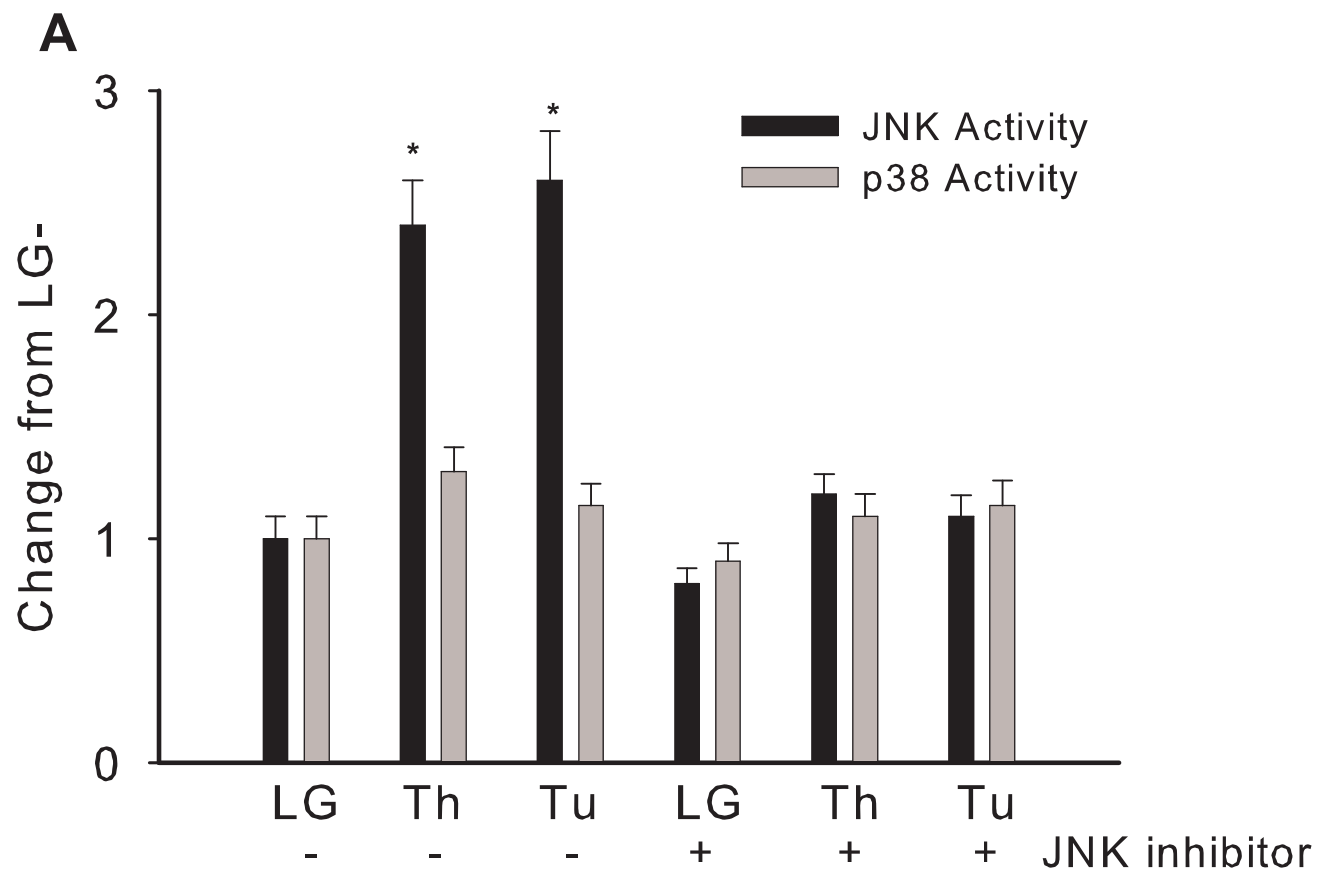


Figure 6

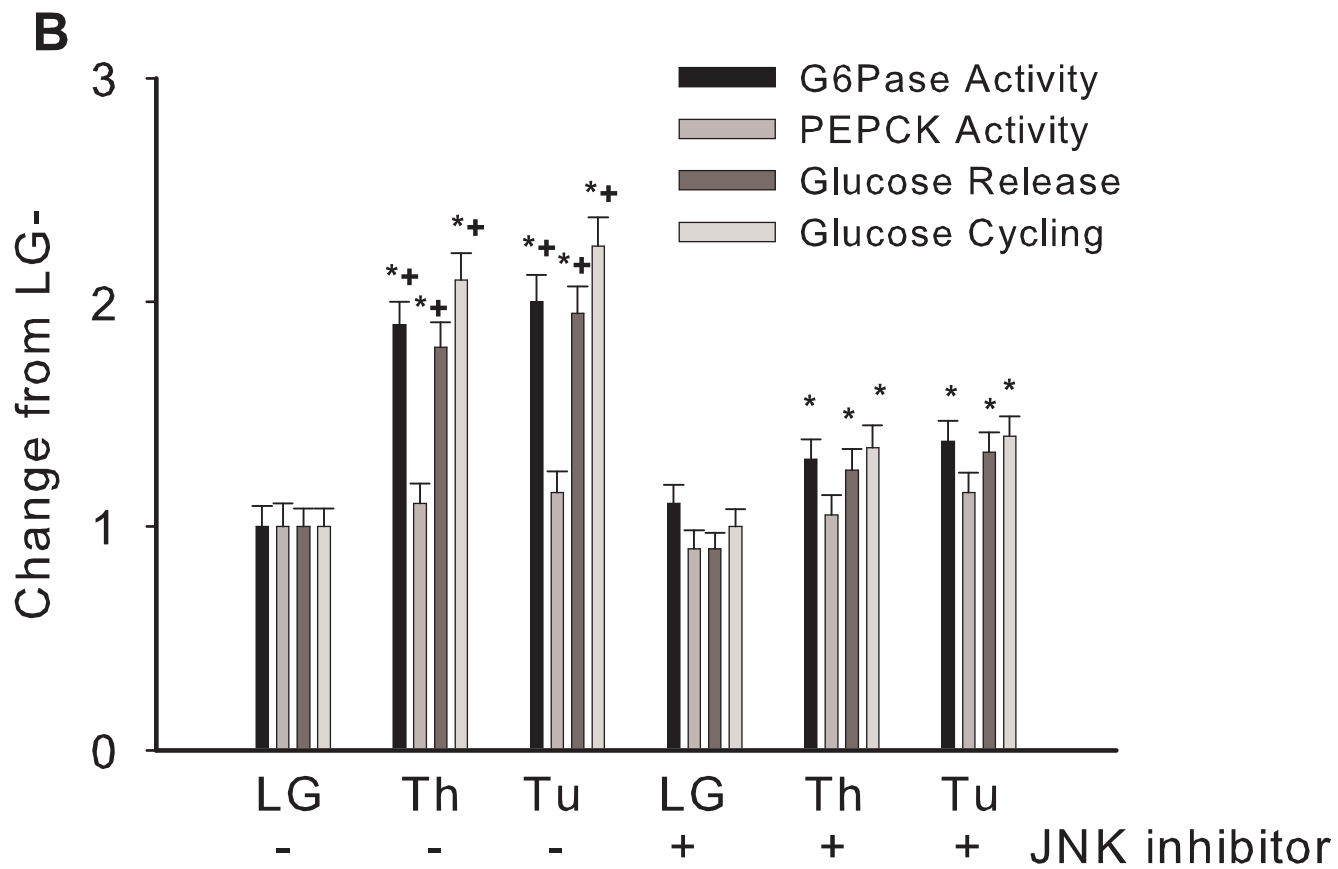
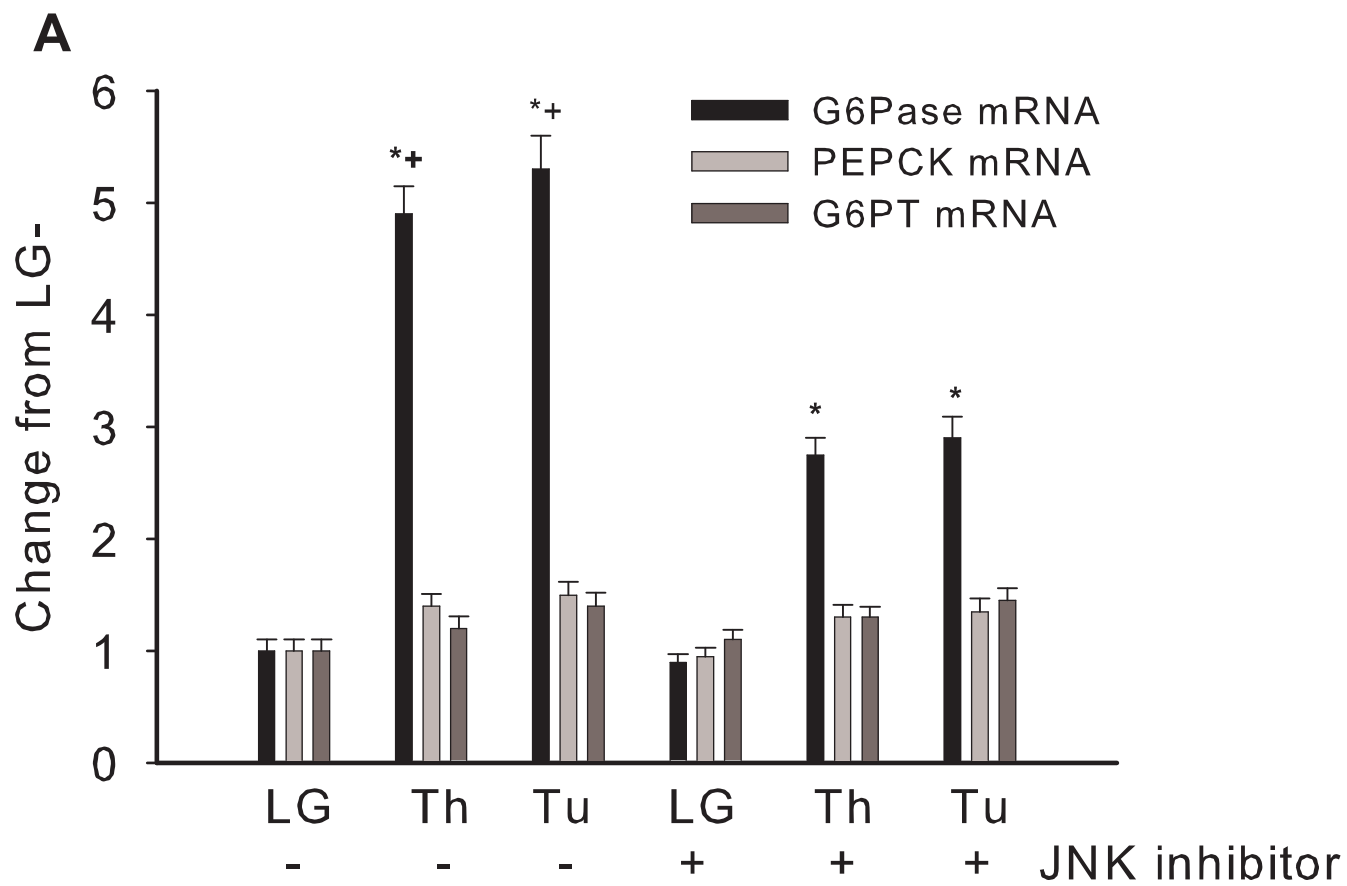


Figure 7