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Insulin Signaling in Regulation of γ-Glutamylcysteine Ligase Catalytic Subunit in Primary Cultured Rat Hepatocytes

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Running Title: PI3K Signaling and γ-Glutamylcysteine Ligase Expression

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Abbreviations: GSH, glutathione; GCL, gamma-glutamylcysteine ligase; GCLC, GCL catalytic subunit; GCLR, GCL regulatory subunit; PI3K, phosphatidylinositol 3-kinase; p70S6K, ribosomal p70 S6 kinase; PKC, protein kinase C; MAPK, mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PD98059, 2′-amino-3′-methoxyflavone; LY294002, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; U0126, 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio) butadiene; SP600125, 1,9-pyrazoloanthrone; AdV-GFP, GFP-expressing adenovirus; AdV-Akt, adenovirus containing a dominant negative kinase-dead mutant of Akt; GST, glutathione S-transferase.

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Abstract

Decreased glutathione (GSH) levels and γ-glutamylcysteine ligase (GCL) activity have been observed in diabetic patients, and insulin reportedly increases GSH synthesis via increased GCL catalytic subunit (GCLC) gene expression. The signaling pathways responsible for mediating insulin effects on GCLC expression and GSH levels, however, are unknown. The signaling pathways involved in the regulation of GSH synthesis in response to insulin were examined in primary cultured rat hepatocytes. GSH levels, GCL activity and GCLC protein and mRNA levels were increased to 140%, 160%, 600% and 340% of that monitored in untreated cells, respectively, in hepatocytes cultured with 100 nM insulin. The phosphatidylinositol 3-kinase (PI3K) inhibitors, wortmannin and LY294002, dominant negative Akt, or rapamycin, an inhibitor of mTOR and ribosomal p70 S6 kinase (p70S6K) phosphorylation, inhibited the insulin-mediated increase in GCLC protein and GSH levels. Although the mitogen-activated protein kinases (MAPKs) ERK, p38 MAPK, and JNK were activated in response to insulin, PD98059, an inhibitor of MEK, SP600125, an inhibitor of JNK and SB203580, an inhibitor of p38MAPK, failed to inhibit the insulin-mediated increase in GCLC protein levels. In conclusion, these data show that insulin signaling pathways involving PI3K/Akt/p70S6K, but not MAPKs, are active in the insulin-mediated regulation of GSH synthesis via increased GCLC expression.

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Reduced glutathione (GSH) serves several vital intracellular functions, including detoxifying electrophiles, scavenging free radicals, maintaining the essential thiol status of proteins, and providing a reservoir for cysteine (Meister and Anderson, 1983; DeLeve and Kaplowitz, 1991; Lu, 1999). Thus, maintenance of GSH levels is pivotal for cellular defense against oxidative injury and for cellular integrity. Although several organs can take up GSH directly from blood, most organs, including liver, depend on *de novo* GSH synthesis to maintain their intracellular GSH content (Ookhtens and Kaplowitz, 1998). The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: the formation of γ-glutamylcysteine from glutamate and cysteine, and formation of GSH from γ-glutamylcysteine and glycine. The first step of GSH biosynthesis is rate-limiting and catalyzed by γ-glutamylcysteine ligase (GCL). GCL consists of a regulatory or light subunit (GCLR) and a catalytic, or heavy subunit (GCLC). Because GCL is a major determinant of GSH synthesis capacity, regulation of GCL subunits has been a topic of extensive research.

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Oxidative stress is increased in diabetic conditions (West, 2000) and is a major factor contributing to chronic complications of diabetes (Baynes and Thorpe, 1999). Oxidative stress has been demonstrated in diabetic rats (Traverso et al, 1998; Sano et al, 1998; Otsyula et al, 2003), and oxidative stress parameters have been reported to be ameliorated upon insulin administration (Sano et al., 1998; Otsyula et al, 2003). Diabetes

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is common in patients with non-alcoholic fatty liver disease and plays a critical role in hepatocellular injury (Neuschwander-Tetri and Caldwell, 2003). Lower concentrations of GSH in the erythrocytes of diabetic patients, relative to healthy subjects, have been reported, and decreased GSH concentrations increased the sensitivity of these cells to oxidative stress (Jain et al, 2000; Yoshida et al, 1995).

It has been reported that GCL activity and GSH levels are increased by insulin in primary cultured rat hepatocytes and the mechanism of insulin-mediated GCL activation appears to be an increase in the transcription of GCLC (Lu et al, 1992; Cai et al, 1995). In contrast, GCLR expression was unaffected by insulin treatment, suggesting that regulation of GCLC expression is critical for GSH homeostasis (Cai et al, 1997). The intracellular signaling pathways involved in insulin-mediated regulation of GCLC expression and GSH synthesis, however, have not been determined.

Insulin receptor signaling results in activation of phosphatidylinositol 3-kinase (PI3K) and a variety of downstream effectors, including Akt, ribosomal p70 S6 kinase (p70S6K) and protein kinase C (PKC) (Farese, 2001; Taha and Klip, 1999). Activation of the insulin receptor also leads to activation of mitogen-activated protein kinase (MAPK) signaling pathways including ERK (Taha and Klip, 1999), p38 MAPK (Sutherland et al, 1997; Kim et al, 2003a; Haussinger et al, 1999) and JNK (Moxham et al, 1996; Miller et al, 1996;

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Desbois-Mouthon et al, 1998). A simplified scheme of insulin receptor signaling is illustrated in Fig. 1.

The objectives of the present study were to determine, using primary cultured rat hepatocytes, the signaling pathways involved in insulin-mediated regulation of GCLC and GSH. The liver, a major target organ of insulin action, plays a central role in a complex interorgan homeostasis of GSH by being the predominant source of plasma GSH (Ookhtens and Kaplowitz, 1998). In the present study, insulin treatment increased GCLC mRNA, protein and activity levels, and GSH content in primary cultured rat hepatocytes. We show that inhibition of PI3K, Akt or p70S6K activity prevents the insulin-mediated increase in GCLC protein and GSH levels. MAPK inhibitors, on the other hand, were without effect on insulin-mediated GCLC expression. These data implicate PI3K, Akt and p70S6K in the insulin-mediated regulation of hepatic GSH synthesis via increased expression of GCLC.

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Materials and Methods

Materials. Modified Chee's medium and L-glutamine were obtained from Invitrogen (Carlsbad, CA). Insulin (Novolin R) was purchased from Novo-Nordisk (Princeton, NJ). Collagenase (type I) was purchased from Worthington Biochemicals (Freehold, NJ). Vitrogen (95-98 % type I collagen, 2-5% type III collagen) was obtained from Cohesion Technologies (Santa Clara, CA). GCLC antibody was purchased from NeoMarkers, Inc. (Freemont, CA). Antibodies against Akt, p70S6K, ERK, JNK, p38 MAPK, phospho-Akt (Ser473), phospho-p70S6K (Thr421, Ser424), phospho-ERK (Thr202, Tyr204), phospho-JNK (Thr183, Tyr185), phospho-p38MAPK (Thr180, Tyr182), and Akt kinase assay kit were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was obtained from BioRad Laboratories (Hercules, CA). Enhanced chemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Wortmannin, LY294002, rapamycin, bisindolylmaleimide, SB203580, PD98059, U0126 and SP600125 were obtained from Calbiochem (La Jolla, CA). Mouse Akt1-K179M (kinase-dead) was obtained from Upstate Biotechnology (Charlottesville, VA). pAdTrack-CMV and pAdEasy were obtained from Dr. B. Vogelstein (The Sidney Kimmel Comprehensive Cancer Center and The Howard Hughes Medical Institute, The Johns Hopkins University Medical Institutions, Baltimore, Maryland). GSH and all other reagents were purchased from Sigma (St. Louis, MO).

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Primary rat hepatocyte culture. Hepatocytes were isolated from the livers of male Sprague-Dawley rats (200-300 g) using collagenase perfusion as described previously (Woodcroft and Novak, 1997; Woodcroft and Novak, 1999). Hepatocytes were plated onto dishes covalently coated with Vitrogen, and modified Chee's medium was fortified as described (Woodcroft and Novak, 1997; Woodcroft and Novak, 1999) and supplemented with 0.1 µM dexamethasone and 1 µM insulin. Cells were plated at a density of $3x10^6$ cells/60 mm dish or $1x10^7$ cells/100 mm dish. Four hours after plating, cells were washed with insulin-free medium several times and cultured for an additional 2 h in insulin-free medium prior to initiation of treatment. Cells were then treated with various concentrations of insulin (0-100 nM). Kinase inhibitors were dissolved in DMSO and added 1.5 h prior to addition of insulin (10 nM). The final DMSO concentration in the medium was 1 µl/ml (0.1%) and this concentration of DMSO did not affect the GCLC protein level, GCL activity or GSH level relative to untreated hepatocytes. Fig. 1 illustrates the site of action of the kinase inhibitors employed in this study. None of the protein kinase inhibitors resulted in increased cell toxicity, as compared to untreated cells, at the concentrations used in this study. Medium was changed every 24 h. For adenovirus infection, 4 h after plating cells were washed with insulin-free medium several times and AdV-Akt (150 MOI; multiplicity of infection) or control virus (AdV-GFP, 15 MOI) was added to the cells in fresh medium. Following overnight infection, medium was changed, and hepatocytes were treated with insulin (10 nM) for 2 days. Hepatocyte viability was monitored by measuring released

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lactate dehydrogenase activity as described previously (Woodcroft and Novak, 1997). The Wayne State University Animal Investigation Committee approved all experimental procedures involving animals.

Preparation of dominant negative Akt adenoviral construct. The kinase-dead form of Akt1 has a point mutation, K179M, removing the ATP-binding site, which results in loss of kinase activity. The coding region of Akt1-K179M containing a Myc-His tag was amplified by PCR using Pfu turbo (Stratagene, La Jolla, CA) and the primers 5'-GCGAGATCTATCCCATGAACGACGTAGCC-3' and 5'-GCGAGATCTAAACTCAATGGTGATGGTGATGAT-3'. The PCR product was A-tailed and ligated into the pGEM-T Easy Vector (Promega, Madison, WI). The kinase-dead Akt was digested from the T Vector with SpeI and NotI and ligated into NotI/XbaI sites in pAdTrack. Homologous recombination of pAdTrack and pAdEasy and infection of 293 cells were performed as described (He et al, 1998). Briefly, pAdTrack-Akt1 (K179M) was linearized with PmeI and cotransformed into BJ5183 cells with the adenoviral backbone plasmid, pAdEasy, by electroporation. Recombinants were selected by kanamycin resistance and confirmed by restriction endonuclease analyses, linearized and transfected into 293 cells. Production of the virus in the packaging cell line was monitored with green fluorescent protein (GFP) encoded by a gene incorporated into the viral backbone. The recombinant dominant negative kinase-dead Akt adenovirus was purified from 293 cells by CsCl

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density centrifugation, collected and dialyzed against 10 mM Tris, pH 8.2, 2 mM $MgCl_2$ and 4% sucrose.

Immunoblot analysis. Whole cell lysates were prepared as described previously (Kim et al, 2003b). For immunoblot analysis of GCLC, lysates (20 µg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose (Bio-Rad, Hercules, CA), blocked in 5% milk powder in PBS-T (0.05% Tween 20 in PBS) and incubated with anti-rat GCLC (1:10,000 in 5% milk powder in PBS-T) overnight at room temperature. To determine the phosphorylation state of Akt, p70S6K, ERK, p38 MAPK and JNK, cell lysates were prepared by scraping cells directly into 500 µL SDS-PAGE sample buffer. Lysates (10 µl) were separated by 10% SDS-PAGE, transferred to nitrocellulose, blocked in 5% milk powder in TBS-T (0.05% Tween 20 in Tris-HCl buffered saline) and probed with phospho-specific antibodies (1:250 in 5% bovine serum albumin in TBS-T) overnight at 4°C. Blots were stripped and re-probed with phosphorylation state-independent antibodies to Akt, p70S6K, ERK, p38 MAPK and JNK. Proteins were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma) and quantified by densitometry with a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences, Piscataway, NJ).

Akt activity assay. Thirty minutes after 10 nM insulin treatment, whole cell lysates were

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prepared as described previously (Kim et al, 2003b). Akt immunoprecipitation and kinase assay were carried out following the procedures in the kit as described by the manufacture (Cell Signaling Technology).

GSH level and GCL activity determination. The concentration of GSH and the activity of GCL were measured as described previously (Kim et al, 2003c). None of the protein kinase inhibitors resulted in interference of determination of GSH.

PCR determination of GCLC mRNA levels. Total hepatocyte RNA was isolated as previously described (Woodcroft and Novak, 1997; Woodcroft and Novak, 1999). Reverse transcription was carried out using 2 µg of total RNA following the protocol for the Taqman Reverse Transcription Master Mix (Applied Biosystems, Foster City, CA). Ten nanograms of cDNA were subjected to PCR using the primer 5'-CATCAGGCTCTTTGCACGATAAC-3' and analyzed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Sybr Green was incorporated using the Sybr Green Master Mix from Applied Biosystems. Cycle threshold (Ct) values were obtained from the ABI Prism 7000 software and fold change was determined.

Statistical analysis. Significant differences between groups were determined by ANOVA followed by the Newman-Keuls multiple comparison test ($p<0.05$). Statistical analysis was

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performed on triplicate cell lysates from a single hepatocyte preparation. Reproducibility of results was confirmed in two to four separate hepatocyte preparations.

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Results

Effects of insulin on GCLC protein and mRNA levels, GCL activity and GSH levels.

In order to examine changes in GSH biosynthesis during culture of primary rat hepatocytes, GCLC protein, GCL activity, GCLC mRNA and GSH levels were monitored in hepatocytes cultured in the absence of insulin (Fig. 2). The levels of GCLC protein and GCL activity were initially increased for 2 days after plating and returned to the levels observed in freshly isolated hepatocytes by 3 days post-plating (Fig. 2A and B), indicating that changes in GCLC protein levels correlate well with GCL activity in primary cultured hepatocytes. GCLC mRNA levels were not changed over 3 days of culture in the absence of insulin (Fig. 2C). GSH levels monitored in Sprague-Dawley rat liver have been reported to be 33.2 ± 2.4 nmol/mg protein (Kim et al, 2003d). GSH levels were markedly decreased to 8.2 ± 0.5 nmol/mg protein during isolation of hepatocytes (Fig. 2D), which may be associated with efflux of GSH. After the 4 h plating period, however, GSH levels increased to 23.3 ± 2.1 nmol/mg protein and recovered to 31.4 ± 0.3 nmol/mg protein 1 day post-plating (Fig. 2D). Subsequently, this tripeptide progressively declined by ~20% over 4 days of culture.

Insulin effects on GCLC protein and mRNA, GCL activity and GSH levels were then examined (Fig. 2). Insulin elevated GCLC protein levels 2.4- to 6.6-fold (Fig. 2A) and GCL activity was increased 160-170% (Fig. 2B), relative to control hepatocytes, over the 4-day

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culture period. Insulin treatment also increased GCLC mRNA levels by 2.5- to 3.5-fold (Fig. 2C) and resulted in a 30-40% increase in GSH levels (Fig. 2D). These results are consistent with previous reports (Lu et al, 1992; Cai et al, 1995).

In order to examine the insulin concentration-dependence of GCLC protein and mRNA, GCL activity and GSH levels, hepatocytes were cultured for 24 h in medium supplemented with 0.1-100 nM insulin (Fig. 3A). GCLC protein, GCLC mRNA, GCL activity and GSH levels were increased in an insulin concentration-dependent manner, and were increased maximally ~210, 270, 165 and 135%, respectively, relative to cells that were not exposed to insulin (Fig. 3A). Significantly increased levels of GCLC protein, GCL activity and GSH were observed at 1 nM insulin, and a further increase was observed in hepatocytes cultured in the presence of 10 nM insulin. In addition, GSH levels determined in medium were also increased from 7.4 $\pm$ 0.4 to 9.1 $\pm$ 0.4 nmol/ml at 24 h following 10 nM insulin treatment. Thus, insulin regulates basal GSH synthesis in cultured hepatocytes.

In order to assess short-term insulin effects on GSH synthesis, insulin (10 nM) was added to hepatocytes for 2, 4, 12 or 24 h (Fig. 3B). Twenty-four hours following initiation of each insulin treatment, GCLC protein, GCL activity and GSH levels were monitored. Insulin treatment for 2 h, followed by 22 h of culture in the absence of insulin, resulted in a significant increase in GCLC protein levels. GCL activity was significantly elevated with 4 h

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of insulin treatment, and the elevation of GSH levels was observed following 12 or 24 h of insulin treatment. These results suggest that the insulin-mediated elevation of GSH levels is associated with increased synthesis of this tripeptide through the induction of GCLC protein expression, and a short treatment with insulin (2 h) is sufficient to initiate this process.

Insulin-mediated phosphorylation of protein kinases in primary cultured rat hepatocytes. Prior to determining which signaling pathway(s) may play a role in insulin regulation of GCLC expression and GSH levels, we examined the insulin activation of protein kinases involved in the PI3K and MAPK signaling pathways in primary cultured rat hepatocytes (Fig. 4). Insulin (10 nM) resulted in a substantial increase (>10-fold) in the phosphorylation of Akt (Ser473) relative to corresponding untreated cells at all time points examined (Fig. 4A). Phosphorylation of p70S6K (Thr421, Ser424) was increased ~1.7-fold within 10 min and the maximal increase of 7.5-fold was observed at 3 h (Fig. 4B). These results demonstrate that insulin results in a marked and prolonged activation of Akt and p70S6K, downstream kinases of PI3K, in primary cultured rat hepatocytes.

Treatment of hepatocytes with 10 nM insulin also increased the phosphorylation of ERK (Thr202, Tyr204) ~3-fold within 5 min and this increase declined after 10 min with a return toward basal levels by 30 min (Fig. 4C). ERK phosphorylation was increased again 3 h

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following initiation of insulin treatment and remained elevated through 24 h. Insulin-mediated elevation of p38MAPK phosphorylation (Thr180, Tyr182) was observed 1 h after insulin addition and was elevated maximally at 3 h (Fig 4D). Phosphorylation of JNK (Thr183, Tyr185) was altered in a time-dependent manner in response to 10 nM insulin (Fig. 4E). Addition of insulin caused a maximal ~3-fold increase in JNK phosphorylation at 3 h. Interestingly, 24 h exposure to 10 nM insulin markedly inhibited the basal phosphorylation of JNK. Thus, all MAPKs monitored in this study were phosphorylated in response to insulin.

Inhibition of ERK, p38MAPK and JNK. To determine whether ERK, p38MAPK or JNK may play a role in insulin regulation of GCLC expression and GSH level, hepatocytes were pretreated with the MEK inhibitors PD98059 and U0126, the p38 MAPK inhibitor SB203580, or the JNK inhibitor SP600125, prior to addition of 10 nM insulin (Table 1). Each of these inhibitors was without effect on the insulin-mediated elevation of GCLC protein levels (Table 1). The p38MAPK inhibitor SB203580 was also without effect on the insulin-mediated elevation of GSH levels. Interestingly, the MEK inhibitor PD98059 and the JNK inhibitor SP600125 both enhanced the insulin effect on hepatocyte GSH levels (Table 1). These results suggest that MAPK pathways activated by insulin in primary cultured rat hepatocytes do not play a role in the insulin regulation of GCLC expression and GSH levels.

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Inhibition of PI3K, PKC, Akt and p70S6K. To determine whether PI3K plays a role in insulin regulation of GCLC expression and GSH levels, hepatocytes were pretreated with wortmannin or LY294002 prior to addition of 10 nM insulin (Fig. 5). We confirmed the PI3K inhibitory action of wortmannin and LY294002 through the observation that the 10 nM insulin-induced phosphorylation of Akt was completely inhibited by 500 nM wortmannin or 10 µM LY294002 (Fig. 5A). In contrast, rapamycin, which inhibits mTOR and consequently the phosphorylation and activation of p70S6K, failed to inhibit insulin-mediated Akt phosphorylation (Fig. 5A). Both wortmannin and LY294002 pretreatment resulted in a concentration-dependent inhibition of the insulin-mediated increase in GCLC protein, with complete inhibition of the insulin effect observed at 500 nM wortmannin or 10 µM LY294002 (Fig. 5B). The insulin-mediated elevation of GSH level was also inhibited by pretreatment of cells with wortmannin or LY294002 (Fig. 5C). Thus, with either wortmannin or LY294002, inhibition of the insulin-mediated elevation in GSH synthesis was accomplished in an inhibitor concentration-dependent manner consistent with inhibition of PI3K signaling.

The possible involvement of PKC in the insulin-mediated effect on GCLC protein and GSH levels was examined using the broad spectrum PKC inhibitor bisindolylmaleimide. GCLC protein levels were 2.2- or 2.5-fold greater in insulin-treated or insulin +

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bisindolylmaleimide-treated hepatocytes, respectively, relative to control cells. GSH levels were 31.1 ± 2.6, 45.3 ± 1.3 and 49.9 ± 5.4 nmol/mg protein in control, insulin-treated and insulin + bisindolylmaleimide-treated hepatocytes, respectively. Thus, bisindolylmaleimide failed to inhibit the insulin-mediated increase in GCLC protein and GSH levels, suggesting that PKC does not contribute to the insulin induction of GCLC or GSH levels.

To determine the effect of inhibition of Akt on insulin-mediated elevation of GCLC expression and GSH level, a dominant negative kinase-dead mutant of Akt1 was expressed by adenoviral infection of primary cultured hepatocytes (Fig. 6). Immunoblot analysis of hepatocytes infected with AdV-Akt (Fig. 6A) indicated expression of a higher molecular weight Akt protein in addition to the endogenous Akt owing to the additional Myc/His tags on the adenovirally introduced Akt. This was confirmed by immunoblotting with a c-Myc antibody (data not shown). To confirm that the overexpressed dominant negative Akt construct was functional, Akt activity was examined in hepatocytes treated with 10 nM insulin for 30 min (Fig. 6B). Akt kinase activity was increased ~10-fold in response to 10 nM insulin and this increase was inhibited ~65% by AdV-Akt. Control AdV-GFP-infected hepatocytes were comparable to uninfected cells in their elevated GCLC protein and GSH levels in response to insulin (Fig. 6C and D). Dominant negative Akt expression resulted in a decline in the insulin-mediated increase in GCLC protein and GSH levels, resulting in a ~50% inhibition of the insulin effect. These results suggest that

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Akt, a downstream effector of PI3K, is involved in the insulin-mediated increase in GCLC protein and GSH levels.

We used rapamycin to determine whether p70S6K plays a role in mediating the insulin effect on GCLC expression and GSH synthesis (Fig. 7). Insulin treatment resulted in increased phosphorylation and retarded migration of p70S6K, which was inhibited by rapamycin pretreatment in a concentration-dependent manner, with complete inhibition of the insulin effect observed at 100 nM rapamycin (Fig. 7A). Also, wortmannin (500 nM) or LY294002 (10 μM) partially inhibited the activation of p70S6K mediated by insulin, confirming that p70S6K activation is downstream of PI3K in primary cultured rat hepatocytes. Pretreatment of cells with rapamycin inhibited the insulin-mediated increase in GCLC protein and GSH levels (Fig. 7B,C), suggesting that p70S6K also plays a role in the insulin-mediated increase in GCLC protein and GSH levels.

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Discussion

GSH levels and expression of GCL are altered in response to pathophysiologic conditions such as diabetes, protein-calorie malnutrition and alcohol consumption as well as hepatic diseases, including steatohepatitis and cirrhosis (Jain et al, 2000; Yoshida et al, 1995; Kim et al, 2003d; Kim et al, 2003e; Lu et al, 1999; Chawla et al, 1984). A number of investigations have shown that GSH level, GCL activity and GCLC expression are under hormonal and growth-related regulation (Lu et al, 1992; Cai et al, 1995). In primary cultures of rat hepatocytes, the activity of GCL, GCLC mRNA level and GSH levels were induced by 1 µg/ml (~174 nM) insulin (supraphysiologic level) treatment (Lu et al, 1992; Cai et al, 1995), and insulin treatment of diabetic rats restored GSH levels and GCL activity (Lu et al, 1992). In the present study significant increases in GCLC protein, GCLC mRNA, GCL activity and GSH levels were observed at 1 nM insulin, and a further increase was observed in hepatocytes cultured in the presence of 10 nM insulin, indicating that insulin can serve as a physiological regulator of GSH homeostasis.

Maximal increases in GCLC protein, GCLC mRNA, GCL activity and GSH levels in response to insulin treatment were ~650, 350, 170 and 140%, respectively, relative to control hepatocytes. Thus, the GCLC protein and mRNA levels were more markedly increased in response to insulin than either GCL activity or GSH levels. One possible explanation for this finding, in addition to feedback inhibition by GSH, is that the

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expression of the regulatory subunit of GCL (GCLR), which regulates GCL activity by promoting high affinity for glutamate and providing sensitivity for feedback inhibition by GSH, is unaffected by insulin treatment (Cai et al, 1997). Thus, the level of GCLR may be a limiting determinant of GCL activity, even when GCLC levels are increased. Synthesis of GSH in hepatocytes is limited not only by GCL activity but also by the availability of cysteine. Cystathionine β-synthase, a key enzyme mediating the first irreversible step in the synthesis of cysteine from homocysteine, has been reported to be decreased by insulin in HepG2 cells and streptozotocin-induced diabetic rats (Ratnam et al., 2002). Thus, cysteine synthesis may be decreased in hepatocytes treated with insulin, which may be a contributing factor in the disparity between GSH levels and GCL activity in response to insulin.

PI3K and a variety of downstream protein kinases, including Akt, p70S6K and PKC, represent one of the major signaling pathways mediating metabolic effects of insulin (Farese, 2001; Taha and Klip, 1999). In the present study, the PI3K signaling pathway was rapidly and markedly activated in response to insulin in primary cultured rat hepatocytes. Insulin receptor signaling also leads to activation of MAP kinases, including ERK, p38 MAPK and JNK in some cell systems (Sutherland et al, 1997; Kim et al, 2003a; Haussinger et al, 1999; Moxham et al, 1996; Miller et al, 1996; Desbois-Mouthon et al, 1998). In the present study ERK, JNK and p38 MAPK phosphorylation were increased by

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insulin treatment but differences in the rapidity and duration of activation were observed. The levels of phosphorylated ERK were increased 5 min after insulin treatment and returned to the level of untreated hepatocytes within 30 min. These results are consistent with previous reports (Ribaux et al, 2002). However, phospho-ERK levels were again increased at 3 h following insulin treatment and maintained over 24 hours. Thus, the activation of ERK mediated by insulin in primary cultured rat hepatocytes appears to be biphasic. Increased phosphorylation of JNK and p38 MAPK was observed from 1 h following insulin treatment, suggesting that the activation of JNK and p38 MAPK induced by insulin may be indirect. Interestingly, 24 hour exposure to 10 nM insulin inhibited the basal phosphorylation of JNK by ~50%. Desbois-Mouthon et al. (1998, 2000) have reported that JNK activation induced by insulin was transient and followed by a sustained inhibition phase through a PI3K- and MAP kinase phosphatase-1-dependent pathway in CHO cells overexpressing human insulin receptors.

The PI3K inhibitors wortmannin and LY294002 completely inhibited the insulin-mediated increases in GCLC protein expression and GSH level, implicating PI3K as an obligatory component in the stimulation of GSH synthesis by insulin. Recently Li et al. (2003) reported that wortmannin prevented the insulin-mediated elevation of GSH levels in cardiac myocytes from rats treated with the GCL inhibitor, buthionine sulfoximine, and the GSH reductase inhibitor, 1,3-bis-(2-chloroethyl)-1-nitrosourea. These authors also showed

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that the up-regulation of GSH by insulin was blocked by PD98059 and SB203580. Thus, it appears that multiple signaling pathways may be involved in insulin-mediated elevation of GSH levels in cardiac myocytes. These results differ from the present results, in which insulin signaling pathways involving PI3K, but not MAPKs, are active in the insulin-mediated elevation of GSH synthesis in primary cultured rat hepatocytes. The reason for this difference is unknown, but may be due, in part, to differences associated with cell context and the use of inhibitors of GCL and GSH reductase.

In the present study overexpression of dominant negative Akt in hepatocytes resulted in a decline in the insulin-mediated increase in GCLC protein and GSH levels, suggesting that Akt is involved in the insulin-mediated increases in GSH synthesis as a downstream protein kinase of PI3K. Rapamycin produced a partial inhibition of the insulin-mediated increase in GCLC protein and GSH levels. A consistent body of evidence indicates that p70S6K plays an important role in regulation of protein synthesis in response to nutrients and hormones (Shah et al, 2000). These results raise the possibility that the insulin effects on GCLC protein and GSH levels may be related to increased translational capacity through activation of p70S6K. Catarzi et al. (2002) reported that PKC activation was involved in platelet-derived growth factor-mediated elevation of GCLC mRNA levels in NIH 3T3 fibroblasts. However, in the present study, the PKC inhibitor bisindolylmaleimide failed to inhibit the insulin-mediated increase in GCLC protein and GSH levels. This difference in

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the role of PKC in regulating GCLC expression may reflect differences associated with the stimulus and cell context. Thus, it appears that insulin signaling pathways involving PI3K/Akt/p70S6K are active in insulin-mediated elevation of GCLC expression and GSH level.

In summary, the results of the present study indicate that insulin regulates GSH synthesis via increased expression of GCLC protein in primary cultured rat hepatocytes, and suggest that the decreased levels of GCLC and GSH in diabetics may be attributed to altered levels of insulin. These results, especially in conjunction with our previous studies demonstrating that insulin increases glutathione S-transferase expression and suppresses CYP2E1 expression (Kim et al, 2003b; Woodcroft et al, 2002), suggest that impairment of the antioxidant defense system may be a contributing factor to the increased oxidative stress and incidence of hepatic disease observed during diabetes. Moreover, this study implicates insulin signaling pathways involving PI3K/Akt/p70S6K in the insulin-mediated regulation of GCLC expression and GSH synthesis. To our knowledge, this is the first report of the identification of the signaling pathways involved in mediating the effect of insulin on GSH synthesis in hepatocytes.

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Figure Legends

Figure 1. Insulin signaling pathways and sites of action of kinase inhibitors.

Figure 2. Insulin effect on GCLC protein (A), GCLC mRNA (B), GCL activity (C) and GSH (D) levels in primary cultured rat hepatocytes. Hepatocytes were maintained in the presence or absence of 100 nM insulin for 1-4 days. GCLC protein and mRNA levels are plotted as a percentage of the level monitored in freshly isolated hepatocytes (0 day, 100%). Data are means ± SD of 3-5 preparations of cell lysates from a single hepatocyte preparation. **,*** Significantly different than levels monitored in corresponding hepatocytes maintained in the absence of insulin, $p<0.01$ or $p<0.001$, respectively.

Figure 3. Concentration-dependent effects of insulin (A) and effects of short-term insulin exposure (B) on GCLC protein, GCLC mRNA, GCL activity and GSH levels in primary cultured rat hepatocytes. A: hepatocytes were cultured in the presence of various concentrations of insulin for 24 h. B: hepatocytes were treated with 10 nM insulin for 2, 4, 12 or 24 h; 24 h after initiation of each insulin treatment, cells were harvested. Data are means ± SD of 3-5 preparations of cell lysates from a single hepatocyte preparation. *,**,*** Significantly different than levels monitored in hepatocytes cultured in the absence of insulin, $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

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Figure 4. Insulin-mediated phosphorylation of Akt (A), p70S6K (B), ERK (C), p38 MAPK (D) and JNK (E) in primary cultured rat hepatocytes. Hepatocytes were cultured in the presence (Ins) or absence (UT) of insulin for the indicated times.

Figure 5. Effect of wortmannin or LY294002 on the insulin-mediated increase in Akt phosphorylation (A), GCLC protein (B) and GSH (C) levels in primary cultured rat hepatocytes. A: hepatocytes were treated with wortmannin (500 nM), LY294002 (10 μM) or rapamycin (1 μM) for 1.5 h before addition of 10 nM insulin for 30 min. B and C: hepatocytes were treated with wortmannin or LY294002 for 1.5 h before addition of 10 nM insulin for 24 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Data are means ± SD of 3-6 preparations of cell lysates from a single hepatocyte preparation. #,##,### Significantly different than levels monitored in untreated hepatocytes, $p<0.05$, $p<0.01$ or $p<0.001$, respectively. *,**,*** Significantly different than levels monitored in hepatocytes treated with insulin only, $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

Figure 6. Effect of dominant negative Akt expression on insulin-mediated GCLC protein and GSH levels in primary cultured rat hepatocytes. A: hepatocytes were infected with 150 MOI AdV-Akt or 15 MOI AdV-GFP and 24 h later, cells were harvested for determination of Akt protein level. B: Following 24 h infection with AdV-Akt or AdV-GFP, hepatocytes were treated with 10 nM insulin for 30 min and assayed for Akt activity. C and

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D: 24 h after infection with AdV-Akt or AdV-GFP, hepatocytes were treated with 10 nM insulin for 2 days. Data are means ± SD of 3-4 preparations of cell lysates from a single hepatocyte preparation. Values with different letters are significantly different from each other, $p<0.05$.

Figure 7. Effect of rapamycin on the insulin-mediated increase in p70S6K phosphorylation (A), GCLC protein (B) and GSH (C) levels in primary cultured rat hepatocytes. A: hepatocytes were treated with rapamycin, wortmannin (500 nM), or LY294002 (10 μM) for 1.5 h before addition of 10 nM insulin for 3 h. B and C: hepatocytes were treated with rapamycin for 1.5 h before addition of 10 nM insulin for 24 h. Untreated (UT) hepatocytes were cultured in the absence of insulin and inhibitor. Data are means ± SD of 3-6 preparations of cell lysates from a single hepatocyte preparation. #,##,### Significantly different than levels monitored in untreated hepatocytes, $p<0.05$, $p<0.01$ or $p<0.001$, respectively. *,**,*** Significantly different than levels monitored in hepatocytes treated with insulin only, $p<0.05$, $p<0.01$ or $p<0.001$, respectively.

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Table 1. Effect of the MEK inhibitors PD98059 or U0126, the p38 MAPK inhibitor SB203580, or the JNK inhibitor SP600125 on the insulin-mediated increase in GCLC protein and GSH levels. Hepatocytes were treated with bisindolylmaleimide (Bis, 5 μM), PD98059 (PD, 50 μM), U0126 (25 μM), SB203580 (SB, 10 μM) or SP600125 (SP, 20 μM) for 1.5 h prior to addition of insulin (10 nM) for 24 h. Control (untreated) hepatocytes were cultured in the absence of insulin and inhibitor.

Treatment	GCLC Protein Level*	GSH Level (nmol/mg protein)
Control	100 ± 11% [a]	30.4 ± 0.1 [a]
+ Insulin	243 ± 7% [b]	43.4 ± 1.6 [b]
+ Insulin + PD	250 ± 16% [b]	60.8 ± 1.4 [c]
+ PD	86 ± 9% [a]	48.3 ± 2.2 [b]
Control	100 ± 10% [a]	32.5 ± 1.9 [a]
+ Insulin	233 ± 6% [b]	43.4 ± 1.6 [b]
+ Insulin + U0126	198 ± 20% [b]	47.9 ± 1.2 [b]
+ U0126	94 ± 8% [a]	33.2 ± 1.2 [a]
Control	100 ± 13% [a]	33.1 ± 1.1 [a]
+ Insulin	176 ± 11% [b]	47.3 ± 2.2 [b]
+ Insulin + SB	168 ± 16% [b]	44.9 ± 3.6 [b]
+ SB	85 ± 7% [a]	35.5 ± 2.3 [a]
Control	100 ± 7% [a]	32.0 ± 3.4 [a]
+ Insulin	226 ± 3% [b]	44.4 ± 0.6 [b]
+ Insulin + SP	259 ± 16% [b]	63.8 ± 4.2 [c]

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+ SP	109 ± 28% [a]	35.2 ± 3.3 [a]

*GCLC protein levels are shown as a percentage of the level monitored in untreated hepatocytes (100%). Data are means ± SD of three preparations of cell lysates from a single hepatocyte preparation. Values with different letters are significantly different from each other, $p<0.05$.

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Figure 1

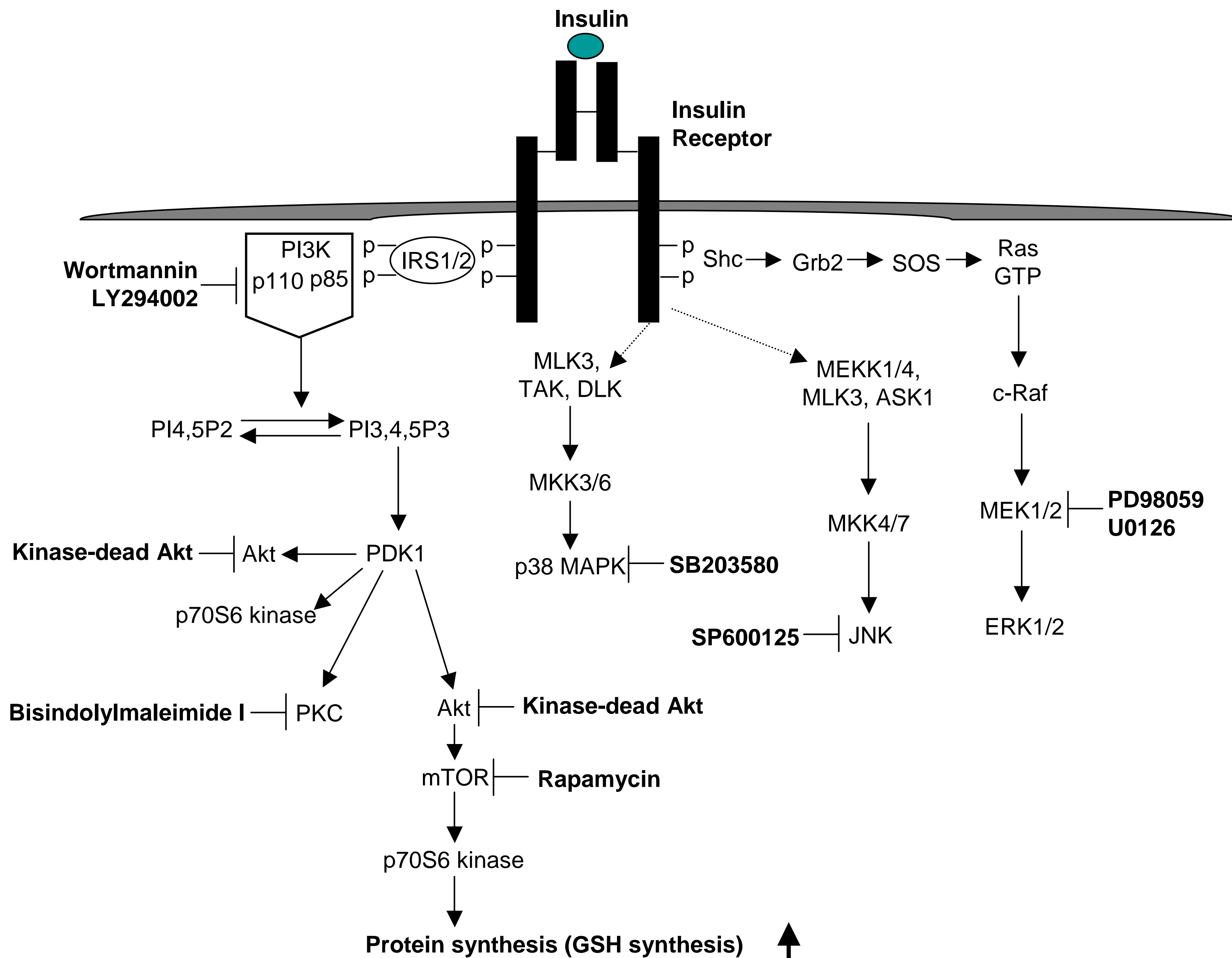

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Figure 2

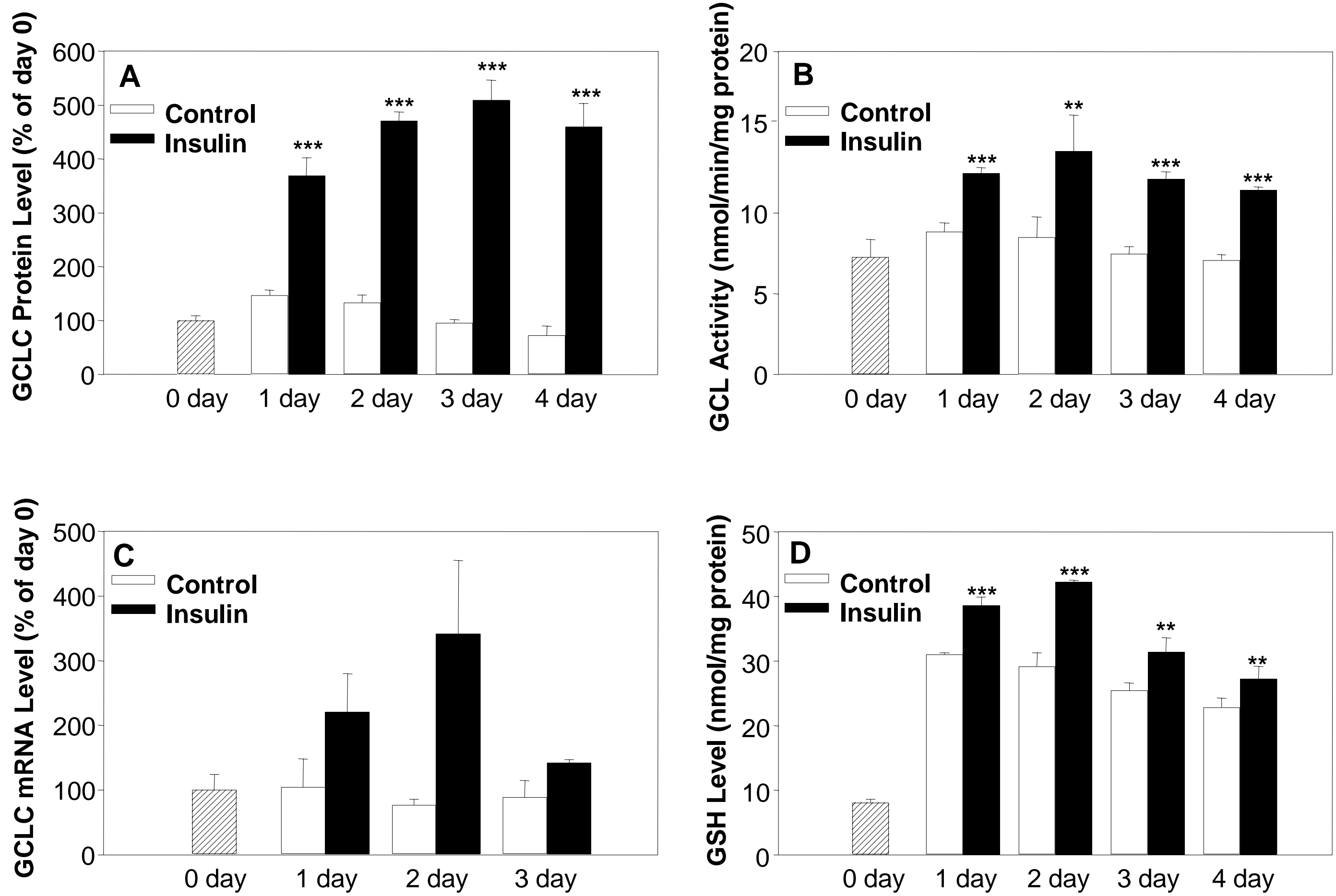

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Figure 3

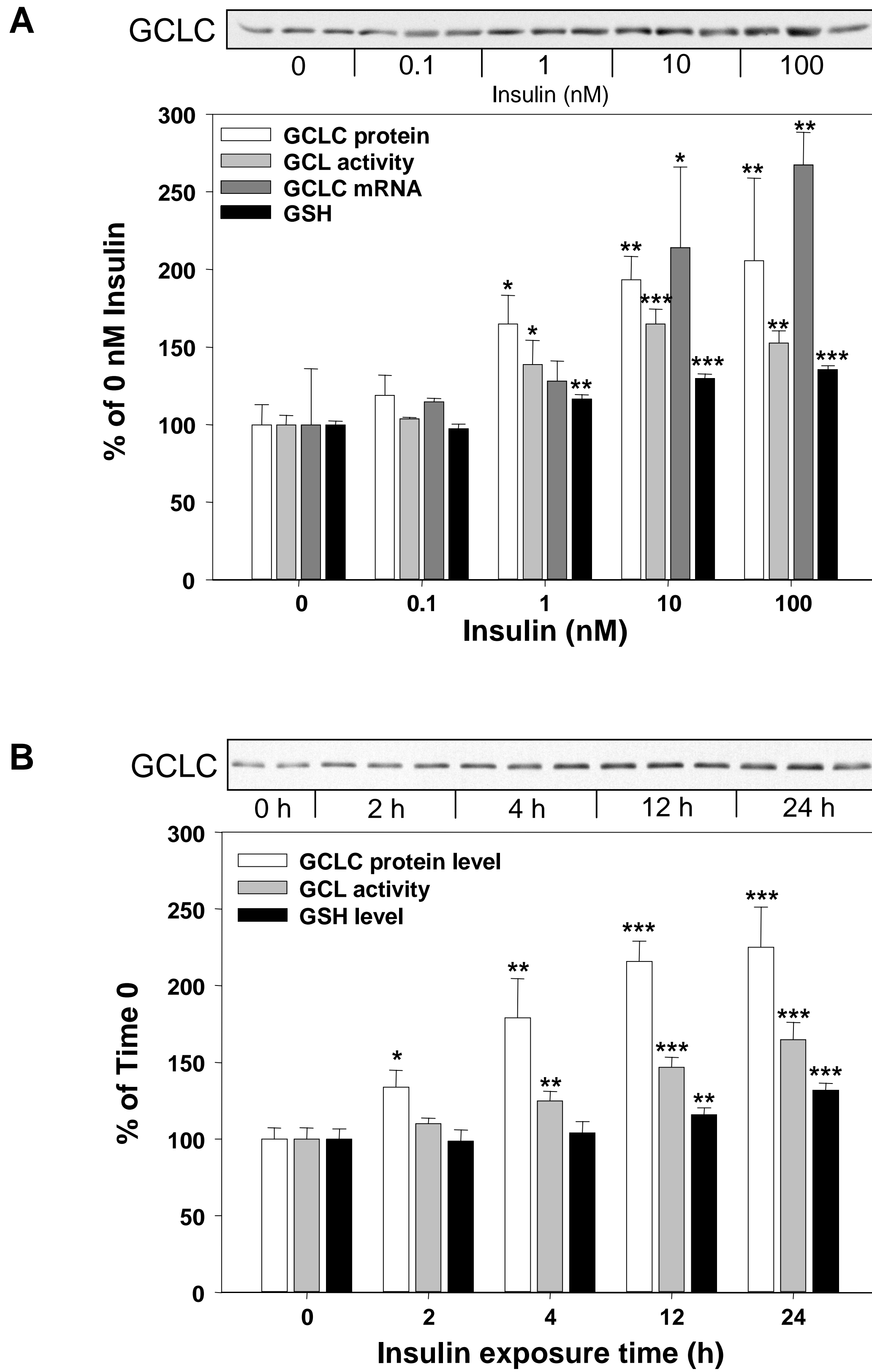

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Figure 4

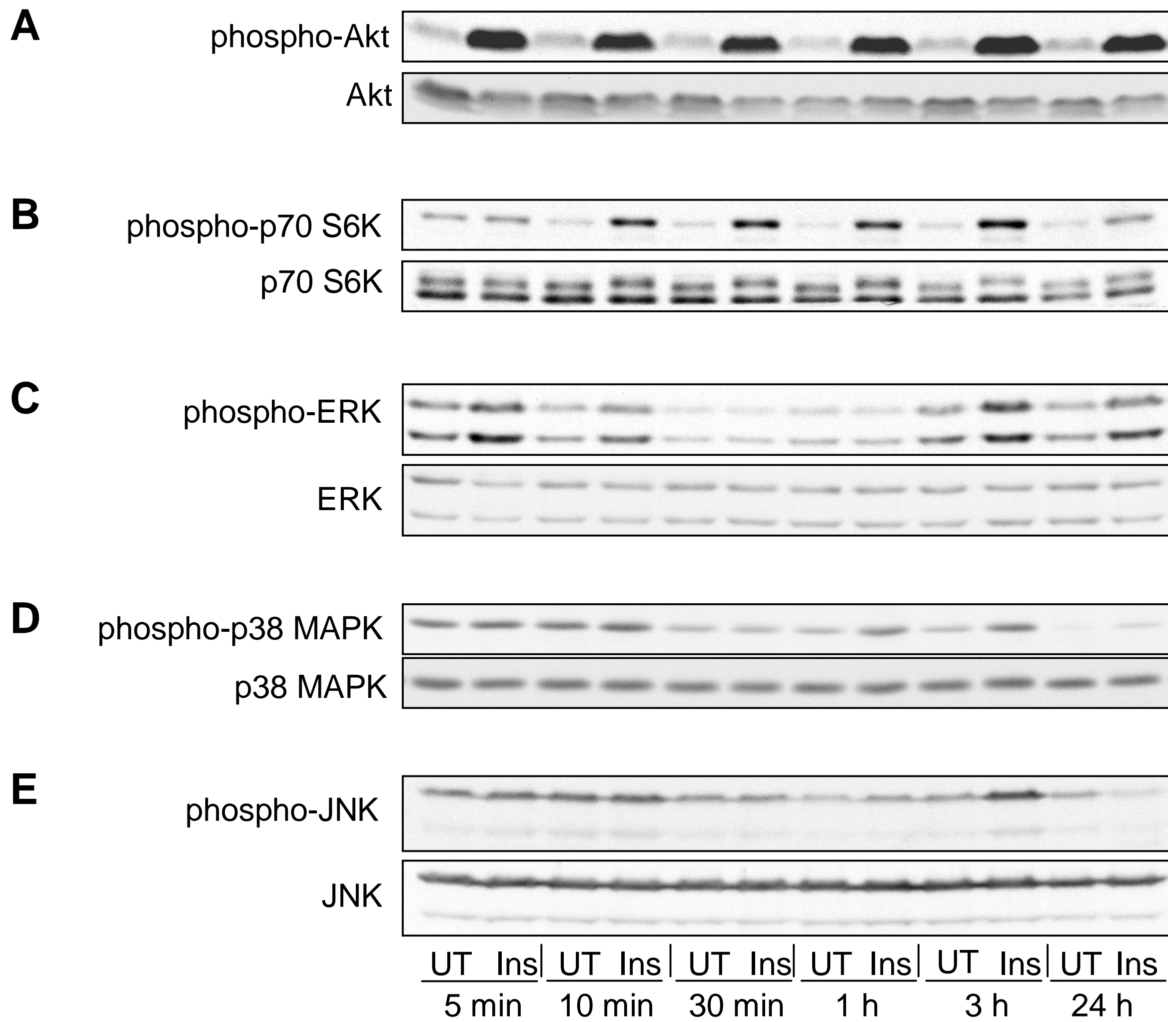

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Figure 5

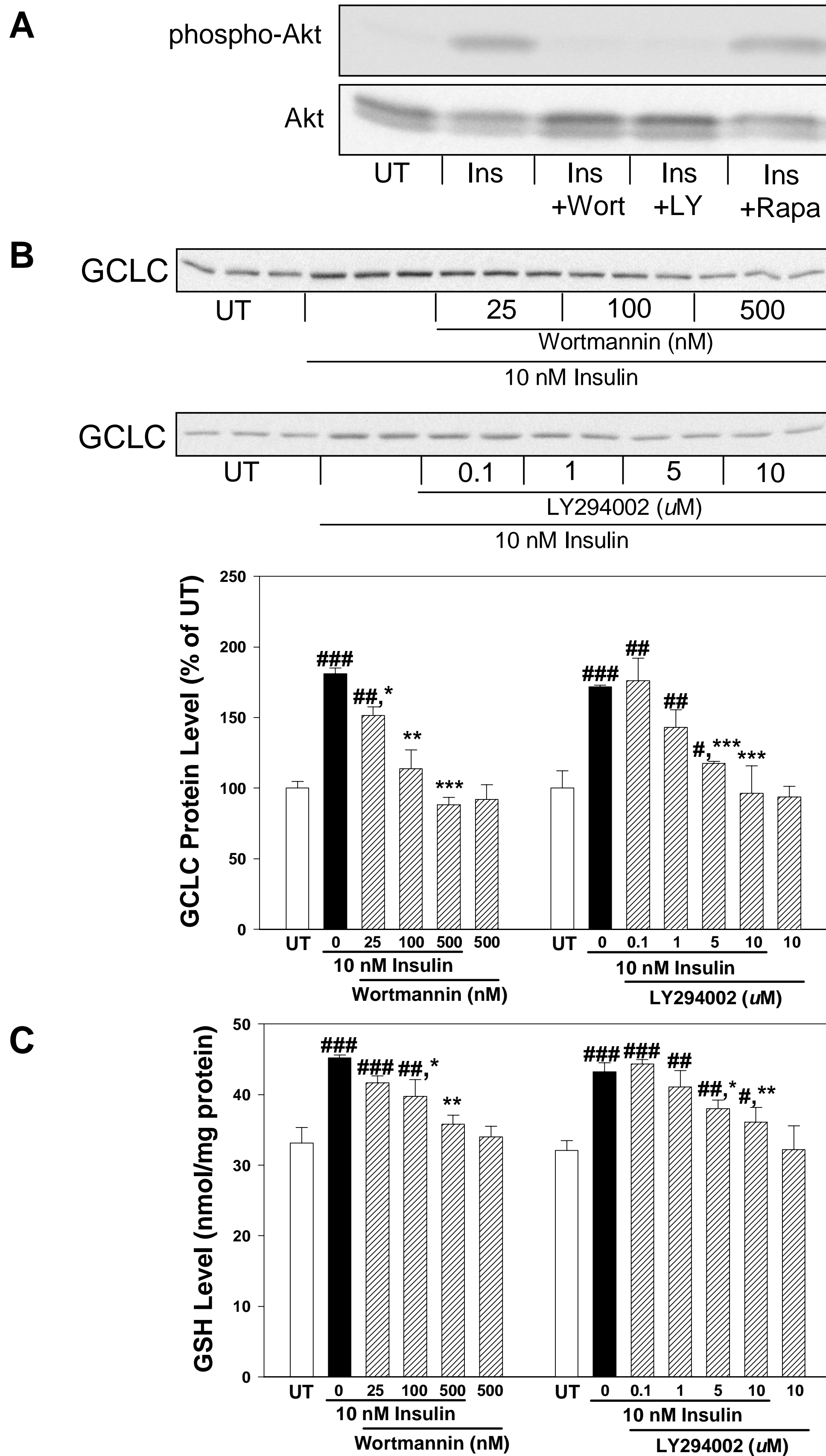

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Figure 6

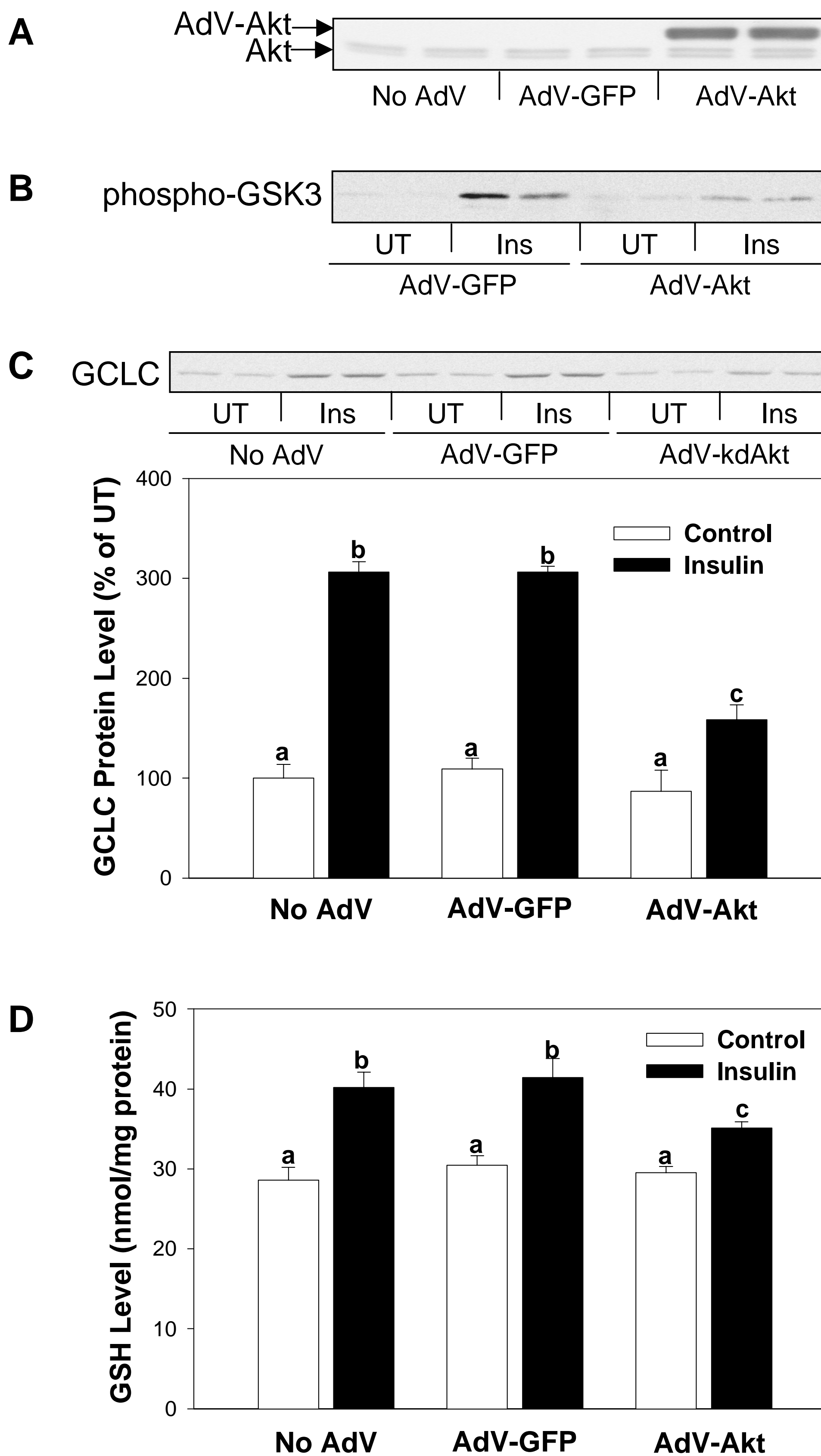

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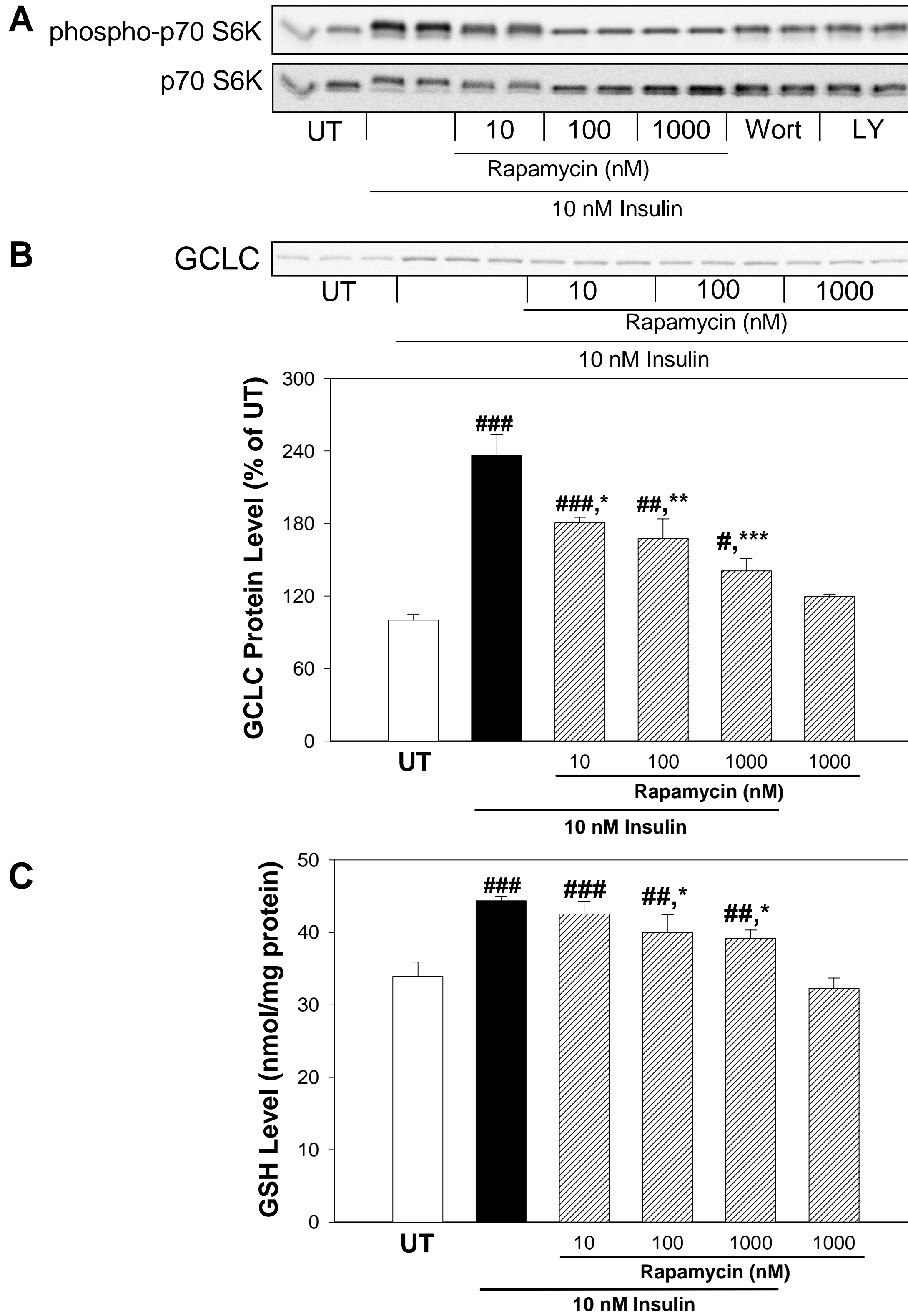
Figure 7



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