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Carrageenan Inhibits Insulin Signaling through GRB10-Mediated Decrease in Phospho(Tyr)-IRS1 and through Inflammation-Induced Increase in Phospho(Ser307)-IRS1

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Running Title: *Carrageenan inhibits insulin signaling by effects on GRB10*

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Background: The common food additive carrageenan inhibits insulin signaling by increasing phospho(Ser307)IRS1, leading to reduced phospho(Ser473)AKT.
Results: Carrageenan also inhibits insulin signaling by increasing expression of GRB10, an inhibitor of phospho(Tyr)IRS1.
Conclusion: Combined effects of carrageenan on phospho(Tyr)IRS1 and phospho(Ser307)IRS1 completely block the insulin-induced increase in phospho(Ser473)AKT.
Significance: Carrageenan impairs glucose tolerance by inflammatory and transcriptional effects that lead to insulin resistance.

ABSTRACT

Inflammation induced by exposure to the common food additive carrageenan leads to insulin resistance by increase in phospho(Ser307)IRS1 and subsequent decline in the insulin-stimulated increase in phospho(Ser473)AKT. Inhibition of carrageenan-induced inflammation reversed the increase in p(S307)IRS1, but did not completely reverse the carrageenan-induced decline in p(S473)AKT. To identify the additional mechanism responsible for the decrease in p(S473)AKT, studies were performed in human HepG2 cells and in C57BL/6J mice. Following carrageenan, expression of Growth Factor Receptor-Bound(GRB)10 protein, an adaptor protein that binds to the insulin receptor and inhibits insulin signaling, increased significantly. GRB10 silencing blocked the carrageenan-induced reduction of the insulin-stimulated increase in p(Tyr)IRS1 and partially reversed the decline in p(S473)AKT. The combination of GRB10 silencing with BCL10 silencing and the ROS-inhibitor Tempol completely reversed the decline in p(S473)AKT. After carrageenan, GRB10 promoter activity was enhanced, due to activation by GATA2. A direct correlation between p(S473)AKT and p(S401)GATA2 was evident, and inhibition of AKT phosphorylation by the PI3K inhibitor LY294002 blocked (S401)GATA2 phosphorylation and the increase in GRB10 expression. Studies indicated that carrageenan inhibited insulin signaling by two mechanisms: through the inflammation-mediated increase in p(S307)IRS1, a negative regulator of insulin signaling, and through a transcriptional

mechanism leading to increase in GRB10 expression and GRB10-inhibition of p(Tyr)IRS1, a positive regulator of insulin signaling. These mechanisms converge to inhibit the insulin-induced increase in p(S473)AKT. They provide an internal feedback, mediated by p(S473)AKT, p(S401)GATA2, and nuclear GATA2, which links the opposing effects of serine and tyrosine phosphorylations of IRS1 and can modulate insulin responsiveness.

Exposure to a low concentration for a short duration of the common food additive carrageenan impaired insulin signaling in HepG2 cells and in C57BL/6J mice and produced insulin resistance and glucose intolerance in the mice [1]. The mechanism of the insulin resistance was attributed to carrageenan-induced inflammation, which proceeds by activation of both innate immune- and reactive oxygen species (ROS) - mediated pathways of inflammation [2-5]. Activation of toll-like receptor (TLR)-4 and B-cell CLL/lymphoma (BCL)-10 leads to NF-κB nuclear translocations involving both canonical and non-canonical pathways. Increase of phospho-inhibitor of nuclear factor κB (IκB) kinase (IKK)-β due to stimulation of the inflammatory cascades leads to increased phosphorylation of Ser307 (in rodent; Ser312 in human)-insulin receptor substrate (IRS)1, a negative mediator of downstream insulin signaling [1,6-8]. Inhibition of the carrageenan-induced inflammation, by the combination of ROS-inhibitor Tempol and TLR4 or BCL10 siRNA, reversed the effects of carrageenan on p(S307)IRS1, as previously reported [1]. Subsequent experiments, presented in this report, have shown that the carrageenan-induced decline in the p(S473)AKT response to insulin was not completely reversed by inhibition of the carrageenan-induced inflammatory cascades. This finding suggested the presence of another mechanism by which carrageenan inhibited insulin signaling. In this report, we present this second mechanism by which carrageenan inhibits insulin signaling in human hepatic cells (HepG2) and C57BL/6J mouse liver. The findings indicate how the carrageenan-induced decline in the insulin-initiated increase in tyrosine phosphorylation of IRS1 and the increase in serine307 phosphorylation of IRS1 combine to inhibit the insulin-induced increase in p(S473)-AKT. The interaction of these mechanisms provides new insight into the regulation of responses to insulin in hepatocytes.

The second mechanism by which carrageenan exposure inhibits phosphorylation of S473-AKT involves the growth factor receptor-bound (GRB)10 protein. GRB10 is an adaptor protein with pleckstrin homology and Src homology 2 (SH2) domains and binds with receptor tyrosine kinases, including the insulin receptor [9,10]. GRB10 acts as a negative regulator of signaling from the insulin receptor [11-16]. GRB10 was identified in genome-wide studies as a candidate gene for Type 2 diabetes [17-19]. GRB10 induced Type 2 diabetes in a non-obese mouse model of diabetes [20], and was implicated in maternal imprinting and heritable effects on glucose tolerance [21-25].

Carrageenan, which is derived from red algae, is composed of sulfated and unsulfated galactose disaccharides linked in alternating alpha-1,3 and beta-1,4 bonds. Carrageenan is widely used as a food additive in processed foods to thicken and solubilize ingredients and improve texture. The amount of carrageenan consumed in the typical American diet is greater than the exposure in the experiments reported previously and in the experiments in this report [1]. The daily consumption of carrageenan in the experimental mice (50 μg / day / 25 g mouse = 2 μg / g = 2 mg / kg) was less than the

amount anticipated to be ingested daily by adults consuming the typical Western diet (~250 mg / day / 60 kg person = ~ 4.2 mg / kg).

The impact of carrageenan on innate immune-mediated inflammation arises from its unusual α-1,3-galactosidic bonds, which are immunogenic in humans and Old World apes [26-28]. Hydrolysis of these bonds by carrageenases reduced the activation of the TLR4-BCL10- mediated inflammation by carrageenan [29]. The activation of the TLR4-mediated pathway of inflammation has been associated with insulin resistance and the onset of diabetes in the non-obese diabetic mouse [30-32]. Hence, the experiments in this study are highly relevant to the emergence of insulin resistance, glucose intolerance, and clinical diabetes.

EXPERIMENTAL PROCEDURES

Cell culture and animal model-HepG2 cells (ATCC HB-8065), a human hepatic adenocarcinoma cell line, were grown under recommended conditions using minimum essential medium (MEM) with 10% FBS and were maintained at 37°C in a humidified, 5% CO_2 environment with media exchange every two days [1]. Confluent cells in T-25 flasks were harvested by EDTA-trypsin, and sub-cultured in multiwell tissue culture plates. In most of the experiments, cells were exposed to λ-carrageenan (1 mg/l) in medium with serum for 20 hours, then to λ-carrageenan (1 mg/l) in medium without serum for 4 hours. Cells were washed with serum-free medium, and fresh serum-free medium with regular human insulin (Humulin U-100; Lilly, Indianapolis, IN; 20 nmol/l) was added for 10 min. Cells were harvested by scraping and frozen at -80°C for subsequent analysis. Some cell preparations were treated with Tempol (1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine; Axxora Life Sciences, San Diego, CA, USA), a superoxide dismutase mimetic, which acts as a free radical scavenger and inhibits reactive oxygen species (ROS). HepG2 cells were treated with Tempol 100 nmol/l in combination with λ-carrageenan (1 mg/l) for 24 h, as previously described [1].

Eight-week-old male C57BL/6J mice (n=18) were purchased (Jackson Laboratories, Bar Harbor, Maine, USA) and housed in the Veterinary Medicine Unit at the Jesse Brown VA Medical Center (JBVAMC, Chicago, IL, USA). All procedures were approved by the Animal Care Committee of the University of Illinois at Chicago and the JBVAMC. Mice were fed a standard diet and maintained with routine light–dark cycles. After acclimation to the environment, the experimental animals received water with carrageenan (λ–κ carrageenan 10 mg/l; Sigma Chemical Co., St Louis, MO, USA; n=6) or without carrageenan (n=6) for nine weeks. Water consumption and weight were measured weekly. These mice were sacrificed following a 2 h fast and insulin injection (2U/kg IP) 10 minutes prior to euthanasia and compared to untreated control (n=6) [1].

Silencing of BCL10, TLR4 and GRB10 by siRNA- Small interfering (si) RNAs for TLR4, BCL10 and GRB10 were obtained commercially (Qiagen, Valencia, CA, USA), and used as described previously [1]. The effectiveness of silencing in the HepG2 cells was demonstrated by Western blotting for TLR4 and by quantitative ELISAs for BCL10 [1, 33] and for GRB10 [Blue Gene, Shanghai, China]. Briefly, cells were grown to 60–70% confluency in 6-well tissue culture clusters, and medium of the growing cells was aspirated and replaced with 2.3 ml of fresh medium with serum. 0.6 µl of 20 µM siRNA (150 ng) was mixed with 100 µl of serum-free medium and 12 µl of HiPerfect Transfection Reagent (Qiagen). The mixture was incubated for 10 min at room temperature to allow the formation of transfection complexes, and then added dropwise onto the cells. The

microplates were swirled gently, and treated cells were incubated at 37°C in a humidified, 5% CO_2 environment. After 24 h, the spent medium was exchanged with fresh growth medium.

Inhibition of AKT Ser473 phosphorylation by LY294002-Phosphatidylinositol 3-kinase (PI3K) is required for phosphorylation of AKT Ser473, thereby enabling AKT activation and downstream effects. LY294002 (50 μM x 24h, Calbiochem, Billerica, MA), an inhibitor of PI3K and mTOR, was used to suppress AKT Ser473 phosphorylation and downstream signaling [34,35].

Western blots for GRB10, p(S401)-GATA2, total GATA2, p(Tyr)IRS1, and IRS-1-Insulin (2U/kg body weight) was administered IP to the C57BL/6J mice at 18 weeks of age, following ingestion of carrageenan for 9 weeks, and a two hour fast. Insulin was injected ten minutes prior to euthanasia by CO_2 inhalation and exsanguination by cardiac puncture, and the liver was immediately harvested and frozen at -80°C. Tissue homogenates were prepared in cell lysis buffer (Cell Signaling, Danvers, MA, USA) with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, Pittsburgh, PA, USA) from carrageenan-exposed and control hepatic tissue of mice that received ante-mortem insulin, and control, as above. Western blots were performed on 10% SDS gels with commercial antibodies to GRB10 [Santa Cruz Biotechnology (SCBT), Santa Cruz, CA], p(S401)GATA2 (Abcam, Cambridge, MA), total GATA (Abcam), p(Tyr)IRS1 (Cell Signaling, Danvers, MA), IRS1 (Cell Signaling), and β-actin (SCBT) to probe for the proteins of interest using established procedures [1]. HepG2 cells exposed to carrageenan and insulin and untreated control cells were also analyzed by Western blots using similar procedures. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham, GE Healthcare, Piscataway, NJ, USA), and Image J software (NIH, Bethesda, MD, USA) was used for densitometry. Density of the protein of interest was compared with β-actin, or IRS-1, from the same specimen, and the densities of treated and control samples were compared.

ELISAs for p(Tyr)IRS1, p(S307)IRS1, p(Tyr)-IR, GRB10, and p(S473)AKT - P(Tyr)IRS1 and p(S307)IRS1 [in rodent; p(S312)IRS1 in human] were determined by commercial sandwich ELISAs (Cell Signaling, Danvers, MA) in mouse liver homogenates and HepG2 cells of untreated controls or following exposure to carrageenan and insulin. In the HepG2 cells, GRB10, BCL10, or TLR4 mRNA were silenced by specific siRNAs prior to carrageenan and insulin treatments, as above. IRS1 in the tissue or cell samples was captured by IRS1 antibody. Phospho-tyrosine or phospho(Ser307) antibodies were used to detect tyrosine or serine phosphorylation of the captured IRS1 protein. Anti-mouse IgG, HRP-linked antibody was then used to detect the bound second antibody. The bound peroxidase activity was determined by adding hydrogen peroxide-TMB substrate, the reaction was stopped by 2(N) H_2SO_4, and the absorbance was measured at 450 nm in a plate reader (FLUOstar, BMG Labtech, Cary, NC). Insulin receptor pan-tyrosine phosphorylation was determined in HepG2 cells post-carrageenan treatment by a commercial phospho(Tyr)IR ELISA (Cell Signaling).

GRB10 in the cell and mouse samples was measured by commercial ELISA (Blue Gene), following the recommended procedures, and the GRB10 concentration in each sample was interpolated from the standard curve.

P(S473)AKT was measured by a cell-based ELISA, as previously (Active Motif, Carlsbad, CA) [1]. Measurements for treated cells were compared with values per mg

protein for the unexposed control cells and expressed as percent of the untreated control.

QPCR for mRNA expression of Grb10 in mouse tissues - C57BL/6J mice were euthanized following IP injection of regular insulin (2U/kg) and exposure to carrageenan in the water supply, as detailed above. Tissues were promptly harvested and immediately frozen. Subsequently, tissue was processed for QPCR. Primers for mouse Grb10 were selected using Primer3 [36] and were: (left) 5´-AGT GTA GCA GAC TTC AGT GGC-3´ and (right) 5´-TCC AAA ACA ACC CTG AGC TGT-3´. QPCR was performed as previously reported [3] and cycle thresholds were calculated and compared with values for β-actin.

GRB10 promoter activity by luciferase reporter assay-Human GRB10 promoter construct in a *Renilla reniformis* luciferase reporter gene (Ren SP) vector was used to detect differences in GRB10 promoter activity following carrageenan treatment of HepG2 cells. Human GRB10 promoter, β-actin promoter construct (positive control), and scrambled sequence R01 (negative control) in *Renilla* luciferase reporters were obtained (LightSwitch Luciferase Assay, SwitchGear Genomics, Menlo Park, CA, USA), and used to determine the effectiveness of transfection and the specificity of the reactions [37]. Transfections were performed with cells at 70% confluence, using FuGENE HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Following transfection, cells were treated with carrageenan (1 mg/l) for 24 hours. Luminescence was measured after incubation at 480 nm in a microplate reader (BMG), and reported as relative luminescence units (RLU).

Nuclear GATA2 determination- GATA2 was identified as a transcription factor which has a binding site in the GRB10 promoter and might regulate GRB10 transcription [38,39]. GATA was measured in the nuclear extract of HepG2 cells treated with carrageenan and/or insulin. Nuclear extracts were prepared (Active Motif), and the nuclear GATA protein was determined in the samples using the TF Filter Plate Assay (Signosis Inc., Santa Clara, CA). A specific biotin-labeled GATA DNA binding sequence was mixed with the nuclear extracts and GATA protein-DNA complexes formed. A filter plate was used to retain the DNA probe with GATA and remove free probe. The bound pre-labeled DNA probe was eluted from the filter and collected for quantitative analysis of nuclear GATA following detection with streptavidin-HRP. Luminescence was reported as RLU.

Chromatin immunoprecipitation (ChIP) assay- For ChIP assay, control, insulin-, carrageenan-, and insulin with carrageenan-treated HepG2 cells and IgG control were fixed with 1% formaldehyde for 10 min at room temperature, followed by shearing of chromatin by sonication (ChIP Assay, Active Motif, Carlsbad, CA, USA) [37]. Sheared DNA was incubated with anti-GATA2 antibody (Abcam) for 1h, as well as with IgG control. Protein–DNA complexes were precipitated by protein A/G-coupled magnetic beads. DNA was purified from the immunoprecipitated complexes by reversal of cross-linking, followed by proteinase K treatment. Then, real-time RT–PCR was performed using Brilliant SYBR Green QRT-PCR master mix (Stratagene, La Jolla, CA, USA) and Mx3000 (Stratagene) to amplify the GRB10 promoter oligonucleotide that encompassed the GATA2 binding site. The primers for the GRB10 promoter were: (left) 5′-CGC CGT CAT TGT CTG AGC-3′ and (right) 5′-AAG ATG GTT CAA GAA TGC ACC-3′. Input DNA of each sample was calculated from the standard curve and expressed as % of total DNA. PCR products were run on a 2.0% agarose gel, and band intensity was compared among the different preparations.

Statistical analysis- Data were analyzed using InStat3 software (GraphPad, La Jolla, CA, USA) and are presented as mean value ± standard deviation (S.D.) of at least three independent biological samples with technical replicates of each determination. The differences between carrageenan-treated and control results were compared by one-way ANOVA with the Tukey–Kramer post-test for multiple comparisons, unless stated otherwise. P-value $\leq$0.05 is represented by *; p$\leq$0.01 by ** and p$\leq$0.001 by ***.

RESULTS

Carrageenan inhibited the insulin-induced tyrosine phosphorylation of IRS1- The insulin-stimulated increase in phospho(Tyr)-IRS1 levels in cultured HepG2 cells (**Fig. 1A**) and in C57BL/6J mouse hepatic tissue *in vivo* (**Fig. 1B**) was detected by ELISA. Carrageenan exposure (1 mg/l x 24 h) alone had no effect on p(Tyr)-IRS1, but carrageenan exposure prior to insulin treatment markedly reduced the insulin-stimulated tyrosine phosphorylation of IRS1 (p<0.001). Representative Western blot also demonstrated the impact of insulin, carrageenan, and insulin and carrageenan in combination on p(Tyr)-IRS1, without change in total IRS1, in the HepG2 cells (**Fig. 1C**). These data indicate that carrageenan inhibits insulin signaling by reducing p(Tyr)-IRS1, which is a positive mediator of insulin signal transduction.

Carrageenan induced the expression of GRB10 in HepG2 cells and C57BL/6J hepatic tissue -GRB10 protein was upregulated following exposure to carrageenan, as shown by Western blots and densitometry in HepG2 cells (**Fig. 2A, 2B**) and in C57BL/6J mouse hepatic tissue (**Fig. 2C, 2D**). Quantitative determination of GRB10 in the HepG2 cells by ELISA also demonstrated increase in GRB10 protein from the control level of 1.20 ± 0.02 ng/mg protein to 2.17 ± 0.09 ng/mg protein (p<0.0001, unpaired t-test, two-tailed) (**Fig. 2E**). QPCR demonstrated increases in Grb10 mRNA expression in hepatic, adipose, and muscle tissue of the mice following exposure to carrageenan and insulin (**Fig. 2F**). Since GRB10 is a known negative regulator of insulin signaling and p(Tyr)-IRS1, these data suggested that the carrageenan-induced decline in p(Tyr)-IRS1 is attributable to increased GRB10 expression.

GRB10 siRNA blocked the carrageenan-induced reduction of the insulin-induced increase in p(Tyr)IRS1- GRB10 was effectively silenced by siRNA in HepG2 cells, the cells were treated with carrageenan, and the GRB10 was quantified by ELISA. GRB10 levels were reduced by >85% by two of the GRB10 siRNAs tested (**Fig. 3A**). The carrageenan-induced reduction of the insulin-stimulated increase in p(Tyr)IRS1 was reversed by GRB10 siRNA (**Fig. 3B**).

The effects of carrageenan on inflammation were inhibited by silencing BCL10 and by Tempol [1-5], but BCL10 in combination with Tempol had no impact on the carragenan-induced reduction of the insulin-induced increase in p(Tyr)IRS1 (**Fig. 3B**). In contrast, BCL10 siRNA and Tempol completely inhibited the carrageenan-induced increase in p(S307)IRS1, whereas GRB10 siRNA had no effect (**Fig. 3C**). These findings are consistent with a distinct effect of carrageenan-induced inflammation increasing p(S307)IRS1, and a second mechanism affecting p(Tyr)IRS1.

The insulin-induced increase in tyrosine phosphorylation of the insulin receptor (IR) was not inhibited by either carrageenan or GRB10 silencing (**Fig. 3D**). These results indicated that the impact of GRB10 was downstream of the IR.

GRB10 siRNA partially inhibited the carrageenan-induced decline in the insulin-stimulated increase in p(S473)AKT- When

HepG2 cells in culture were treated with control siRNA, BCL10 siRNA, TLR4 siRNA, or Tempol, or combinations of BCL10 siRNA or TLR4 siRNA and Tempol to suppress the carrageenan-induced inflammation, there was partial reversal of the impact of carrageenan on the insulin-induced increases in p(S473)AKT (**Fig. 4A**). The combination of GRB10 siRNA with BCL10 siRNA and Tempol completely reversed the carrageenan-induced inhibition (**Fig. 4B**). These findings revealed that both carrageenan-induced inflammatory effects and effects on GRB10 expression were required for the complete inhibition of p(S473)-AKT following insulin.

Carrageenan stimulated GRB10 promoter activity through GATA2-Treatment of HepG2 cells with carrageenan (1 mg/l) for 24 hours increased the GRB10 promoter activity significantly ($p<0.001$), as shown by increase in the *Renilla reniformis* luciferase reporter signal to more than two times the baseline level (**Fig. 5A**). A GATA2 consensus sequence (ACAAT) was identified in the GRB10 promoter [38,39]. The impact of carrageenan treatment on binding of nuclear GATA2 protein to the GRB10 promoter DNA that encompassed this site was determined in nuclear extracts of HepG2 cells and in C57BL/6J hepatic tissue by oligonucleotide binding assays. GATA2 binding increased to 3.41 ± 0.22 times the basal level ($p<0.0001$; unpaired t-test, two-tailed; n=6) in the HepG2 cells, and to 2.94 ± 0.15 times the control value in the mouse liver ($p<0.0001$; unpaired t-test, two-tailed; n=6) (**Fig. 5B**) following carrageenan and insulin exposure, compared to insulin alone.

Adherence of sheared DNA from HepG2 nuclear extracts to GATA2 was determined by chromatin immunoprecipitation assay (ChIP). Following carrageenan, the amount of GATA2-bound DNA was significantly increased compared to control ($p<0.001$). Insulin significantly reduced the amount of GATA2-bound DNA detected ($p<0.001$) (**Fig. 5C**). Agarose gel confirmed the increase in GATA2-bound DNA following carrageenan (**Fig. 5D**). The intensity of the bands from the carrageenan-treated and carrageenan with insulin-treated cells was much greater than from the controls or the insulin-only treated cells. These data demonstrated that carrageenan exposure increased the GRB10 promoter activation through a GATA2 binding site.

Carrageenan inhibited the insulin-induced phosphorylations of AKT and GATA2- Phospho(S307)-AKT was reported to phosphorylate GATA2 [40], leading to consideration of the association between p(S473)AKT, p(Ser401) GATA2, and GATA2 in the HepG2 cells. Fast-activated cell-based ELISAs were performed for both p(S473)AKT and p(S401)GATA2 and demonstrated more than 400% increase in S473 phosphorylation of AKT and more than 160% increase in S401 phosphorylation of GATA2 post-insulin stimulation ($p<0.001$) (**Fig. 6A**). Carrageenan treatment completely inhibited the responses to insulin, but carrageenan alone had no effect on the phosphorylations. The percentages of p(S473)AKT and p(S401)GATA2 present under different conditions (control, insulin, carrageenan, insulin and carrageenan) showed a strong positive correlation ($r = 0.97$).

Western blots of p(S401)GATA2 and GATA2 demonstrated that insulin treatment induced the phosphorylation of GATA2, and prior carrageenan treatment completely inhibited the stimulatory effect of insulin on p(S401)GATA2 (**Fig. 6B**). The immunoreactive band for p(S401)GATA2 was much stronger than control after insulin exposure, and carrageenan treatment, either alone or in combination with insulin, reduced the band intensity. Neither insulin nor carrageenan alone or in combination with insulin modified the intensity of the cellular

GATA2 band. These results indicated a direct relationship between p(Ser473)-AKT and p(S401)GATA2.

Inhibition of AKT phosphorylation increased GRB10 expression- LY294002, an inhibitor of PI3K and mTOR, completely blocked phosphorylation of (S473)AKT (**Fig. 7A**) and (S401)GATA2 in the HepG2 cells (**Fig. 7B**) ($p<0.001$). Carrageenan again blocked the insulin-induced increases in p(S473)AKT and p(S401) GATA2, but to a lesser extent than LY294002. Consistent with the demonstrated impact of GATA2 on GRB10 expression, LY294002 increased the mRNA (**Fig. 7C**) and protein expression (**Fig. 7D**) of GRB10 in the HepG2 cells. These findings further support an inverse association between p(S473)AKT and GRB10 expression, mediated through p(S401)GATA2 and GATA2.

DISCUSSION

This study demonstrates the upregulation of the adaptor protein GRB10 when HepG2 cells and C57BL/6J mice are exposed to the food additive carrageenan. The increase in GRB10 led to declines in the insulin-stimulated increases in p(Tyr)IRS1 and p(S473)AKT. The effects on GRB10 and on p(Tyr)IRS1 were independent of carrageenan-initiated inflammation. Blockade of the inflammatory effects of carrageenan by TLR4 siRNA, BCL10 siRNA, and Tempol did not completely inhibit the carrageenan-mediated inhibition of insulin-stimulated p(S473)AKT or inhibit the carrageenan-induced reduction in the insulin-induced increase in p(Tyr)IRS1. The inflammatory responses stimulated by carrageenan treatment interfered with insulin signaling by increasing p(S307)IRS1, which inhibits the propagation of insulin signaling [1], but did not affect the carrageenan-induced decline in the insulin-induced increase in p(Tyr)IRS1, which propagates insulin signaling.

The carrageenan-induced decline in p(Tyr)IRS1 is a second mechanism by which carrageenan contributes to insulin resistance. Carrageenan-initiated inflammatory pathways and GRB10 upregulation both impede insulin signaling upstream of AKT phosphorylation and inhibit the insulin-induced S473 phosphorylation of AKT. These pathways and their crosstalk are presented in **Figure 8.** Overall, studies demonstrate how inflammation-induced increase in p(S307)IRS1 leads to decline in p(S473)AKT, and how decline in p(S473)AKT can lead to increase in GRB10 expression and decline in p(Tyr)IRS1. Thus, insulin resistance following carrageenan is mediated through inverse effects on p(S307) (increase)- and p(Tyr) (decrease) -IRS1. The effect on p(Tyr)-IRS1 is enabled by the transcriptional response of GRB10 to changes in p(S473)AKT which are transmitted through p(S401)GATA2 and GATA2.

These insulin signaling pathways provide critical links between carrageenan-induced inflammation, which leads to interaction of p-IKKβ with p(S307)IRS1, and subsequent phosphorylations of (S473)AKT and (S401)GATA2. These reactions lead to increased nuclear GATA2 and transcriptional activation of GRB10, which feeds back to inhibit p(Tyr)IRS1. These interactions provide mechanistic links between increase in p(S307)IRS1 and decline in p(Tyr)IRS1, and help to clarify the relationship between p(S307)IRS1 and p(Tyr)IRS1 [41]. Phosphorylations of IRS1 and their impact on the regulation of insulin sensitivity and propagation of insulin signaling have been considered in detail [41-44]. In this report, we have presented a transcriptional mechanism whereby carrageenan exposure leads to increased mRNA expression of GRB10 by GATA2 activation of the GRB10 promoter, with attention to S307 of IRS1. Multiple sites of serine/threonine phosphorylation of IRS1

have been identified [41], and might also participate in the cascade of reactions described in this report, but have not yet been evaluated.

Insulin was previously reported to induce GATA2 S401 phosphorylation by a phosphoinositide 3-kinase (PI3K)/AKT-dependent mechanism in cultured preadipocytes [40]. The interaction between p(S473)AKT and p(S401)GATA2 links the propagation of insulin signaling with a transcriptional mechanism.

Two distinct mechanisms of insulin resistance follow carrageenan exposure: 1) the previously reported inflammatory-derived signaling mechanism, whereby the p(S473)AKT response to insulin is reduced by carrageenan-induced inflammation leading to increased p(S307)IRS1 which inhibits downstream signaling [1]; and 2) a transcriptional mechanism initiated by decline in p(Ser307)AKT, with associated decline in p(S401)GATA2 and increase in nuclear GATA, leading to increased GRB10 expression and to GRB10 inhibition of p(Tyr)IRS1 and p(S473)AKT. The synergy between these mechanisms may lead to sustained insulin resistance following inflammation, as well as fine-tuning of responses to insulin in response to other exogenous stimuli.

The molecular mechanism by which carrageenan increased the mRNA expression of GRB10 expression was attributable to activation of the GRB10 promoter by increased nuclear GATA2. Increase in p-(S401)GATA2 was highly correlated ($r = 0.97$) with increase in p(S473)AKT, without change in total GATA2. The transcription factor GATA2 has been reported to be activated and to translocate to the nucleus when dephosphorylated and to be degraded in the proteasome when phosphorylated [45]. By increasing the phosphorylation of GATA2, insulin-induced increase in p(S473)AKT reduced the GATA2 that could translocate to the nucleus and bind to the GRB10 promoter and thereby stimulate GRB10 expression. In contrast, carrageenan-induced inflammation inhibited the insulin-induced p(S473)AKT and p(S401)GATA2, enabling more unphosphorylated GATA2 to translocate to the nucleus and bind with the GRB10 promoter and stimulate GRB10 expression. Insulin alone reduced the binding of GATA2 to the GRB10 promoter, as shown by ChIP assay, consistent with insulin exposure leading to increases in p(S473)AKT and p(S401)GATA2. The increase in GRB10 can be sustained through a signaling loop, whereby reduction in p(S473)AKT, initiated by inflammation, induces a second mechanism of insulin resistance mediated by increase in GRB10. Increase in GRB10 can then lead to further or persistent decline in p(S473)AKT. The interaction between carrageenan-induced inflammation and enhanced GRB10 expression through p(S473)AKT indicates how two mechanisms that cause insulin resistance can act together. By causing inflammation, carrageenan, or other activators of inflammation, initiate a cascade by which insulin resistance can become sustained through the ongoing impact of GRB10 on p(S473)AKT. Since carrageenan is contained in so many food products, the consumption of carrageenan may contribute to the prevalence of insulin resistance and glucose intolerance associated with the Western diet. A recommendation to avoid ingestion of carrageenan-containing food products should be considered as part of routine nutritional counseling in patients with insulin resistance. Also, other sources of inflammation, such as infection, may also activate both the inflammatory and the GRB10 transcriptional mechanisms of insulin resistance and thereby lead to impaired insulin sensitivity and to glucose intolerance.

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FIGURE LEGENDS

Figure 1. Carrageenan inhibits the insulin-induced increase in p(Tyr)-IRS1.
A. Cultured HepG2 cells were treated with carrageenan (1 mg/l x 24 h, including 4 hr in serum-free media) and insulin (20 nmol/l x 10 min). P(Tyr)IRS1 was measured by ELISA. Insulin increased the tyrosine phosphorylation of IRS1 to more than five times the baseline, and carrageenan significantly inhibited the stimulatory effect of insulin (*** for $p<0.001$; n=3).
B. Hepatic p(Tyr)IRS1 was determined by ELISA in carrageenan-exposed mice (10 mg/l in drinking water x 9 weeks) that received ante-mortem insulin (2U/kg IP x 10 minutes). Values increased to 515 ± 56% the baseline value of the control animals. Carrageenan exposure reduced this increase to 315 ± 20% of the baseline value (*** for $p<0.001$; n=6).
C. Representative Western blot of p(Tyr)IRS1 in the HepG2 cells shows decline in the band intensity following carrageenan. Total IRS1 was unchanged.
[CGN=carrageenan; IRS=insulin receptor substrate]

Figure 2. GRB10 expression increases following carrageenan exposure in HepG2 cells and hepatic tissue.
A. Representative Western blot demonstrates increased density of GRB10 protein in HepG2 cells following exposure to carrageenan.
B. Densitomeric analysis indicated statistically significant increase in GRB10 protein in the HepG2 cells following carrageenan (100 ± 6.0 % vs. 152.2 ± 8.8 %; p=0.0011, unpaired t-test, two-tailed; n=3).
C. Representative Western blot shows increased density of the Grb10 band in liver tissue of C57BL/6J mice exposed to carrageenan and ante-mortem insulin.
D. Densitometric analysis confirmed the increased density of the predominant Grb10 band following carrageenan exposure (p=0.04, unpaired t-test, two-tailed; n=9).

E. GRB10 protein in control and carrageenan-treated HepG2 cells was quantified by ELISA. Carrageenan increased the GRB10 concentration to 2.17 ± 0.09 ng/mg protein from the baseline level of 1.20 ± 0.02 ng/mg protein ($p<0.0001$; unpaired t-test, two-tailed, n=3).
F. Grb10 mRNA expression was significantly increased in the hepatic, gastrocnemius, and adipose tissue of the C57BL/6J mice following exposure to carrageenan and insulin (*** for $p<0.001$; n=3).
[CGN=carrageenan; GRB=growth factor receptor-bound protein]

Figure 3. Impact of silencing GRB10 on p(Tyr)IRS1, p(S307)IRS1, and phospho(Tyr)-IR.
A. HepG2 cells in culture were transfected with control siRNA and GRB10 siRNAs. Twenty-four hours after transfection, cells were treated with 1 mg/l of carrageenan for another 24 h. Cells were harvested, and GRB10 in the cell extracts was measured by ELISA. GRB10 siRNA reduced the GRB10 protein post-carrageenan by >85% (*** for $p<0.001$, n=3).
B. HepG2 cells in culture were treated with carrageenan (1 mg/l x 24 h) after silencing GRB10. Other cells were treated with BCL10 siRNA and Tempol 100 nM x 24 h to inhibit the carrageenan-initiated inflammatory cascades. The insulin-induced increase in p(Tyr)IRS1 was inhibited by GRB10 siRNA (*** for $p<0.001$; n=3), but not by the combination of Tempol and BCL10 siRNA.
C. In contrast, GRB10 siRNA had no impact on the carrageenan-induced increase in p(S307)IRS1 in HepG2 cells. BCL10 siRNA and Tempol did significantly inhibit the carrageenan-induced increase in p(S307)IRS1, as previously reported [1].
D. Neither carrageenan nor GRB10 siRNA had any effect on the insulin-induced increase in tyrosine phosphorylation of the IR.
[BCL=B-cell CLL/ymphoma; GRB=growth factor receptor-bound protein; IR=insulin receptor; IRS=insulin receptor substrate; N.D.=no difference]

Figure 4. GRB10 siRNA partially reverses the effect of carrageenan on the insulin-induced increase in p(S473)AKT.
A. HepG2 cells in culture were treated with control siRNA, BCL10 siRNA, TLR4 siRNA, or Tempol, or combinations to suppress the carrageenan-induced inflammatory cascades. Cell-based ELISA demonstrated partial reversal of the impact of carrageenan on the insulin-induced increases in p(S473)AKT (*** for $p<0.001$, n=3).
B. The combination of GRB10 siRNA and inhibitors of the inflammatory effects of carrageenan completely reversed the carrageenan-induced inhibition of the insulin-induced increase in p(S473)AKT (*** for $p<0.001$, n=3).
[AKT=protein kinase B; BCL=B-cell CLL/ymphoma; CGN=carrageenan; GRB=growth factor receptor-bound protein; N.D.=no difference; TLR=toll-like receptor]

Figure 5. Carrageenan treatment increases GRB10 promoter activity in HepG2 cells.
A. Treatment of HepG2 cells with carrageenan after transfection of the cells with the GRB10 promoter in a *Renilla reniformis* luciferase reporter demonstrated an increase to more than twice the baseline in the luciferase signal (*** for $p<0.001$, n=6).
B. Oligonucleotide binding assay showed that the binding of nuclear GATA protein to the GRB10 promoter oligonucleotide which encompasses the GATA2 binding site increased to 341 ± 21.5% and 294 ± 15.3% of the baseline levels in carrageenan-treated HepG2 cells and mouse hepatic tissue, respectively (*** for $p<0.0001$; unpaired t-test, two-tailed; n=6).
C. Chromatin Immunoprecipitation (ChIP) assay was performed with specific GATA2 antibody following carrageenan (1 mg/l x 24 hr; with 4 hr in serum-free media) or insulin (20 nmol/l x 10

minutes) or combined carrageenan and insulin in the HepG2 cells. Carrageenan either alone or in combination with insulin increased the GATA2-bound DNA. DNA recovery increased from baseline control value of 3.82 ± 0.42% to 6.90 ± 0.73% after carrageenan exposure (*** for $p<0.001$; n=6) and declined following insulin exposure to 2.00 ± 0.08 % (*** for $p<0.001$, n=6).
D. Agarose gel electrophoresis of the PCR product demonstrates increased band density following carrageenan either with or without insulin exposure.
[CGN = carrageenan; con=control; GRB = growth factor receptor-bound protein; Ins=insulin]

Figure 6. Insulin-induced increases in AKT and GATA2 phosphorylations are inhibited by carrageenan.

A. P(S473)AKT and of p(S401)GATA2 were detected by FACE assay after the HepG2 cells were treated with insulin (20 nmol/l x 10 minutes), carrageenan (1mg/l x 24 h, with 4 h in serum-free media), and the combination of insulin and carrageenan. Carrageenan significantly inhibited the insulin-induced phosphorylations of AKT and GATA2 (*** for $p<0.001$, n=6).
B. Insulin induced and carrageenan inhibited phosphorylation of GATA2 in HepG2 cells, with no impact on total GATA2, as shown by representative Western blot. The insulin-induced increase in p(S401)GATA2 was inhibited by carrageenan exposure.
[AKT = protein kinase B; CGN = carrageenan; FACE=fast-activated cell-based ELISA]

Figure 7. Inhibitor of PI3K-induced AKT S473 phosphorylation increases GRB10 expression.

A. P(S473)AKT was reduced in the HepG2 cells following exposure to LY294002 (50 μM x 24 h), an inhibitor of PI3K-mediated phosphorylation of AKT (*** for $p<0.001$; n=3).
B. Decline in S401 GATA2 phosphorylation was also evident following exposure to LY294002 (*** for $p<0.001$; n=3). This is consistent with the observed positive correlation between p(S401)GATA2 and p(S473)AKT.
C. GRB10 mRNA expression, detected by QPCR, increased significantly following inhibition of AKT phosphorylation by LY294002 (*** for $p<0.001$; n=6).
D. GRB10 protein, determined by ELISA, increased significantly following treatment with LY294002 (*** for $p<0.001$, n=3).
[CGN=carrageenan; LY=LY294002]

Figure 8. Schematic illustration of effects of carrageenan on insulin signaling, mediated through GRB10 and inflammation.

When inflammation is present, phospho-IKKβ leads to phosphorylation of Ser307 of IRS1, an inhibitor of insulin signaling. This leads to decline in p(S473)AKT, with associated decline in p(S401)GATA2, increase in nuclear GATA2, and increase in mRNA expression of GRB10. The increase in GRB10 affects the downstream insulin signaling due to binding of GRB10 with the IR, leading to inhibition of tyrosine phosphorylation of IRS-1. Hence, feedback is initiated that leads to upstream inhibition of propagation of insulin signals and further insulin resistance when p(S473)AKT is reduced by inflammation.
[AKT=protein kinase B; BCL=B-cell CLL/lymphoma; CGN=carrageenan; GRB=growth factor receptor-bound protein; Hsp=heat-shock protein; IKK=IκB kinase; IκB=inhibitor of kappaB; IL-8=interleukin-8; IR=insulin receptor; IRS=insulin receptor substrate; NFκB=nuclear factor kappa B; ROS=reactive oxygen species; TLR=toll-like receptor; ub=ubiquitin]

Figure 1. Carrageenan inhibits the insulin-induced increase in phospho(Tyr)-IRS1.

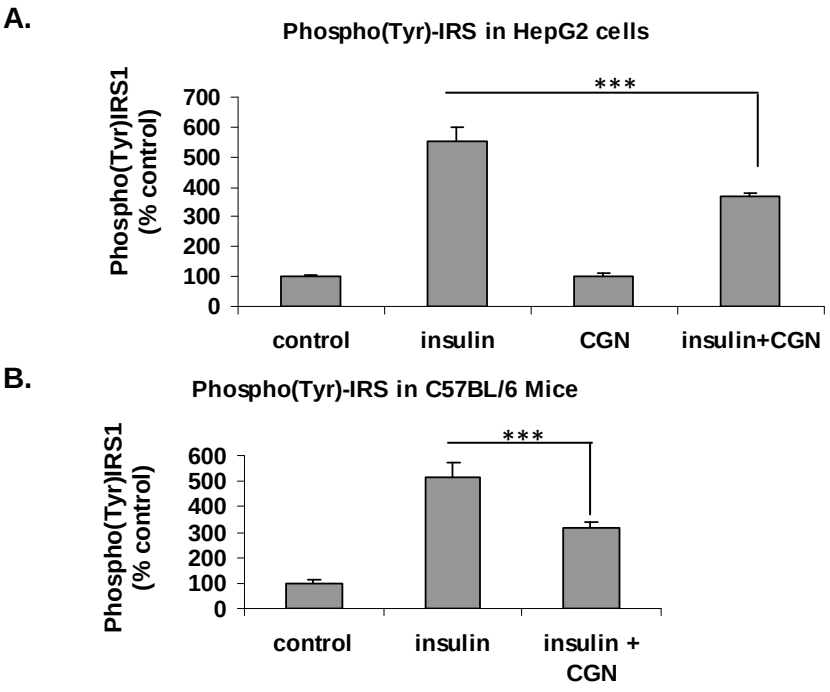
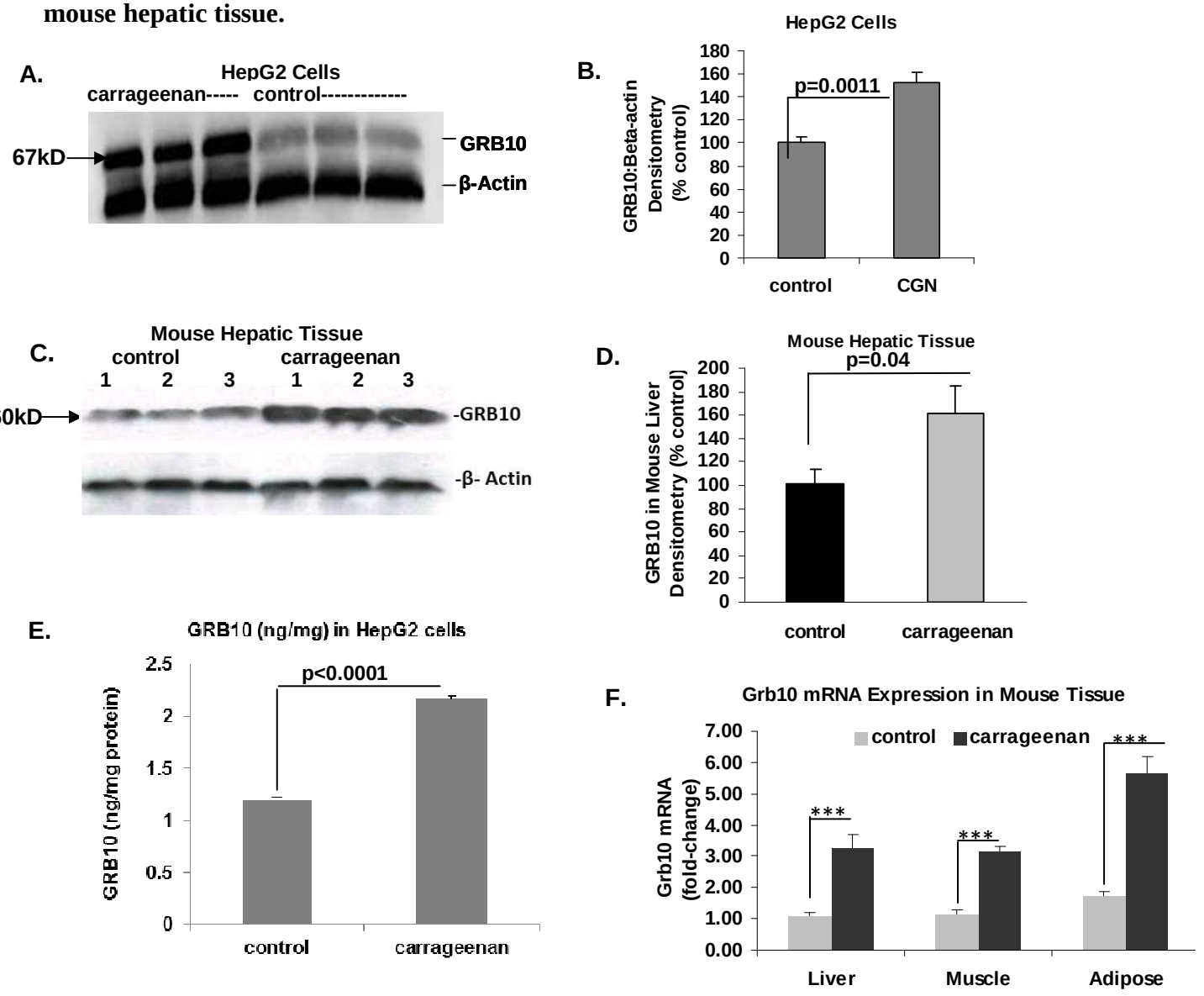


Figure 2. GRB10 expression increases following carrageenan exposure in HepG2 cells and mouse hepatic tissue.



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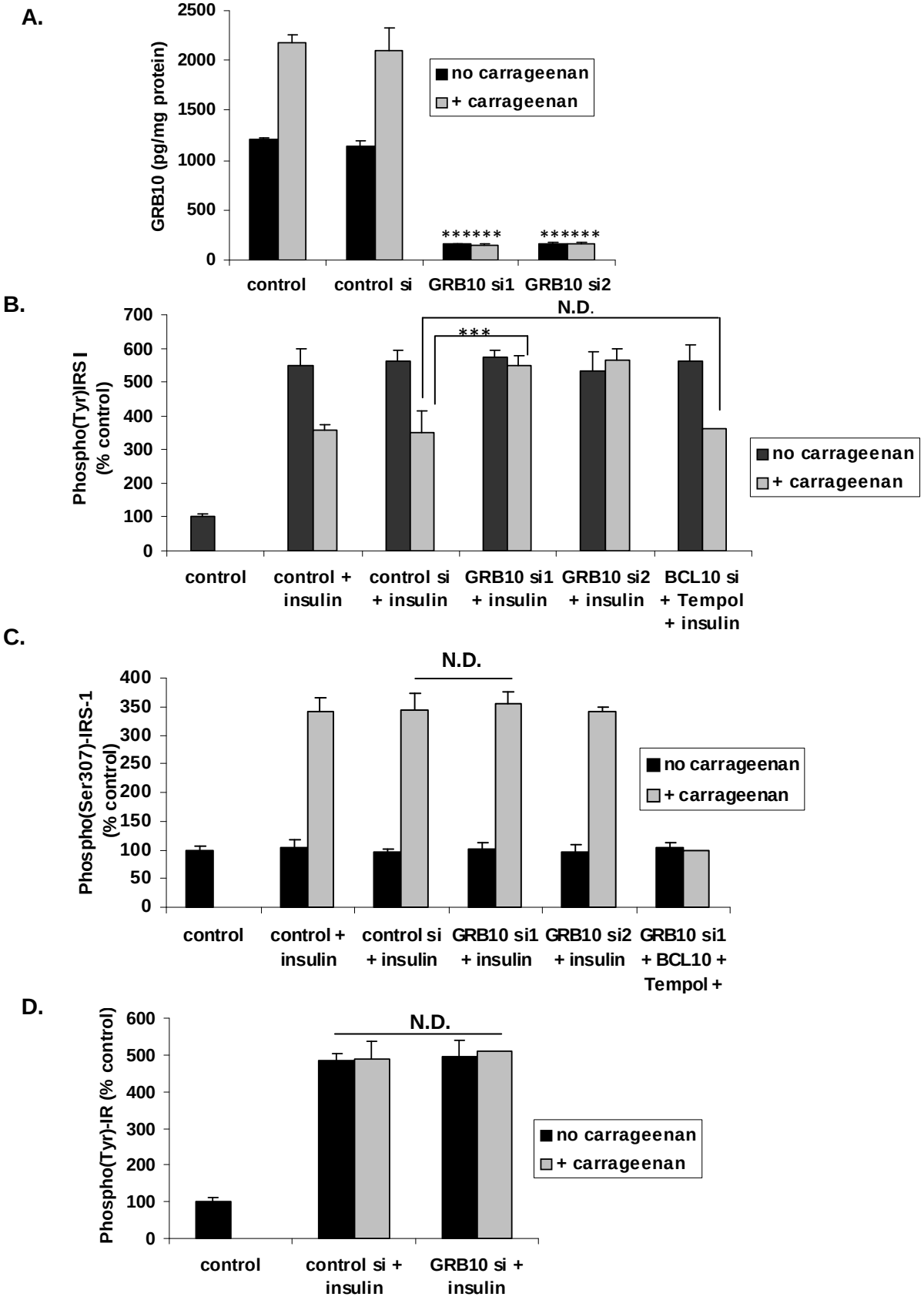

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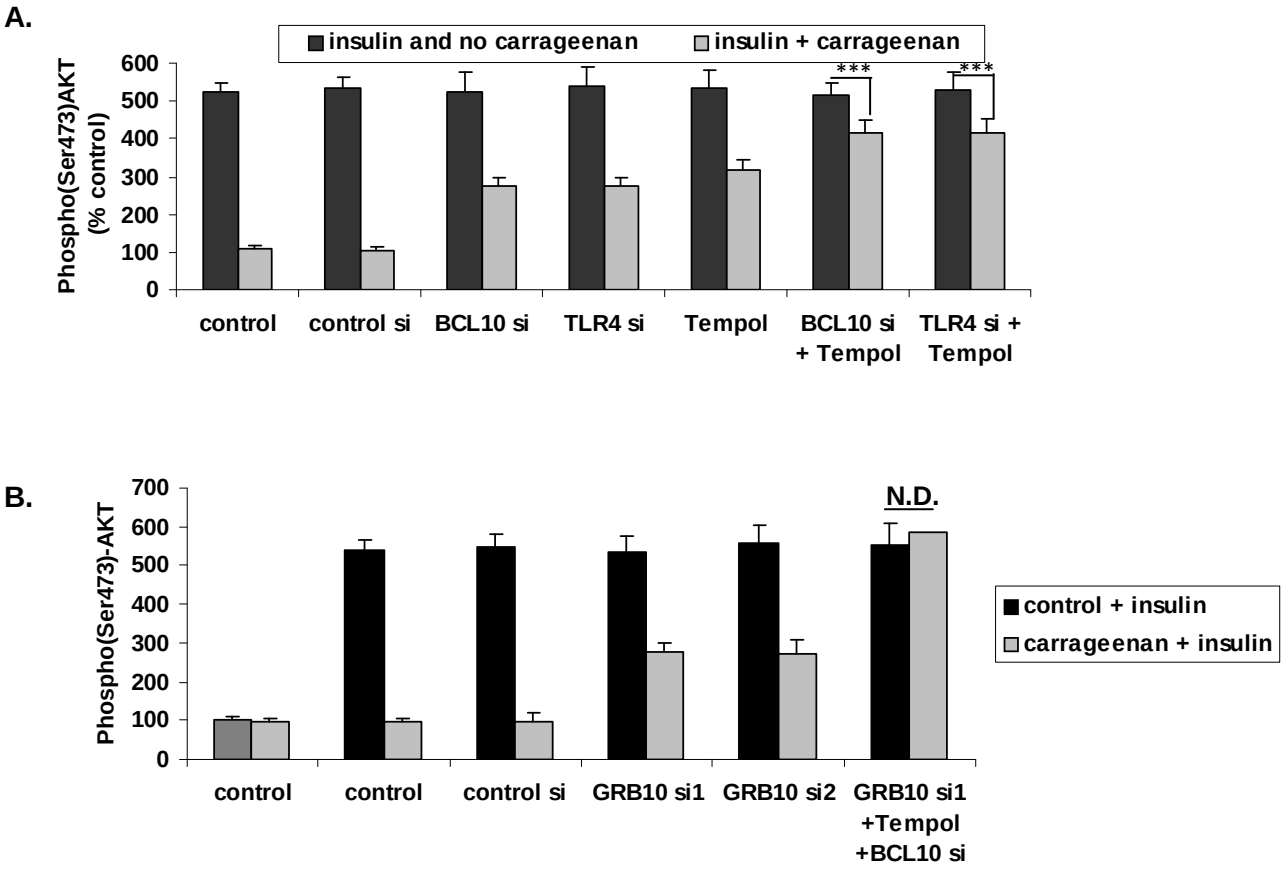

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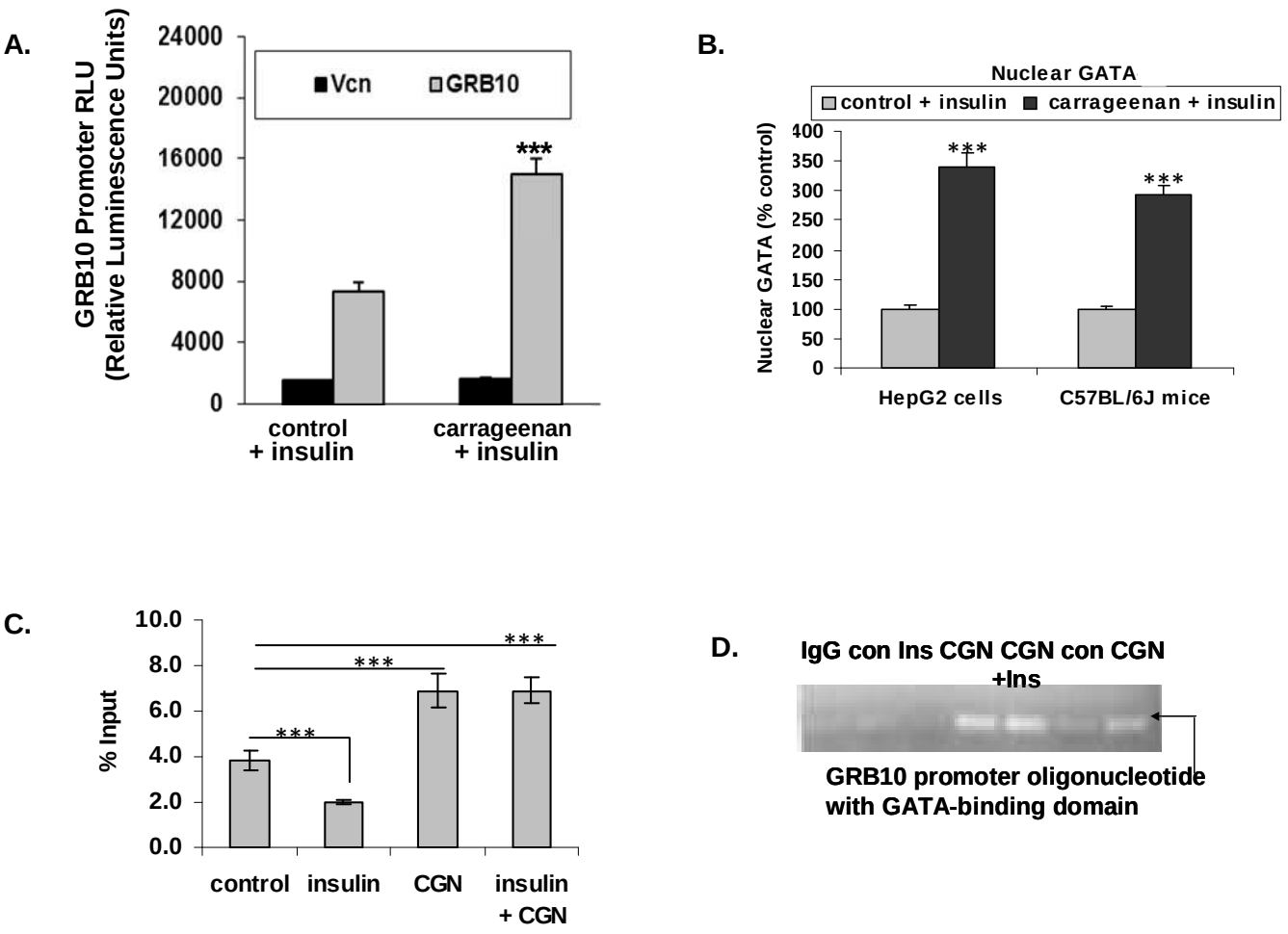

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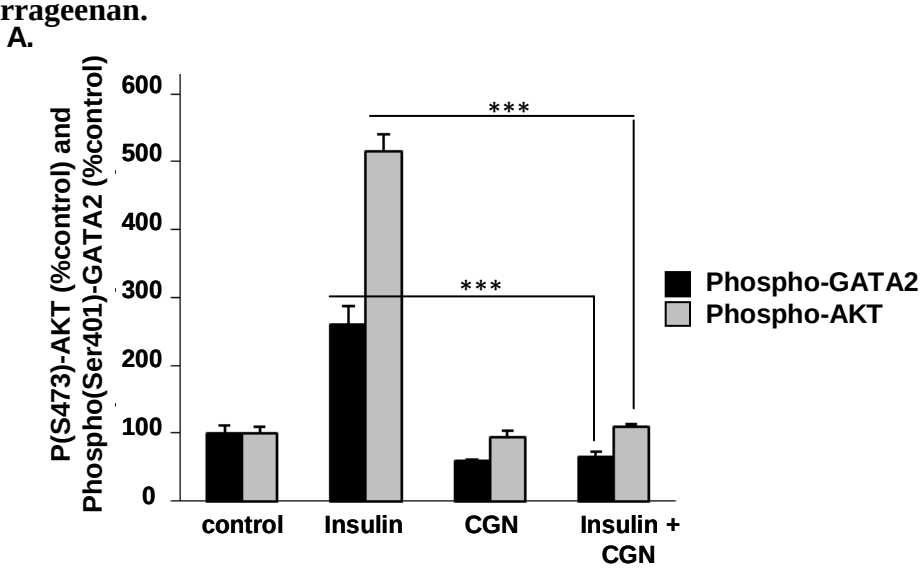


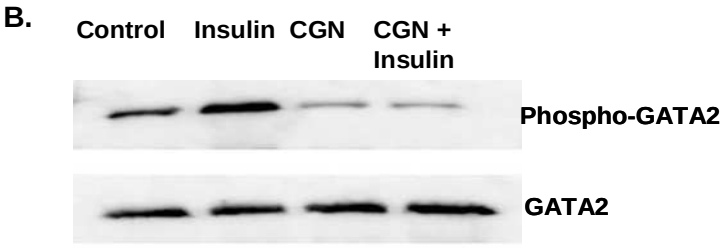
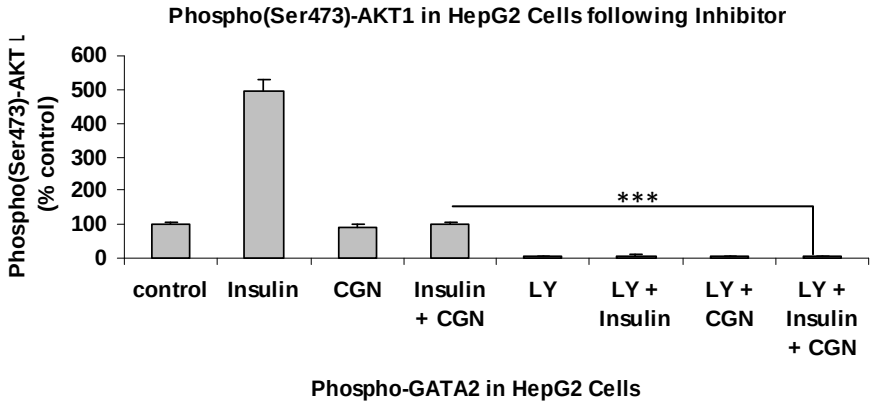
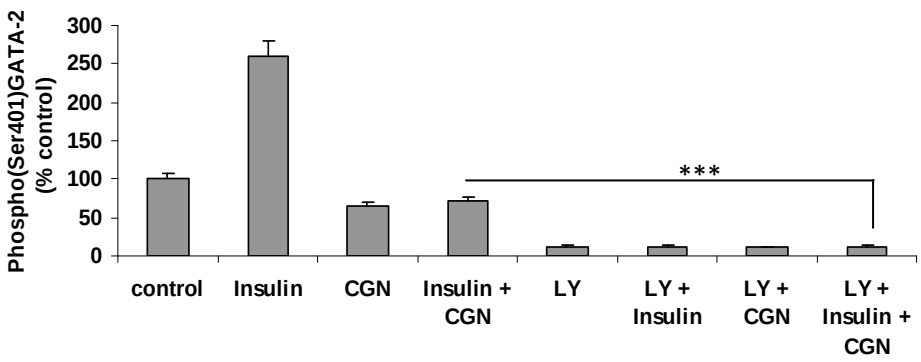


Figure 7. Inhibitor of PI3K-induced (S473)AKT phosphorylation increases GRB10 expression.

A.





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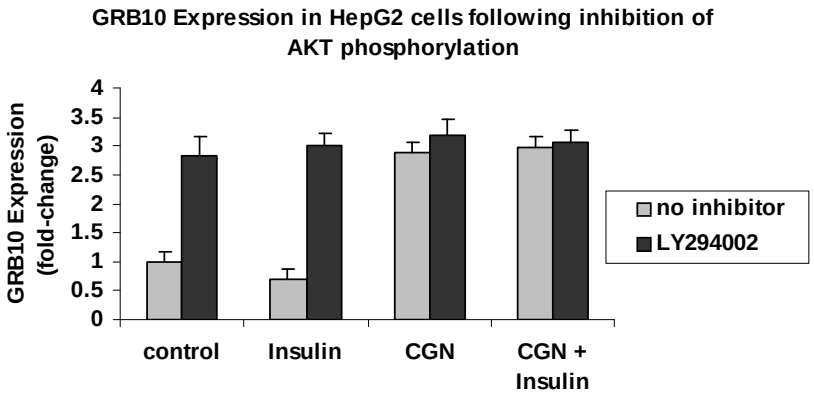

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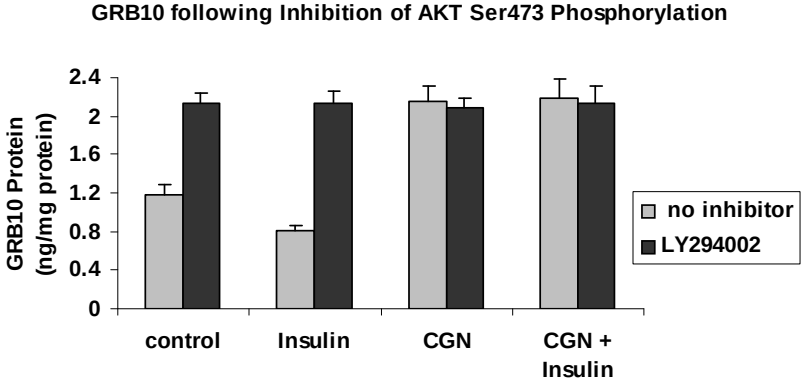

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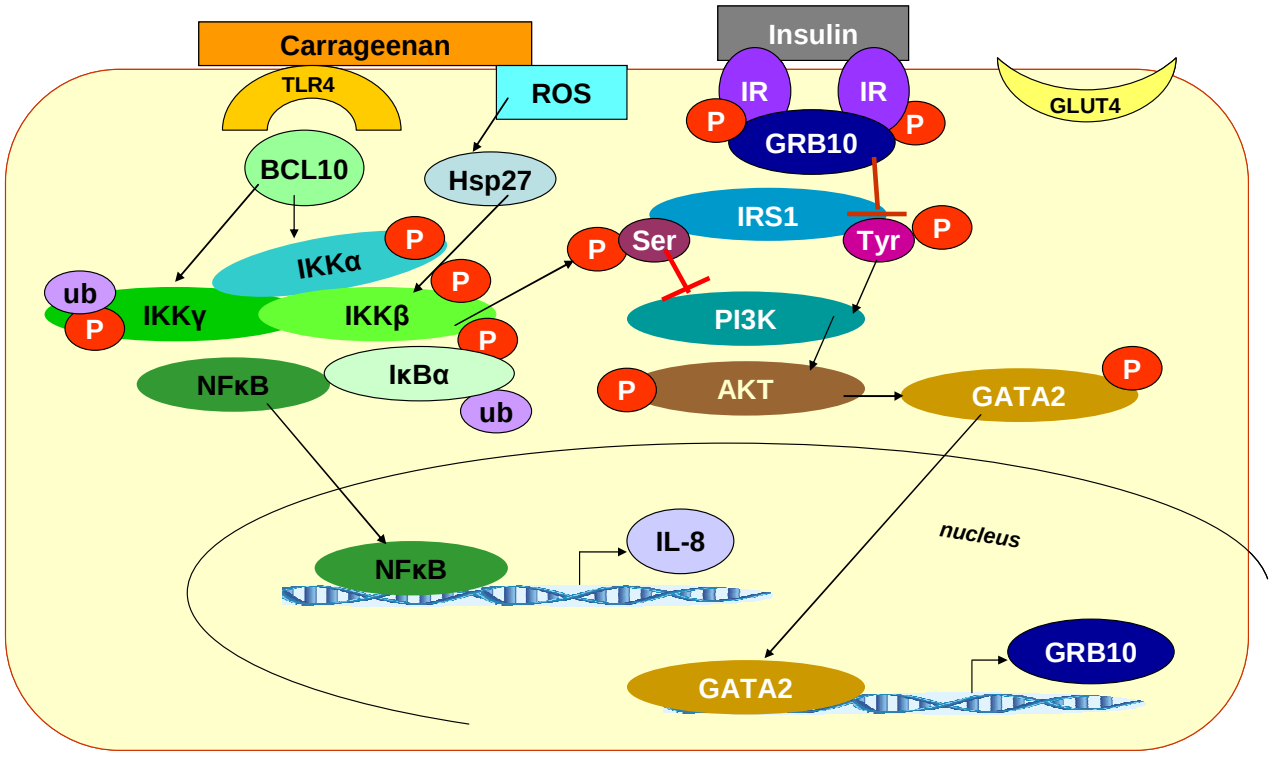

Molecular Bases of Disease:

Carrageenan Inhibits Insulin Signaling through GRB10-Mediated Decrease in Phospho(Tyr)-IRS1 and through Inflammation-Induced Increase in Phospho(Ser307)-IRS1

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