

# Exposure to the common food additive carrageenan leads to glucose intolerance, insulin resistance and inhibition of insulin signalling in HepG2 cells and C57BL/6J mice

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## Abstract

**Aims/hypothesis** The aim of this study was to determine the impact of the common food additive carrageenan (E407) on glucose tolerance, insulin sensitivity and insulin signalling in a mouse model and human hepatic cells, since carrageenan is known to cause inflammation through interaction with toll-like receptor (TLR)4, which is associated with inflammation in diabetes.

**Methods** Male C57BL/6J mice were given carrageenan (10 mg/l) in their drinking water, and underwent a glucose tolerance test (GTT), an insulin tolerance test (ITT) and an ante-mortem intraperitoneal insulin injection. HepG2 cells were exposed to carrageenan (1 mg/l × 24 h) and insulin. Levels of phospho(Ser473)-protein kinase B (Akt), phospho (Ser307)-IRS1, phosphoinositide 3-kinase (PI3K) activity and phospho(Ser32)-inhibitor of  $\kappa$ B ( $I\kappa$ B $\alpha$ ) were determined by western blotting and ELISA.

**Results** Glucose tolerance was significantly impaired in carrageenan-treated 12-week-old mice compared with untreated controls at all time points ( $n=12$ ;  $p<0.0001$ ). Baseline insulin and insulin levels at 30 min after taking

glucose during the GTT were significantly higher following carrageenan treatment. During the ITT, glucose levels declined by more than 80% in controls, but not in carrageenan-treated mice. Carrageenan exposure completely inhibited insulin-induced increases in phospho-(Ser473)-Akt and PI3K activity in vivo in mouse liver and in human HepG2 cells. Carrageenan increased phospho(Ser307)-IRS1 levels, and this was blocked when carrageenan-induced inflammation was inhibited.

**Conclusion** This is the first report of the impact of carrageenan on glucose tolerance and indicates that carrageenan impairs glucose tolerance, increases insulin resistance and inhibits insulin signalling in vivo in mouse liver and human HepG2 cells. These effects may result from carrageenan-induced inflammation. The results demonstrate extra-colonic manifestations of ingested carrageenan and suggest that carrageenan in the human diet may contribute to the development of diabetes.

**Keywords** Carrageenan · Glucose tolerance · Insulin resistance · Phospho-Akt · Phospho-IRS1 · Phosphoinositide 3-kinase

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## Abbreviations

Akt	Protein kinase B
BCL10	B cell (CLL-lymphoma)10
GTT	Glucose tolerance test
IGF-1	Insulin growth factor-1
$I\kappa$ B $\alpha$	Inhibitor of $\kappa$ B
ITT	Insulin tolerance test
JECFA	Joint (Food and Agriculture Organization of the United Nations [FAO]/WHO) Expert Committee on Food Additives
LCGN	$\lambda$ -Carrageenan
NF $\kappa$ B	Nuclear factor $\kappa$ B
PI3K	Phosphoinositide 3-kinase

ROS Reactive oxygen species  
TLR Toll-like receptor

## Introduction

The common food additive carrageenan (E407) is used in a wide variety of processed foods to improve texture and solubility. Carrageenan has also been used for decades in thousands of animal and cell-based experiments to induce inflammation [1]. Carrageenan induces inflammation through an innate immune pathway mediated by toll-like receptor (TLR)4 and a reactive oxygen species (ROS)-mediated pathway [2–4]. These cascades lead to activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) by both canonical and non-canonical cascades involving  $\nu$ -rel reticuloendotheliosis viral oncogene homologue A (avian) (RelA) and avian reticuloendotheliosis viral ( $\nu$ -rel) oncogene related B (RelB) [5]. Carrageenan activates an innate immune-mediated pathway of inflammation through TLR4 and B cell (CLL-lymphoma) 10 (BCL10), leading to increased phospho-inhibitor of  $\kappa$ B (I $\kappa$ B $\alpha$ ), thereby enabling the nuclear translocation of NF $\kappa$ B and the subsequent transcriptional events required for inflammation, including increased production of interleukin-8 (IL-8). The immune-related effects of carrageenan may be attributable to its unusual  $\alpha$ -1,3-galactosidic linkage, which is recognised as an immune epitope in human cells, since humans lack the  $\alpha$ -1,3-galactosyltransferase enzyme [6–8]. Unlike the naturally occurring sulphated glycosaminoglycans that contain galactose (keratan sulphate) or *N*-acetylgalactosamine (chondroitin sulphate and dermatan sulphate), carrageenan, which is derived from red algae, has this unusual disaccharide. Hydrolysis of the  $\alpha$ -1 $\rightarrow$ 3 galactosidic link was found to be associated with reduced production of IL-8, whereas hydrolysis of the  $\beta$ -1 $\rightarrow$ 4 link increased carrageenan-associated inflammation [9].

Since diabetes is associated with activation of TLR4-mediated inflammatory pathways [10–12], and exposure to carrageenan induces inflammation through a TLR4-mediated cascade [3], an investigation of the impact of carrageenan on glucose tolerance was undertaken. Unexpected and highly significant effects occurred, affecting glucose tolerance, insulin sensitivity and insulin signalling. These effects occurred at concentrations that were less than anticipated from the average daily intake of carrageenan in the human diet. Average daily intake of carrageenan in the diet in the USA was calculated to be 108 mg in the 1970s, and more recent estimates indicate average daily intake to be about 250 mg/day, or even higher [13–16]. The use of carrageenan in processed foods has increased in recent years, since by improving solubility and texture, carrageen-

an can compensate for reduced fat in dairy products, including yogurt, ice cream, whipped cream and soya milk, and in other foods, such as processed meats, infant formula, pet foods and dietetic beverages. In addition to uses in human food products, carrageenan is also used in pet food, room air fresheners, cosmetics and pharmaceuticals. The Joint (Food and Agriculture Organization of the United Nations and WHO) Expert Committee on Food Additives (JECFA) has recommended that carrageenan be excluded from infant formula and that a re-evaluation of carrageenan content in the diet be undertaken [17].

In this report, the impact of carrageenan exposure on glucose tolerance, insulin resistance and insulin signalling, as manifested by changes in the phosphorylation of Akt and phosphoinositide 3-kinase (PI3K) activity in mouse liver and human HepG2 cells, is presented. These effects are linked to carrageenan-induced inflammation, since Ser307 phosphorylation of IRS1 inhibits insulin signalling and also crosstalks with the I $\kappa$ B kinase (IKK) signalosome [18], which carrageenan activates through a TLR4-mediated pathway and through ROS [2–5]. In the studies that follow, previously identified inhibitors of carrageenan-induced inflammatory cascades, including *Tlr4* siRNA, *Bcl10* siRNA and 1-oxy-2,2,6,6-tetramethyl-4-hydroxypiperidine (Tempol), a ROS scavenger, are shown to reduce both phosphorylation of IRS1 (at S307) and of I $\kappa$ B $\alpha$  (at S32) in HepG2 cells, thereby affecting both propagation of insulin signalling and inflammation.

## Methods

**Animal care and carrageenan exposure** Eight-week-old male C57BL/6J mice ( $n=12$ ) were purchased (Jackson Laboratories, Bar Harbor, Maine, USA) and housed in the Veterinary Medicine Unit at the Jesse Brown VA Medical Center (Chicago, IL, USA). Principles of laboratory animal care were followed, and all procedures were approved by the Animal Care Committee. Mice were fed a standard diet and maintained in individual cages with routine light–dark cycles. After acclimatising to the environment, the water supply was changed to ddH<sub>2</sub>O with undegraded carrageenan ( $\lambda$ - $\kappa$  carrageenan 10 mg/l; Sigma Chemical Co., St Louis, MO, USA;  $n=6$ ) or without carrageenan ( $n=6$ ). Weight and water consumption were measured weekly; animals ate standard mouse chow (65% carbohydrate, 20% protein and 15% fat) and drank ad libitum. No effects of carrageenan on body weight, activity, or well-being were observed. The mice underwent a glucose tolerance test (GTT) with determination of insulin, an insulin tolerance test (ITT) and an ante-mortem insulin injection, as detailed below. An additional group of eight C57BL/6J mice of similar age were exposed to carrageenan ( $n=5$ ) or served as

untreated controls ( $n=3$ ) for studies of inflammation and insulin signalling, and were killed without ante-mortem insulin injection.

**Cell culture of HepG2 cells** HepG2 cells, a human hepatic adenocarcinoma cell line (ATCC HB-8065), were grown in minimum essential medium (MEM) with 10% FBS and maintained at 37°C in a humidified, 5% CO<sub>2</sub> environment with media exchange every 2 days. Confluent cells in T-25 flasks were harvested by EDTA-trypsin, and sub-cultured in multiwell tissue culture plates under similar conditions. In the majority of experiments, cells were exposed to undegraded  $\lambda$ -carrageenan (1 mg/l  $\times$  20 h) in media with serum, then to  $\lambda$ -carrageenan (1 mg/l  $\times$  4 h) in serum-free media. Cells were then washed, supplied with fresh serum-free media and treated with regular human insulin (Humulin U-100; Lilly, Indianapolis, IN, USA; 2, 10, or 20 nmol/l) for 10 min, harvested by scraping and frozen at  $-80^{\circ}\text{C}$  for subsequent analysis.

**GTT in C57BL/6J mice** GTTs were performed in 12-week-old male C57BL/6J mice ( $n=12$ ); six of the mice had been exposed to carrageenan for 18 days. Following an 18 h overnight fast, a small tail cut was made, and whole blood glucose was measured by glucometer (One Touch Ultra 2, LifeScan, Milpitas, CA, USA) from the second drop of blood expressed at time 0 and at 15, 30, 60 and 90 min following injection of dextrose (2 g/kg i.p. in filtered PBS).

**Insulin levels and ITT** Plasma insulin levels were measured by ELISA (Alpco, Salem, NH, USA) in blood samples collected in heparinised capillary tubes at 0 and 30 min during the GTT performed at 12 weeks. Optical density (O.D.) was measured (FLUOstar, BMG, Cary, NC, USA) and compared with a standard curve, and insulin was quantified as ng/ml.

ITTs were performed in the same C57BL/6J male mice at 14 weeks of age, when six of the mice had been treated with carrageenan (10 mg/l) in their water for 33 days. Food was removed for 2 h prior to insulin treatment (0.75 U/kg i.p. diluted 1:1,000 in diluents; Lilly), which was administered at time 0, following measurements of weight and baseline glucose. The tail was nicked laterally for blood sampling at 0, 15, 30, 60, 90 and 120 min.

Insulin (2 U/kg body weight or 10 U/kg body weight) was administered i.p. to the same C57BL/6J mice at 18 weeks, following ingestion of carrageenan for 9 weeks, and a 2 h fast. Insulin was injected 10 min prior to killing by CO<sub>2</sub> inhalation and exsanguination by cardiac puncture, and the liver was immediately harvested and frozen at  $-80^{\circ}\text{C}$ .

**Western blots for phospho(Ser473)-Akt, total Akt and phospho(Ser307)-IRS1** Tissue homogenates were prepared

from liver tissue 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) of carrageenan-exposed mice that had received insulin load prior to death, and controls that did not receive insulin. Western blots were performed on 10% SDS gels with commercial antibodies to phospho(Ser473)-Akt, total Akt, and phospho(Ser307)-IRS1 (Cell Signaling), tubulin (Sigma) and  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to probe for the proteins of interest by established procedures [2, 5]. HepG2 cells exposed to carrageenan and insulin and untreated control cells were also analysed by western blots performed by similar procedures, and immunoreactive bands were visualised using enhanced chemiluminescence (Amersham, GE Healthcare, Piscataway, NJ, USA). Image J software (NIH, Bethesda, MD, USA) was used for densitometry. Density of the protein of interest was compared with either  $\beta$ -actin or total Akt from the same specimen, and the density of treated and control samples was then compared.

**Cell-based ELISA for phospho(Ser473)-Akt in HepG2 cells** Phospho(Ser473)-Akt was measured by cell-based ELISA in HepG2 cells grown in 12-well plates. Cells were exposed to  $\lambda$ -carrageenan and insulin, as described above. The cells were then fixed with 4% formaldehyde in PBS for 20 min, washed with PBS, treated with quenching buffer and blocking buffer, and incubated overnight at 4°C with phospho(Ser473)-Akt antibodies. The phospho-Akt was detected by secondary antibody bound to horseradish peroxidase (HRP), and hydrogen peroxide-tetramethylbenzidine (TMB) was used for colour development. The reaction was stopped by 1,000 mmol/l H<sub>2</sub>SO<sub>4</sub>. O.D. was measured in an ELISA plate reader (FLUOstar, BMG) at 450 nm, cells were harvested, and protein determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA), using BSA as a standard. Measurements were compared with readings for the unexposed control cells per mg protein.

**ELISA for phospho-I $\kappa$ B $\alpha$  in liver tissue of carrageenan-exposed mice and HepG2 cells** Commercial ELISA for phospho(Ser32)-I $\kappa$ B $\alpha$  (Cell Signaling) was used to determine the extent of carrageenan-induced inflammation that was present, following carrageenan exposure in the mouse liver tissue and in HepG2 cells. An established procedure was followed [2–4], and the phospho-I $\kappa$ B $\alpha$  was expressed as per cent of control.

**Serum keratinocyte chemokine (KC) for measurement of inflammation** KC, the mouse homologue of IL-8, was measured in blood samples from the carrageenan-treated mice and the controls by ELISA (R&D, Minneapolis, MN, USA), using established methods, to determine the systemic

inflammation induced by carrageenan exposure. Results were expressed as  $\text{ng/l} \pm \text{SD}$ .

**PI3K activity in mouse liver tissue and HepG2 cells** PI3K activity was determined in the mouse liver tissue following *in vivo* carrageenan (10 mg/l  $\times$  60 days) and insulin exposure (2 or 10 U/kg *i.p.* ante-mortem), in the HepG2 cells following carrageenan (1 mg/l  $\times$  24 h) and insulin (10 nmol/l  $\times$  10 min) and in controls, using a commercial competitive ELISA (Echelon Biosciences, Salt Lake City, UT, USA) that assayed the production of phosphatidylinositol (3,4,5)-triphosphate (PI [3–5]P3) from phosphatidylinositol 4,5-bisphosphate (PI [4, 5]P2). The PI3K reaction was set up by adding 25  $\mu\text{g}$  of cell lysate in kinase reaction buffer to an equal volume of 8  $\mu\text{mol/l}$  of PIP2 substrate, and then incubated at 37°C for 2 h. The reaction was stopped by adding an equal volume of stop solution, containing PIP3 detector and EDTA. A 50  $\mu\text{l}$  aliquot of this mixture was then added to the wells of a microtitre plate that was coated with PIP3, and then incubated at room temperature for 1 h. The primary PIP3 detector bound to the plate was further detected by a peroxidase-linked secondary detector. The binding of the primary detector to the coated plate was inversely proportional to the amount of PIP3 produced in the kinase reaction in the liver tissue or HepG2 cells, because the PIP3 produced in the reaction competed with the coated PIP3 for the primary detector. The bound peroxidase activity was determined by adding hydrogen peroxide-TMB substrate. The reaction was stopped by 1,000 mmol/l  $\text{H}_2\text{SO}_4$  and absorbance was measured at 450 nm in a plate reader (FLUOstar, BMG). The sample values were extrapolated from a standard curve of O.D. vs known PIP3 concentration, and activity was expressed as  $\text{pmol} (\text{mg protein})^{-1} \text{h}^{-1}$  or per cent of control.

**Inhibition of carrageenan-induced inflammation** Tempol (Axxora Life Sciences, San Diego, CA, USA) is a free radical scavenger that inhibits ROS. HepG2 cells were treated with Tempol 100 nmol/l in combination with  $\lambda$ -carrageenan (1 mg/l)  $\times$  24 h, as previously used [4]. Small interfering (si) RNAs for *Tlr4* and *Bcl10* were obtained commercially (Qiagen, Valencia, CA, USA), and were used as described previously [2–5]. The effectiveness of silencing in the HepG2 cells was demonstrated by western blotting for TLR4 and by quantitative ELISA for BCL10.

**Statistical analysis** Data were analysed using InStat3 software (GraphPad, La Jolla, CA, USA). Mean values  $\pm$  SD were calculated, and differences between carrageenan-treated and control results were compared by one-way ANOVA with a Tukey–Kramer post-test for multiple comparisons, or by paired or unpaired *t* tests, two-tailed. Unless stated otherwise in the text or figure legends,

statistical analysis was by one-way ANOVA with Tukey–Kramer post-test. At least three independent samples with technical replicates were analysed for each experiment. Measurements are expressed as mean  $\pm$  SD.

## Results

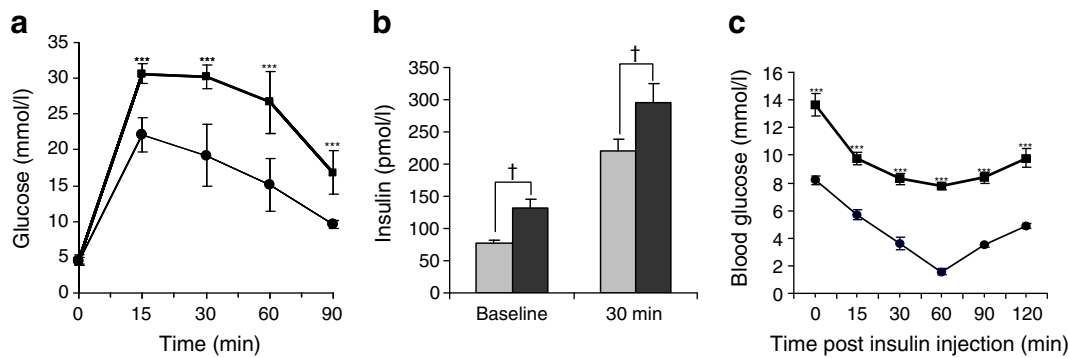
**Impaired glucose tolerance with insulin resistance following exposure to carrageenan** GTT at 12 weeks, following treatment with carrageenan 10 mg/l in the drinking water for 18 days, demonstrated marked differences in responses to glucose load (dextrose 2 g/kg *i.p.*) between the carrageenan-treated ( $n=6$ ) and control ( $n=6$ ) mice ( $p < 0.001$  for 15, 30, 60 and 90 min; Fig. 1a). Glucose peaked at a mean of  $30.6 \pm 1.4$  mmol/l in the carrageenan-treated mice and at  $22.1 \pm 2.4$  mmol/l in the controls at 15 min, from similar fasting levels (control:  $4.4 \pm 0.6$  mmol/l; carrageenan-treated:  $4.7 \pm 0.7$  mmol/l). Differences between weight-matched pairs were also highly significant ( $0.0037 \leq p \leq 0.015$ , paired *t* test, two-tailed). Weights were similar at the time of the GTT in the carrageenan-treated ( $27.1 \pm 0.9$  g) and control mice ( $26.5 \pm 1.2$  g). Weights were also similar at the onset of carrageenan exposure and at killing at 18 weeks, indicating lack of an association between glucose intolerance and weight in the carrageenan-treated mice.

Insulin levels were determined at baseline and at 30 min following glucose load in the GTT (Fig. 1b). Baseline and glucose-stimulated insulin levels were significantly higher in the carrageenan-treated mice, indicating that impaired glucose tolerance in the carrageenan-treated mice was not attributable to reduced insulin secretion ( $p < 0.0001$  at baseline and 30 min; unpaired *t* test, two-tailed).

Following exogenous insulin (0.75 U/kg *i.p.*) at 14 weeks, blood glucose declined more than 80% in the control mice, from a mean of  $8.2 \pm 0.3$  to  $1.6 \pm 0.2$  mmol/l at 60 min (Fig. 1c). In contrast, in the mice exposed to carrageenan (10 mg/l in their drinking water for 33 days), insulin injection did not produce hypoglycaemia. The maximum decline in glucose level in the carrageenan-treated mice was 43% at 60 min, with baseline blood glucose of  $13.6 \pm 0.8$  mmol/l declining to  $7.7 \pm 0.2$  mmol/l ( $p < 0.001$ ).

**Carrageenan inhibits phospho(Ser473)-Akt response to insulin in mouse liver *in vivo* and in HepG2 cells** Insulin (2 or 10 U/kg) was administered *i.p.* 10 min prior to death at 18 weeks of age, following continued carrageenan exposure for 9 weeks, in six carrageenan-treated and five surviving control C57BL/6J mice. Representative western blots demonstrated no increase in phospho(Ser473)-Akt in the carrageenan-treated mice, in contrast with the control





**Fig. 1** Impaired glucose tolerance in C57BL/6J mice at 12 weeks. **a** GTT: six male C57BL/6J mice were exposed to carrageenan (10 mg/l) in their water for 18 days prior to GTT (dextrose, 2 g/kg i.p.), and beginning at 9.5 weeks of age. Glucose in whole blood samples from tail incisions was measured at baseline, 15, 30, 60 and 90 min in carrageenan-treated and age-matched controls ( $n=6$ ). Results were significantly less at all time points following injection in the carrageenan-treated mice. ( $***p<0.001$  at each time point; overall  $p<0.0001$ , one-way ANOVA with Tukey–Kramer post-test). Black squares and thick line, carrageenan-treated mice; black circles, control mice. **b** Insulin levels during GTT: when GTT was performed (as above) at 12 weeks following carrageenan treatment for 18 days, blood was collected in heparinised tubes for determination of insulin levels at

baseline and 30 min post-dextrose administration. Insulin levels were markedly greater in the mice exposed to carrageenan (10 mg/l) in their water, compared with untreated mice ( $\dagger p<0.0001$  at baseline and 30 min; unpaired  $t$  test, two-tailed). Grey bars, control mice; black bars, carrageenan-treated mice. **c** ITT: regular insulin (0.75 U/kg) was administered i.p. to the carrageenan-treated ( $n=6$ ) and control ( $n=6$ ); one control mouse died during the ITT) mice at 14 weeks, following carrageenan treatment for 33 days. Maximum glucose decline was 43% at 60 min in the mice exposed to carrageenan, in contrast with the controls, in which glucose declined by over 80% ( $***p<0.0001$  at each time point; overall  $p<0.0001$ ; one-way ANOVA with Tukey–Kramer post-test). Black squares and thick line, carrageenan-treated mice; black circles, control mice

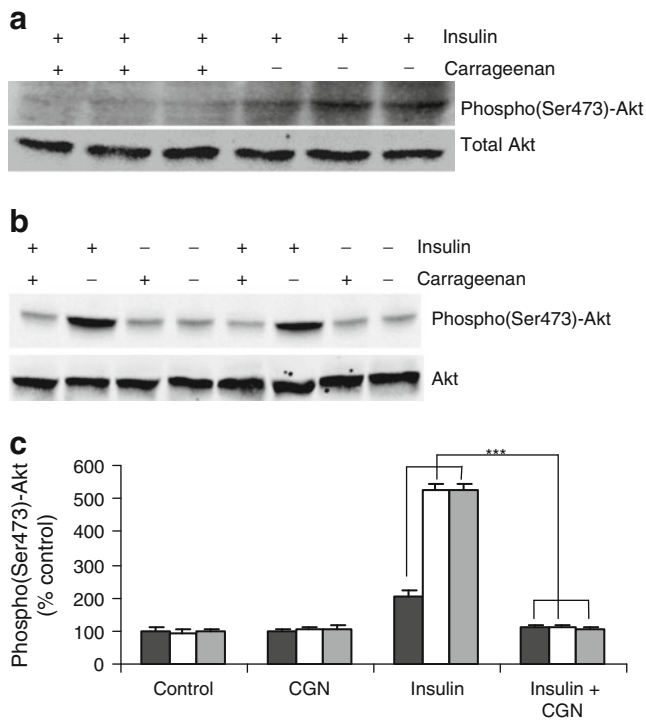
mice, in which a phospho(Ser473)-Akt band was prominent (Fig. 2a). Total Akt demonstrated no change in intensity. Densitometry confirms the visual impression of marked decline in insulin-induced increase in phospho-Akt following carrageenan exposure.

When HepG2 cells were exposed to  $\lambda$ -carrageenan (1 mg/l  $\times$  20 h in media with serum and  $\times$  4 h in serum-free media) and then to regular insulin (2, 10, or 20 nmol/l) for 10 min, a marked decline in the phospho(Ser473)-Akt response to insulin occurred in the carrageenan-exposed cells, as detected by western blot (Fig. 2b). Total Akt demonstrated no change in intensity between control and carrageenan-treated cells. Densitometry confirmed significant differences in phospho-Akt band intensity. Similarly, cell-based ELISA indicated that carrageenan exposure markedly diminished the expected insulin-induced increase in Akt Ser473 phosphorylation (Fig. 2c). The expected increases in phospho-Akt were totally inhibited by exposure to carrageenan, demonstrating a profound effect of carrageenan on downstream insulin signalling ( $p<0.001$ ; one-way ANOVA with Tukey–Kramer post-test).

*Carrageenan inhibited insulin-induced increase in PI3K activity in mouse liver in vivo and in HepG2 cells* Insulin-induced activation of PI3K is required upstream for subsequent phosphorylation of Akt in the insulin signalling pathway. Carrageenan exposure inhibited the expected increase in PI3K activity following insulin exposure in mouse liver (Fig. 3a) and in HepG2 cells (Fig. 3b), when determined by a competitive ELISA-based method. PI3K

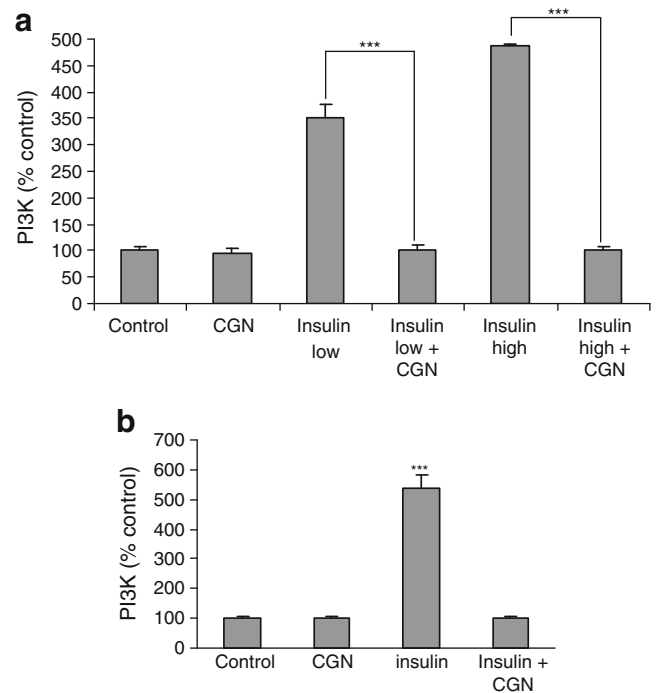
activity declined to baseline activity, from the insulin-induced increases of  $3.50\pm 0.26$  and  $4.89\pm 0.03$  times baseline, with low (2 U/kg) and high (10 U/kg) insulin exposure, respectively ( $p<0.001$ ). Similarly, in the HepG2 cells, carrageenan exposure reduced the insulin-induced increase in PI3K activity from  $\sim 5.4$  times the baseline back to the baseline activity ( $p<0.001$ ).

*Carrageenan exposure increased phospho(Ser307)-IRS1 levels in mouse liver in vivo and in HepG2 cells* Phospho (Ser307)-IRS1 acts to inhibit downstream insulin signalling, in contrast with other phosphorylations of IRS1, which are activating. Carrageenan exposure increased phospho(Ser307)-IRS1 in mouse liver, as shown by western blot (Fig. 4a). Densitometry confirms the visual impression of increase by carrageenan. The effectiveness of siRNA silencing of TLR4 and BCL10 in the HepG2 cells was demonstrated by western blot (Fig. 4b) and quantitative ELISA (Fig. 4c). ELISA for phospho(Ser307)-IRS1 demonstrated the increase following carrageenan in the HepG2 cells (Fig. 4d;  $p<0.001$ ). Carrageenan-induced increase in phospho(S307)-IRS1 level is a mechanism by which carrageenan can inhibit the propagation of insulin signalling. When carrageenan-induced inflammatory cascades were inhibited by TLR4 siRNA, BCL10 siRNA, or the ROS scavenger Tempol in HepG2 cells, the increases in phospho(Ser307)-IRS1 levels declined, and the combination of TLR4 or BCL10 knockdown with Tempol produced declines to baseline (Fig. 4d;  $p<0.001$ , one-way ANOVA with Tukey–Kramer post-test).



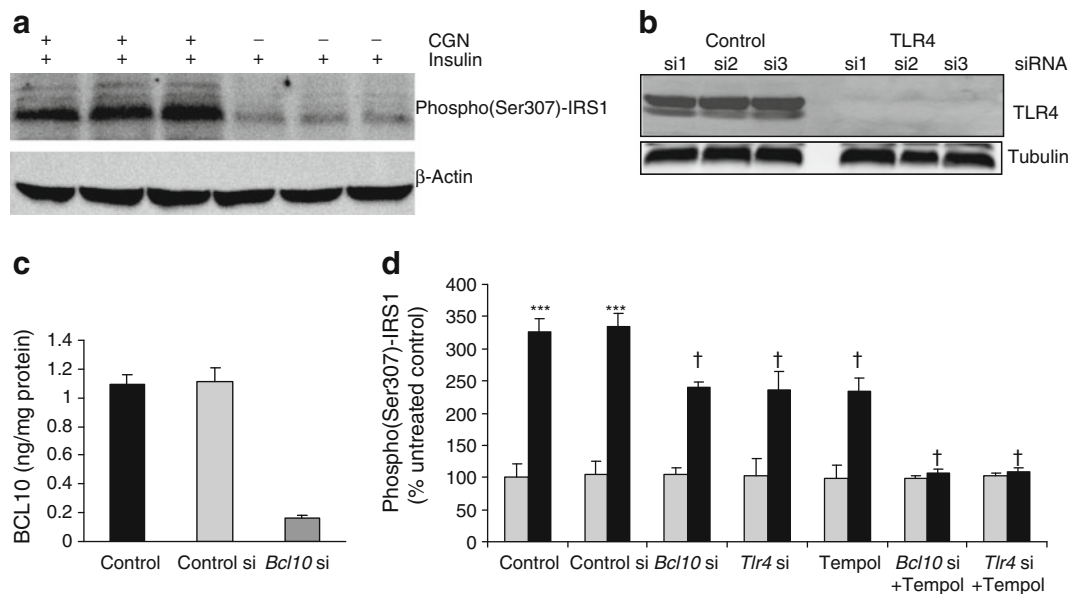
**Fig. 2** Inhibition of insulin-induced increase in phospho(Ser473)-Akt by carrageenan (CGN) in mouse liver *in vivo* and in HepG2 cells. **a** Phospho(Ser473)-Akt in mouse liver *in vivo*: 18-week-old C57BL/6J mice were treated with regular insulin (2 U/kg *i.p.*) 10 min prior to death, following a 2 h fast. Carrageenan-treated mice had received 10 mg/l of carrageenan in their water for 9 weeks. Phospho(Ser473)-Akt production was markedly reduced, as evident by the representative western blot and densitometry ( $p=0.007$ , unpaired  $t$  test, two-tailed). Total Akt was unchanged in intensity between carrageenan-treated and control samples. Densitometry: carrageenan + insulin  $21.7\pm 2.0$ ; insulin  $100\pm 26.7$ . **b** Phospho(Ser473)-Akt in HepG2 cells: HepG2 cells were treated with  $\lambda$ -carrageenan (1 mg/l  $\times$  24 h, including 4 h of serum deprivation) and insulin (10 nmol/l  $\times$  10 min), then harvested. The western blot of the HepG2 cells also demonstrates the profound effect of carrageenan exposure on phosphorylation at Ser473 on Akt, without any change in total Akt. Densitometry confirmed the visual impression of marked inhibition of insulin signalling by carrageenan ( $p<0.01$ ). Densitometry (pAkt/total Akt): carrageenan + insulin  $107\pm 21$ ; insulin  $365\pm 61$ ; carrageenan  $119\pm 20$ ; control  $100\pm 24$ . **c** Phospho(Ser473)-Akt in HepG2 cells by ELISA. HepG2 cells were treated with  $\lambda$ -carrageenan (1 mg/l  $\times$  24 h, including 4 h in serum-free media), then treated with regular insulin (2 nmol/l, 10 nmol/l or 20 nmol/l) for 10 min. Cell-based ELISA was used to detect phospho(Ser473)-Akt. Exposure to carrageenan completely inhibited the insulin-induced increases in phospho(Ser473)-Akt ( $***p<0.001$ ). Black bars, 2 nmol/l insulin; white bars, 10 nmol/l; grey bars, 20 nmol/l

*Effect of carrageenan on inflammation in the C57BL/6J mice and HepG2 cells* KC, the mouse homologue of IL-8, was increased in the blood samples of C57BL/6J mice that had been exposed to carrageenan in their water for 9 weeks. KC content was  $94.9\pm 8.0$  ng/l in the controls and  $182.2\pm 0.3$  ng/l in the carrageenan-treated mice ( $p<0.0001$ , unpaired  $t$  test, two-tailed).



**Fig. 3** Reduction of insulin-induced increase in PI3K activity following carrageenan (CGN) treatment in mouse liver *in vivo* and in HepG2 cells. **a** PI3K activity in mouse liver *in vivo*: mice were stimulated for 10 min ante-mortem with insulin (low dose, 2 U/kg; high dose, 10 U/kg). The PI3K activity was significantly reduced in the liver tissue of the carrageenan-treated mice (10 mg/l  $\times$  9 weeks;  $n=6$ ) vs the insulin-stimulated controls that had not received carrageenan ( $n=5$ ) and age-matched control mice that received neither insulin nor carrageenan ( $n=3$ ) or carrageenan alone ( $n=5$ ). Exposure to carrageenan reduced the insulin-stimulated PI3K peaks from  $350\pm 26\%$  (low insulin) and  $489\pm 3\%$  (high insulin) of the baseline level to baseline ( $***p<0.001$ ). **b** PI3K activity in HepG2 cells: HepG2 cells were treated with insulin (10 nmol/l) for 10 min, and cells were harvested and lysed. PI3K activity was determined as described in the methods section. Activity was markedly reduced in the cells that had been pre-treated with carrageenan (1 mg/l), declining from an insulin-induced increase of  $537\pm 46\%$  of the baseline level to the baseline ( $***p<0.001$ )

Phospho(Ser32)-I $\kappa$ B $\alpha$  level was measured by ELISA in hepatic tissue of C57BL/6J mice that had been treated with carrageenan (10 mg/l) in their water for 9 weeks and in HepG2 cells following exposure to carrageenan 1 mg/l  $\times$  24 h and untreated controls. Significant increases in phospho-I $\kappa$ B $\alpha$  followed exposure to carrageenan in the mouse liver (Fig. 5a) and in the HepG2 cells (Fig. 5b;  $p<0.001$ ). Similarly to the effects on phospho(S307)-IRS1 levels, siRNA for TLR4 or BCL10 and Tempol markedly reduced the carrageenan-induced increases in phospho(S32)-I $\kappa$ B $\alpha$  levels. These findings are consistent with activation of an inflammatory cascade by carrageenan in the mouse liver and in the HepG2 cells, and inhibition of the carrageenan-induced inflammation by knockdown of TLR4 or BCL10 and Tempol. Exposure to insulin had no effect on phospho-I $\kappa$ B $\alpha$ .



**Fig. 4** Production of phospho(Ser307)-IRS1 increased following carrageenan (CGN) in mouse liver in vivo and in HepG2 cells, and is reduced by inhibition of carrageenan-induced inflammation. **a** Phospho(Ser307)-IRS1 in mouse liver in vivo: in mouse liver tissue harvested 10 min after i.p. injection of insulin (2 U/kg), western blot for phospho(Ser307)-IRS1 indicated a marked increase in the mice that were exposed to carrageenan (10 mg/l $\times$ 9 weeks). Densitometry confirmed significant differences ( $p < 0.0001$ , unpaired  $t$  test, two-tailed). Densitometry: CGN 100 $\pm$ 3.6; no CGN 23 $\pm$ 3.9. **b** Effective silencing of TLR4 in HepG2 cells by *Tlr4* siRNA: the western blot demonstrates the effectiveness of silencing of TLR4 in the HepG2 cells. **c** Effective silencing of BCL10 in HepG2 cells by *Bcl10* siRNA: BCL10 protein production was effectively silenced by siRNA in the HepG2 cells, as demonstrated by quantitative ELISA. **d** Phospho

(Ser307)-IRS1 in HepG2 cells: phospho(Ser307)-IRS1 was measured by ELISA following exposure to carrageenan in the HepG2 cells. Following exposure to carrageenan, phospho(Ser307)-IRS1 increased significantly (\*\* $p < 0.001$ ). When carrageenan-induced inflammatory cascades were inhibited in the HepG2 cells by treatment with either *Tlr4* siRNA, *Bcl10* siRNA or Tempol, the production of phospho(Ser307)-IRS1 was partially inhibited ( $^{\dagger}p < 0.001$ ). When the cells were treated with carrageenan and the combination of Tempol with either *Tlr4* siRNA or *Bcl10* siRNA, the carrageenan-induced increase in phospho(Ser307)-IRS1 was completely inhibited, consistent with inhibition of insulin signalling by carrageenan-induced inflammation ( $^{\dagger}p < 0.001$ ). Grey bars, no carrageenan treatment; black bars, carrageenan-treated

## Discussion

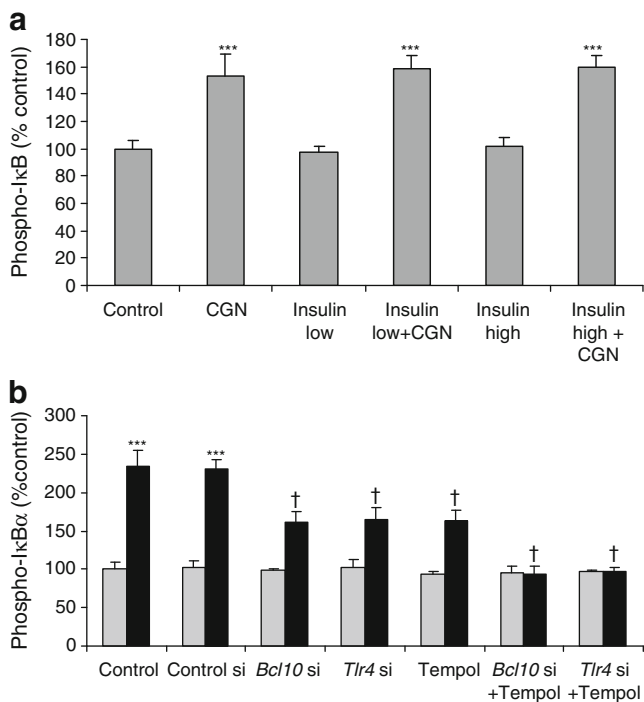
The results of this study suggest that dietary exposure to carrageenan may contribute to the increasing worldwide prevalence of glucose intolerance and insulin resistance. The prevalence of diabetes in the USA is highly correlated with published data about carrageenan consumption (mg [person] $^{-1}$  day $^{-1}$ ) in the USA during recent decades, since the correlation coefficient ( $r$ ) for the prevalence of diabetes mellitus from 1963 to 2004 (per cent of total population) and the per capita consumption of carrageenan (mg [person] $^{-1}$  day $^{-1}$ ) in the USA is 0.86, increasing to  $r = 0.88$  with a 10-year lag period [19, 20]. As intake of low-fat processed foods has increased in recent decades, carrageenan has increasingly been used to improve the texture of a wide variety of food products, including infant formula, low-fat processed meats and nutritional supplements.

The JECFA recommended the elimination of carrageenan from infant formula and further study of the actual consumption of carrageenan in the current diet [17]. In the average diet in the USA, carrageenan consumption is reported to be 250 mg/day [14, 15], far exceeding the therapeutic dosage of

glycoside pharmaceuticals such as digoxin, which is prescribed in doses of well under 1 mg/day.

In the studies in this report, carrageenan exposure induced glucose intolerance and insulin resistance in C57BL/6J mice, and inhibition of insulin signalling in the liver of the C57BL/6J mice and in HepG2 cells. The effect of carrageenan on glucose homeostasis is attributable to impaired insulin action, although a direct effect on beta cell secretion and/or insulin production cannot be ruled out. Further investigation is required to determine precisely the functional responses of beta cells to glucose stimulation following carrageenan and the longer term effect on beta cell mass of exposure to carrageenan.

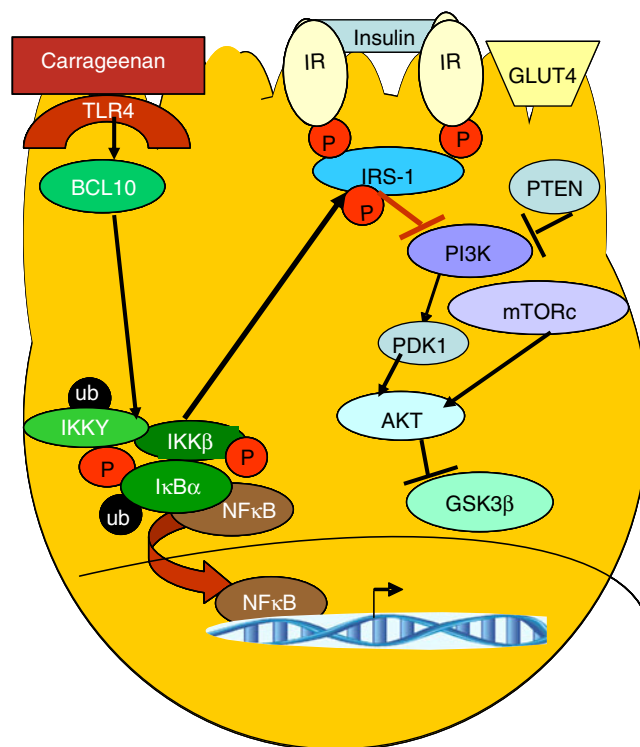
The concentration of carrageenan used in the cell-based experiments in this report and in other studies [2–5] was 1 mg/l, 50-fold lower exposure than would be anticipated from a daily carrageenan intake of 250 mg/day in the human diet (e.g. 250 mg in gastrointestinal contents of 5 l = 250 mg/5 l = 50 mg/l). In the experiments in this report, mice weighing <30 g were given carrageenan in their water at a concentration of 10 mg/l and consumed  $\sim$ 5 ml/day, thereby comparable to a 100 mg daily consumption in a



**Fig. 5** Phospho(Ser32)-IκBα is increased following carrageenan (CGN) exposure in mouse liver in vivo and in HepG2 cells. **a** Phospho(Ser32)-IκBα in mouse liver in vivo: following carrageenan treatment, phospho-IκBα was significantly increased in the mouse liver tissue obtained at 18 weeks (\*\*\*) ( $p < 0.001$ ). Phospho-IκBα was unaffected by ante-mortem insulin exposure, since there was no difference between the insulin-exposed samples and the non-insulin-exposed controls. **b** Phospho(Ser32)-IκBα in HepG2 cells: phospho-IκBα was significantly increased in the HepG2 cells following exposure to carrageenan (1 mg/l × 24 h), consistent with activation of inflammation in these cells (\*\*\*) ( $p < 0.001$ ). The inhibitors of carrageenan-induced inflammation that had been identified in colonic epithelial cells also inhibited the production of phospho-IκBα in HepG2 cells: BCL10 siRNA or TLR4 siRNA, in combination with Tempol, completely inhibited the carrageenan-induced increase in phospho-IκBα (†) ( $p < 0.001$  for differences in phospho [Ser32]-IκBα following treatment with the inhibitors either singly or in combination). Grey bar, no carrageenan treatment; black bar, carrageenan-treated

60 kg human, and lower than the measured average daily intake in the mid-1970s [21].

Recent experimental work demonstrated the presence of an NFκB binding site in the BCL10 promoter, suggesting the potential for ongoing inflammatory effects in epithelial cells, similar to the effects in immune cells, once BCL10 is activated [22–24]. Hence, the inflammatory cascade initiated by carrageenan may have a sustained impact on insulin signalling as well. The studies in this report indicate that the effects of oral carrageenan are not confined to the intestine, since altered insulin signalling was shown in the mouse liver. The mechanisms whereby carrageenan might elicit these extra-colonic effects remain to be elucidated, but macrophage uptake of carrageenan at sites of intestinal



**Fig. 6** Schematic representation of crosstalk between carrageenan-induced inflammation and inhibition of insulin signalling. The schematic drawing illustrates the relationships between the inflammatory cascades involving carrageenan, including the IKK signalosome, composed of IKKα, IKKβ and IKKγ, and the phosphorylations of IκBα (Ser32) and IRS1 (Ser307). The phosphorylation of IRS by IKKβ acts as an inhibitory signal, halting subsequent insulin signal transduction. The phosphorylation of IκBα enables the nuclear translocation of NFκB, and the subsequent transcriptional events of inflammation. IR, insulin receptor; ub, ubiquitin; PTEN, phosphatase and tensin homologue; GSK, glycogen synthase kinase

inflammation, as suggested by previous animal studies [1, 25–27], may lead to deposition in extra-colonic sites, with results such as those shown in the mouse liver. Increased circulating KC in the carrageenan-treated mice also indicates the presence of systemic inflammation.

The finding that phosphorylation of Ser307 on IRS1 was increased by carrageenan provides insight into one mechanism whereby carrageenan exposure can impair insulin signalling. Since phospho(Ser307)-IRS1 inhibits downstream transduction of insulin signalling, reductions in PI3K activity and in phosphorylation of (Ser473)-Akt follow, as presented schematically (Fig. 6). The demonstration that reducing carrageenan-associated inflammation by either silencing TLR4 or BCL10, or by Tempol, a ROS scavenger, produced a decline in phospho(Ser307)-IRS1, is consistent with the association between carrageenan-induced inflammation, manifested by increases in KC and phospho-IκBα, and inhibition of insulin signalling. The IKK signalosome component IKKβ has been identified as a



key modulator of phospho(Ser307)-IRS1, as well as being required for phosphorylation of I $\kappa$ B $\alpha$ , and thereby enabling the nuclear translocation of NF $\kappa$ B [5, 18, 28]. Whether or not carrageenan interferes with glucose uptake or insulin secretion by other mechanisms, the impact of carrageenan-induced inflammation at the level of the IKK signalosome produces a significant restraint on hepatic insulin signalling.

Although carrageenan has been widely shown to cause inflammation in animal and cell-based models of inflammation, this is the first report of the effects of carrageenan on glucose intolerance, insulin resistance, inhibition of insulin signalling and propensity to diabetes. Preliminary work, including cDNA microarray [29] and western blot studies (not published), has identified effects of carrageenan on increased expression of growth factor receptor-bound protein 10 (GRB10), recently recognised as a target of mammalian target of rapamycin (mTORC1) and involved in the inhibition of insulin signalling [30, 31]. Future work may provide additional insights into mechanisms by which the effects of carrageenan are manifested at extra-intestinal sites and contribute to glucose intolerance, insulin resistance and insulin signalling. Furthermore, reduced intake of carrageenan may help control the increase in incidence and prevalence of diabetes.

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**Duality of interest** The authors declare that there is no duality of interest associated with this manuscript.

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