Casein glycomacropeptide-derived peptide IPPKKNQDKTE ameliorates high glucose-induced insulin resistance in HepG2 cells via activation of AMPK signaling

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Scope: Recently, casein glycomacropeptide (GMP)-derived peptide was found to possess potent antioxidant and anti-inflammatory activities. In this study, the improvement effects and underlying molecular mechanisms of GMP-derived peptide on hepatic insulin resistance were investigated.

Methods and results: The peptide IPPKKNQDKTE was identified from GMP papain hydrolysates by LC-ESI-MS/MS. Effects of IPPKKNQDKTE on glucose metabolism and expression levels of the hepatic insulin signaling proteins in high glucose-induced insulin-resistant HepG2 cells were evaluated. Results showed that IPPKKNQDKTE dose-dependently increased glucose uptake and intracellular glycogen in insulin-resistant HepG2 cells without affecting cell viability. IPPKKNQDKTE increased the phosphorylation of Akt and GSK3\textbeta\, and decreased the expression levels of p-GS, G6Pase and PEPCK. These IPPKKNQDKTE-mediated protection effects were reversed by PI3K/Akt inhibitor LY294002, showing the mediatory role of PI3K/Akt. Moreover, treatment with IPPKKNQDKTE reduced IRS-1 Ser307 phosphorylation and increased phosphorylation of AMPK. Knockdown AMPK using siRNA in HepG2 cells increased Ser307 phosphorylation of IRS-1 and reduced Akt phosphorylation in IPPKKNQDKTE-treated insulin-resistant cells.

Conclusion: IPPKKNQDKTE prevents high glucose-induced insulin resistance in HepG2 cells by modulating the IRS-1/PI3K/Akt signaling pathway through AMPK activation, indicating that IPPKKNQDKTE plays a potential role in the prevention and treatment of hepatic insulin resistance and type 2 diabetes.

Keywords: Akt / AMPK / Casein glycomacropeptide / HepG2 cells / Insulin resistance

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Abbreviations: Akt, protein kinase B; AMPK, AMP-activated protein kinase; GMP, glycomacropeptide; GSK 3\textbeta, glycogen synthase kinase 3\textbeta; GS, glycogen synthase; G6Pase, glucose-6-phosphatase; IRS-1, insulin receptor substrate-1; MAPKs, mitogen-activated protein kinases; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA

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1 Introduction

Type 2 diabetes mellitus as a complex metabolic disorder has become a major global public health problem. The prevalence and incidence of type 2 diabetes are increasing at an explosive rate worldwide over the past three decades [1]. It has been estimated that more than 382 million people suffer from type 2 diabetes in year 2013 and this number is estimated to increase to 592 million by the year 2035 [2]. Type 2 diabetes is associated with high rates of mortality because of the presence of many severe diabetes-related complications, such as nephropathy, retinopathy, cardiovascular disease and peripheral vascular disease [3, 4]. Insulin resistance in peripheral tissues is indispensable for the development of type 2 diabetes. Liver, skeletal muscle and adipose tissue are major tissues responsive to insulin [5, 6]. Hepatic insulin resistance contributes to fasting hyperglycaemia and type 2 diabetes, while prolonged hyperglycaemia leads to the development of insulin resistance [7–9]. Therefore, it is important to prevent hepatic insulin resistance.

In order to improve hepatic insulin resistance and increase hepatic insulin sensitivity, a variety of synthetic drugs have been utilized, such as thiazolidinediones and metformin. However, these existing agents are often associated with adverse side-effects, including weight gain, bone loss and gastrointestinal side effects [10, 11]. In this regard, there is an urgent need to develop natural ingredients which improve hepatic insulin resistance with negligible side effects. To date, a variety of food-derived natural products, such as polysaccharide, flavonoid and protein hydrolysates, have been proposed to relieve insulin resistance. These derived macropeptide (GMP), a κ-casein-derived macropeptide f(106–169) comprised of 64 amino acids, has been reported to possess many bioactivities, such as reducing weight gain, decreasing cardiovascular disease risk markers, and improving intestinal flora [15–17]. In our previous study, GMP reduces body weight of obese Sprague-Dawley (SD) rats induced by high-fat diet, and inhibits proliferation and differentiation of preadipocytes isolated from SD rats in vitro [18, 19]. Furthermore, our study shows that papain-generated peptides from GMP suppress lipopolysaccharide-induced inflammatory response in murine RAW 264.7 macrophages, and protect macrophages from hydrogen peroxide-induced oxidative stress [20, 21]. However, the effects of GMP-derived peptides against hepatic insulin resistance have not been tested.

The purpose of this study was to identify the amino acid sequences of GMP-derived peptides and investigate the protective effects of the identified peptide against insulin resistance in high glucose-induced insulin-resistant HepG2 cells.

2 Materials and methods

2.1 Materials

Casein glycomacropeptide (Lactoprodan® CGMP-20, purity > 95%) was obtained from Arla Foods Ingredients (Viby, Denmark). Papain (EC 3.4.22.21, from papaya latex, 0.5–2.0 units per milligram) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxy-D-glucose (2-NBDG) was obtained from Life Technologies (Carlsbad, CA, USA). The peptide IPPKKN-QDKTE (purity > 95%) was supplied by Nanjing Leon Biological Technology Co. Ltd. (Nanjing, China). The antibodies to β-actin, GSK3β, GS and p-GS (Ser641) were purchased from Abcam (Cambridge, UK). The anti-AMPKα, anti-p-AMPKα (Thr172), anti-Akt, anti-p-Akt (Ser473), anti-IRS-1, anti-p-IRS-1 (Ser307), p-GSKβ (Ser9) antibodies, horseradish peroxidase-conjugated anti-rabbit secondary antibodies and LY294002 were supplied by Cell Signaling Technology (Beverly, MA, USA). The siRNA targeting human AMPKα1/2 and scrambled control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used in the study were of analytical grade.

2.2 Preparation and identification of GMP hydrolysates

GMP was dissolved in distilled water at a concentration of 5% (w/v). The solutions were adjusted to pH 6.0 and preincubated at 55°C. The papain enzyme was added at an enzyme/substrate mass ratio of 1/20 to hydrolyze GMP for 1 h at constant temperature and pH. The protease in hydrolysate samples was inactivated at 85°C for 20 min. The supernatants were collected by centrifugation at 4000 × g at 4°C for 20 min with TGL-20M high-speed centrifuge (Pingfan Instrument Co. Ltd., Changsha, China) and freeze-dried. The GMP hydrolysates were separated using high performance liquid chromatography (HPLC). The samples were injected into a column (Eclipse XDB-C18, 4.6 mm × 250 mm, 5 μm, Agilent Tech. Inc., China) and eluted with a linear gradient of acetonitrile (10–90% (v/v) in 30 min) and 0.1% trifluoroacetic acid (v/v) in H2O under a flow rate of 1 mL/min at 30°C. Peptide fragments were identified by liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) with a nanoAcquity nano HPLC system (Waters, Milford, MA, USA), which was directly interfaced with a Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA, USA).

2.3 Cell culture and treatments

Human hepatic carcinoma HepG2 cells were obtained from American Type Culture Collection (Rockville, MD, USA).
HepG2 cells were cultured in Minimum Essential Medium (MEM), which was supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acids, 100 μg/mL streptomycin and 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37°C in an atmosphere of 5% CO₂ and 95% humidity, and passaged every 3 days by trypsinization.

A cell model for high glucose-induced insulin resistance was established by exposing HepG2 cells to 30 mM glucose for 24 h, as described previously [22, 23]. Cells were cultured in a medium with normal (5.5 mM) or high (30 mM) glucose in the presence of different concentrations of IPPKKNQDKTE (0, 125, 250 and 500 μM) for 24 h. After incubation, cells were stimulated with 100 nM insulin for 10 min and then harvested for further analysis.

### 2.4 Measurement of cell viability

Cells were seeded into 96-well plates at a density of 5 × 10⁴ cells per well for 12 h. The cells were treated with various concentrations of IPPKKNQDKTE (0, 125, 250 and 500 μM) in the presence of 30 mM glucose for 24 h. Afterwards, 20 μL 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT) solution was added to obtain a final concentration of 0.5 mg/mL and incubated for 4 h at 37°C. Subsequently, the dark blue formazan crystals formed in each well were dissolved with 200 μL dimethyl sulphoxide. The absorbance at 570 nm was monitored with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was expressed as a percentage of the optical density of each treatment group relative to the optical density of control group.

### 2.5 Glucose uptake assay

Cellular glucose uptake was determined using fluorescent 2-NBDG reagent, as described previously [24]. In brief, cells were plated into 6-well plates at a density of 3 × 10⁵ cells per well. After treatments, cells were washed twice with phosphate-buffered saline (PBS) buffer (pH 7.4). Then, 2-NBDG was added to each well at a final concentration of 10 μM and incubated at 37°C. After 1 h, cells were washed twice with PBS buffer to remove the unabsorbed probe. The cell suspension was subjected to flow cytometric analyses using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Data were analysed by FlowJo 7.6.1 software (TreeStar, Ashland, OR, USA).

### 2.6 Determination of intracellular glycogen content

Intracellular glycogen was tested using a Glycogen Assay Kit (BioVision, Mountain View, CA, USA), according to the manufacturer’s instructions. Briefly, cells were seeded in 10-cm dishes (1.0 × 10⁶ cells per dish). After treatments, cells were homogenized with 200 μL of dH₂O on ice. Homogenates were boiled for 10 min to inactivate enzymes, followed by centrifugation at 18000 × g for 10 min. The supernatants were collected and assayed for glycogen content.

### 2.7 Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cultured cells by Trizol reagent (Tiangen Biotech, Beijing, China). After RNA isolation, first strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA in a 20 μL reaction volume using EasyScript Plus cDNA Synthesis Kit (ABM, Richmond, BC, Canada) according to the manufacturer’s instructions in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The SYBR green-based RT-qPCR assay was carried out with a Tecne Quantica real-time PCR detection system (Tecne, Stone, Staffordshire, UK). The RT-qPCR program was as follows: 95°C for 2 min, 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 30 s. The primers for glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were showed in Supporting Information Table 1. The mRNA levels of target genes were normalized to the mRNA expression of the housekeeping gene GAPDH. Data were presented as the fold change of each sample group relative to the control group.

### 2.8 Western blot analysis

Cells were collected and lysed in cell lysis buffer (Beyotime, Haimen, Jiangsu, China) containing 1 mM phenylmethylsulfonylfluoride. The samples were centrifuged at 12 000 × g for 15 min at 4°C and supernatants were collected. After quantification by the BCA method, equal amounts of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) on a wet transfer apparatus (Bio-Rad, Hercules, CA, USA). The PVDF membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 2 h, followed by incubation with primary antibodies overnight at 4°C. Subsequently, the membranes were washed three times, each for 5 min with TBS-T, before incubation with peroxidase conjugated secondary antibody for 1 h at room temperature. After three 5-min washes with TBS-T, the protein bands were detected using enhanced chemiluminescence reagent (Millipore, Bedford, MA, USA) and autoradiographic film. The band intensities were determined by ImageJ software (National Institutes of Health, Bethesda, MD, USA).
Figure 1. (A) Liquid chromatography-electrospray ionisation-tandem mass spectrometry spectrum of the peptide IPPKNQDKTE identified in glycomacropeptide (GMP) papain hydrolysates. The x-axis represented the m/z at which the fragment (b, y) ions were detected. The y-axis showed the relative intensity of ions. (B) Mature amino acid sequences of Bos taurus κ-casein (UniProt KB database accession number P02668). Peptide sequences identified in GMP papain hydrolysates were bold and underlined.

2.9 RNA interference

Human AMPKα1/2 small interfering RNA (siRNA), a non-specific control siRNA and Lipofectamine® 2000 were diluted in Opti-MEM reduced serum medium (Life Technologies, Carlsbad, CA, USA). HepG2 cells, grown to 50% confluence, were transfected using Lipofectamine® 2000 reagent with AMPKα1/2 siRNA or a non-specific control siRNA according to the manufacturer’s instructions.

2.10 Statistics

All assays in the present study were performed at least in triplicate and data were expressed as means ± standard deviations (SD). Significant differences (p < 0.05) between means were evaluated with one way ANOVA, followed by Duncan’s multiple-comparison test using SPSS software (version 20.0, IBM Inc., Chicago, IL, USA).

3 Results

3.1 IPPKKNQDKTE was identified from GMP hydrolysates

To identify the amino acid sequences of the peptides in GMP hydrolysates, the GMP hydrolysates were separated by HPLC and analysed by LC-ESI-MS/MS. The peptide with a molecular mass of 1298.69 Da was determined in GMP hydrolysates. Following sequence interpretation and database searching, the MS/MS spectrum of ion with m/z 649.85 was matched to sequence IPPKKNQDKTE (Fig. 1A), which matched Bos taurus κ-casein f(129–139) (Fig. 1B). The peptide IPPKKNQDKTE was synthesized for further studies.

3.2 IPPKKNQDKTE increased glucose uptake of insulin-resistant HepG2 cells

To understand the potential roles of IPPKKNQDKTE on glucose uptake in high glucose-induced insulin-resistant HepG2 cells, the cellular glucose uptake was determined using fluorescent 2-NBDG reagent. As shown in Fig. 2A and B, compared with the control group, high glucose stimulation significantly decreased the cellular glucose uptake (p < 0.05), which was prevented by IPPKKNQDKTE treatment dose-dependently. Moreover, the effects of IPPKKNQDKTE on glucose uptake were not associated with cytotoxicity, as the MTT assay showed no toxicity at the tested concentrations (Fig. 2C). These results suggested that IPPKKNQDKTE increased glucose uptake in insulin-resistant HepG2 cells induced by high glucose.

3.3 IPPKKNQDKTE down-regulated expressions of key gluconeogenic enzymes in insulin-resistant HepG2 cells

To investigate the effects of IPPKKNQDKTE on gluconeogenesis of insulin-resistant HepG2 cells, the expression levels of key enzymes related to gluconeogenesis were...
assayed. As revealed in Fig. 3, compared with the control group with only normal glucose (5.5 mM) treatment, the mRNA expression levels of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) were significantly increased by high glucose (30 mM) stimulation ($p < 0.05$). However, this effect was repressed by IPPKKNQDKTE treatment in high glucose-stimulated cells. When 500 $\mu$M IPPKKNQDKTE was present, the mRNA expression levels of G6Pase and PEPCK were decreased by 36.06 and 73.97%, respectively, compared with the high glucose group. These data suggested that IPPKKNQDKTE might contribute to modulate the glucose gluconeogenesis through down-regulation of key enzymes involved in hepatic gluconeogenesis.

### 3.4 IPPKKNQDKTE regulated phosphorylation levels of key glycogenic proteins and increased intracellular glycogen in insulin-resistant HepG2 cells

To investigate the effects of IPPKKNQDKTE on glycogenesis of insulin-resistant HepG2 cells, the levels of phosphorylated glycogen synthase kinase (GSK) $\beta$, phosphorylated glycogen synthase (GS) and intracellular glycogen were determined. The related original film from western blot experiment was shown in the supplementary information. Figure 4A and B showed that the levels of p-GSK$\beta$ in the cells cultured in high glucose conditions were remarkably lower than those in the cells grown in normal glucose conditions ($p < 0.05$). Compared with high glucose groups, treatment with IPPKKNQDKTE induced significant increase of p-GSK$\beta$ level ($p < 0.05$) in a concentration-dependent manner. On the other hand, high glucose treatment elevated the phosphorylated levels of GS in comparison to the control group ($p < 0.05$), but IPPKKNQDKTE reduced p-GS level ($p < 0.05$). In addition, compared with control group, high glucose treatment decreased glycogen levels ($p < 0.05$). However, the reduction of intracellular glycogen was recovered by IPPKKNQDKTE treatment (Fig. 4C). Our study showed that the peptide IPPKKNQDKTE increased intracellular glycogen by regulating the phosphorylation levels of key proteins of glycogen synthesis in insulin-resistant HepG2 cells.

![Figure 2. Effect of IPPKKNQDKTE on glucose uptake in insulin-resistant HepG2 cells.](image-url)
3.5 IPPKKNQDKTE regulated expression levels of key gluconeogenic and glycogenic proteins via activation of the PI3K/Akt signaling in insulin-resistant HepG2 cells

As shown in Fig. 5A and B, the phosphorylation of protein kinase B (Akt) was reduced in high glucose-treated HepG2 cells \((p < 0.05)\), which was dose-dependently increased by IPPKKNQDKTE treatment. To evaluate the potential roles of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways in the regulation of IPPKKNQDKTE on expression levels of G6Pase, PEPCK, p-GSK3β and p-GS associated with gluconeogenesis and glycogen synthesis, cells were pretreated with PI3K/Akt inhibitor LY294002 (10 μM) for 1 h, followed by IPPKKNQDKTE treatment in the presence of 30 mM glucose (30 mM) or high (30 mM) glucose culture medium in the absence or presence of IPPKKNQDKTE at indicated concentrations (0, 250 and 500 μM) for 24 h, and further exposed to 100 nM insulin for 10 min. The mRNA expressions of G6Pase (A) and PEPCK (B) were determined by RT-qPCR. Values are means ± SD from three separate determinations. * \(p < 0.05\) vs. control cells; # \(p < 0.05\) vs. only high glucose-treated cells.
Figure 5. Effect of IPPKKNQDKTE treatment on PI3K/Akt signaling pathway in high glucose-induced insulin-resistant HepG2 cells. HepG2 cells were incubated with 10 μM PI3K/Akt inhibitor LY294002 for 1 h prior to the treatment of 24-h glucose (5.5 and 30 mM) and different concentrations (0, 250 and 500 μM) of IPPKKNQDKTE, and further exposed to 100 nM insulin for 10 min. A representative blot for p-Akt (Ser473) and total Akt was depicted on panel A and densitometric results from three different assays were shown in panel B. Panel C showed the mRNA expression of G6Pase and PEPCK determined by RT-qPCR. Panel D and E showed a representative blot for p-GSK3β (Ser9) and p-GS (Ser641) and densitometric results from three different assays, respectively. Values are means ± SD from three separate determinations. *p < 0.05 vs. control cells; #p < 0.05 vs. only high glucose-treated cells; ##p < 0.05 vs. cells treated with high glucose plus IPPKKNQDKTE.
Figure 6. Effects of IPPKNNQDKTE treatment on phosphorylation of IRS-1 and AMPK in high glucose-induced insulin-resistant HepG2 cells. HepG2 were cultured in normal (5.5 mM) or high (30 mM) glucose medium in the absence or presence of IPPKNNQDKTE at indicated concentrations (0, 250 and 500 μM) for 24 h, thereafter stimulated by 100 nM insulin for 10 min. Cell lysates were analyzed by western blot for p-IRS-1 (Ser307) and p-AMPKα (Thr172). (A) Western blot bands of representative experiments. (B) Fold change in optical density relative to controls. Values are means ± SD from three separate determinations. *p < 0.05 vs. control cells; #p < 0.05 vs. only high glucose-treated cells.

3.6 IPPKNNQDKTE regulated phosphorylation of IRS-1 and AMPK in insulin-resistant HepG2 cells

To determine whether the activation of PI3K/Akt signaling pathways by IPPKNNQDKTE is due to the alternation of phosphorylation state of insulin receptor substrate-1 (IRS-1) and AMP-activated protein kinase (AMPK), Ser307 phosphorylation level of IRS-1 and Thr172 phosphorylation level of AMPK in insulin-resistant HepG2 cells were assayed. The related original film from western blot experiment was shown in the Supporting Information. As indicated in Fig. 6A and B, high glucose promoted the activation of Ser307 phosphorylation of IRS-1 in HepG2 cells, whereas IPPKNNQDKTE treatment decreased serine phosphorylation of IRS-1. Moreover, Thr172 phosphorylation levels of AMPK were decreased in high glucose-exposed HepG2 cells compared with those of HepG2 under normal glucose conditions. Treatment with IPPKNNQDKTE dose-dependently prevented the suppression of phosphorylation of AMPK induced by high glucose. These results indicated that the activation of PI3K/Akt signaling pathways by IPPKNNQDKTE in insulin-resistant HepG2 cells was associated with the state of IRS-1 Ser307 phosphorylation and AMPK Thr172 phosphorylation.

3.7 Regulation of IPPKNNQDKTE on phosphorylation of IRS-1 and Akt depended on AMPK activation in insulin-resistant HepG2 cells

In order to further confirm that regulation of IPPKNNQDKTE on phosphorylation of IRS-1 and Akt was related to AMPK phosphorylation, AMPK expression in HepG2 cells was inhibited by siRNA methods. As shown in Fig. 7A, AMPK protein expression was knocked down by transfecting cells with AMPK siRNA, in contrast to the control group or control siRNA group. The decreasing level of phosphorylated IRS-1 Ser307 and increasing phosphorylation level of Akt Ser473 were observed by IPPKNNQDKTE treatments in the presence of high glucose without AMPK siRNA transfection (Fig. 7B and C). However, IPPKNNQDKTE partially failed to reduce the phosphorylation of IRS-1 at Ser307 and increase the phosphorylation of Akt Ser473 in AMPK siRNA-treated cells. Compared with AMPK-siRNA cells treated with high glucose plus IPPKNNQDKTE, cells treated with AMPK siRNA without peptide exhibited higher level of IRS-1 serine 307 phosphorylation and lower level of Akt Ser473 phosphorylation. These results suggested that the activation of AMPK may be essential to the regulatory effects of IPPKNNQDKTE on phosphorylation of IRS-1 and Akt in insulin-resistant HepG2 cells.

4 Discussion

Liver plays a critical role in control of glucose homeostasis and maintenance of normal blood glucose levels by regulating the balance between gluconeogenesis, glycogen synthesis and glycogenolysis [25]. It has been reported that hepatocytes in insulin resistant states always exhibit impaired glucose utilization, which is associated with alteration of glucose metabolism, including glucose uptake, glycogen
synthesis and gluconeogenesis [26, 27]. Elevated gluconeogenesis, which is due to overexpression of two key gluconeogenic enzymes PEPCK and G6Pase is involved in hepatic insulin resistance [28, 29]. Moreover, the phosphorylation level of GSK3, a key enzyme involved in glycogen synthesis, is suppressed in insulin-resistant liver, which subsequently inactivates GS by phosphorylation and reduces glycogen synthesis [30]. Selective inhibition of GSK3β has been proposed as a novel therapeutic target for type 2 diabetes by improving insulin resistance in muscle or liver or both [31]. In the present study, the treatment of IPPKKNQDKTE improved glucose uptake of insulin-resistant HepG2 cells cultured in high glucose conditions. IPPKKNQDKTE also prevented high glucose-induced expression of PEPCK and G6Pase, suggesting IPPKKNQDKTE possessed the ability to suppress hepatic gluconeogenesis in insulin-resistant HepG2 cells. Furthermore, IPPKKNQDKTE promoted glycogenesis via regulating phosphorylation levels of GSK3β and GS in high glucose-induced insulin-resistant HepG2 cells. Similarly, the glucosamine-induced increase in expressions of PEPCK and G6Pase and phosphorylation level of GS, and decrease in the phosphorylation of GSK3 are ameliorated by irisin in insulin-resistant HepG2 cells [9]. These results suggested that IPPKKNQDKTE improved impaired glucose metabolism in insulin-resistant HepG2 cells states partially through promoting glycogenesis and inhibiting gluconeogenesis.

Insulin-mediated promotion of glycogenesis and inhibition of gluconeogenesis are associated with the activation of IRS/P13K/Akt pathway [32]. Hepatic IRS-1/P13K/Akt signaling is impaired in livers of type 2 diabetic rodents and hepatocytes of type 2 diabetic patients [33]. The impaired insulin signaling and the defect in downstream PI3K/Akt signal transduction are related to the phosphorylation of IRS on serine and threonine residues [34, 35]. The Ser307 site of IRS-1 is phosphorylated in an insulin-resistant state, followed by reduction of IRS-1 tyrosine phosphorylation and suppression of IRS-1 binding to IR, which results in down-regulation of PI3K/Akt signaling pathway and insulin-stimulated glucose utilization [36]. Previous study shows that astaxanthin treatment promotes the IRS-1/P13K/Akt pathway of insulin signaling in liver of insulin-resistant mice via decreasing serine phosphorylation of IRS proteins and improving glucose metabolism [37]. In the current study, we found that IPPKKNQDKTE increased the phosphorylation levels of Akt and decreased IRS-1 serine 307 phosphorylation in HepG2 cells with an insulin-resistant state. Moreover, the improvement of IPPKKNQDKTE on high glucose-induced insulin resistance was reversed by P13K/Akt inhibitor. These data showed that the peptide IPPKKNQDKTE attenuated high glucose-induced insulin resistance by the regulation of IRS/P13K/Akt signaling pathway.

Apart from impaired IRS-1/P13K/Akt signaling pathway, dysregulation of AMPK in insulin-sensitive tissues is associated with insulin resistance and distorted glucose metabolism [38]. Activated AMPK improves insulin signaling...
Beneficial effects of the peptide IPPKKNQDKTE on high glucose-induced insulin resistance in HepG2 cells. The peptide IPPKKNQDKTE activated IRS/PI3K/Akt signaling pathway by increasing phosphorylation levels of AMPK in high glucose-induced insulin resistant HepG2 cells. This ultimately modulated high glucose-induced abnormal gluconeogenesis and glycogen synthesis. Red solid line arrows represented changes in response to high glucose; Blue dotted line arrows represented changes in high glucose-stimulated cells receiving peptide intervention.

![Diagram showing beneficial effects of the peptide IPPKKNQDKTE on high glucose-induced insulin resistance in HepG2 cells.](image)

Figure 8. Beneficial effects of the peptide IPPKKNQDKTE on high glucose-induced insulin resistance in HepG2 cells. The peptide IPPKKNQDKTE activated IRS/PI3K/Akt signaling pathway by increasing phosphorylation levels of AMPK in high glucose-induced insulin resistant HepG2 cells. This ultimately modulated high glucose-induced abnormal gluconeogenesis and glycogen synthesis. Red solid line arrows represented changes in response to high glucose; Blue dotted line arrows represented changes in high glucose-stimulated cells receiving peptide intervention.

via inhibiting the Ser phosphorylation of IRS-1 and activating PI3K/Akt signaling, which increases insulin sensitivity and modulates glucose homeostasis [32]. Thus, AMPK activation is an attractive target for treating insulin resistance and type 2 diabetes [39]. In this study, high glucose-induced decrease in phosphorylation of AMPK was increased by IPPKKNQDKTE treatment. Moreover, the ability of IPPKKNQDKTE to inhibit Ser307 phosphorylation of IRS-1 and increase Akt phosphorylation was reversed by AMPK siRNA, suggesting that AMPK activation was required for the effects of IPPKKNQDKTE against high glucose-induced insulin resistance in HepG2 cells. In agreement with our results, rosmarinic acid prompts insulin signaling and consequently modulates glucose metabolism via the AMPK pathway, which improves insulin-sensitizing effects in livers of diabetic rats and insulin-resistant HepG2 cells [40]. Additionally, mitogen-activated protein kinases (MAPKs) have been identified as important proteins involved in high glucose-induced oxidative stress, phosphorylation of serine residues of IRS proteins and subsequently defective insulin signaling [41]. Whether high glucose-induced insulin resistance in HepG2 cells was improved by IPPKKNQDKTE treatment through decreasing the activation of MAPK needs to be further investigated.

Food protein-derived bioactive peptides have been found to possess multifunctional properties, such as lipid-lowering, antioxidative, antihypertensive and immunomodulatory activities. Interaction of peptides with cells is the basis for exerting bioactivities of peptides. It is reported that the peptides IAVPGV, IAVPGV, and LPYP are able to enter into HepG2 cells to modulate cholesterol metabolism [42]. By contrast, the peptide SLDSTHTHAPWP binds to HepG2 cells via specific binding receptors on cell membrane [43]. However, the interaction mode of the peptide IPPKKNQDKTE identified in our study with HepG2 cells need to be further investigated. Besides, the bioactivities of peptides are considered to be related to the amino acid composition and sequence of peptides [44, 45]. It has been shown that proline-containing peptides reveal the hypoglycemic and hypolipidemic effects in experimental models of diabetes mellitus [46, 47]. Milk-derived tripeptides containing proline improves insulin sensitivity and inhibits adipose inflammation in diet-induced obese mice [48]. Moreover, three proline-containing peptides from soy glycmin, including LPYP, IAVPGV, and IAVPGV enhance the capacity of glucose uptake and modulate cholesterol metabolism in HepG2 cells [42, 49]. In this study, the identified peptide IPPKKNQDKTE also contained proline, suggesting proline may be associated with beneficial effects of IPPKKNQDKTE on insulin-resistant state. In addition, the peptides containing isoleucine, proline, lysine, glutamine, aspartic acid and glutamic acid have been shown to possess antioxidant capacity and prevent oxidative stress [21, 44]. Suppression of oxidative stress attenuates hepatic insulin resistance in high-fat-diet-treated mice [50]. In this study, abundant amino acids with antioxidant property existed in the peptide IPPKKNQDKTE, which may contribute to the improvement effects of IPPKKNQDKTE on insulin resistance.

In summary, GMP-derived peptide IPPKKNQDKTE showed beneficial effects on insulin-resistant state of HepG2 cells under high glucose conditions. As depicted in Fig. 8, these beneficial effects were involved with the modulation of IPPKKNQDKTE on the high glucose-induced abnormal gluconeogenesis and glycogen synthesis via activation of IRS/PI3K/Akt signaling pathway. Furthermore, IPPKKNQDKTE-induced phosphorylation of AMPK was essential for activation of IRS/PI3K/Akt signaling. This study suggests that IPPKKNQDKTE may be a potential candidate for prevention and amelioration of hepatic insulin resistance and type 2 diabetes.

J.-J.S, Q.W., M.D., T.-G.L, B.C and X.-Y.M. were involved in the study design. J.-J.S conducted the experiments and analyzed the data. J.-J.S, M.D. and X.-Y.M. wrote the manuscript. X.-Y.M. had primary responsibility for the final content. All authors have read and approved the final manuscript.

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The authors have declared no conflict of interest.

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