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Research Article

A novel antidiabetic peptide GPAGAP from *Andrias davidianus* collagen hydrolysates: screening, action mechanism prediction and improving insulin resistance in HepG2 cells

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Abstract: In this study, a novel hypoglycemic peptide Gly-Pro-Ala-Gly-Ala-Pro (GPAGAP) was screened from skin collagen hydrolysates of *Andrias davidianus* by network pharmacology and bioinformatics, and its hypoglycemic mechanism was predicted. Meanwhile, the improvement of insulin resistance (IR) in HepG2 cells were detected. Through network pharmacology screening, GPAGAP had good drug-like properties, and 105 targets of GPAGAP overlap with diabetes mellitus type 2 (T2DM) targets. These targets were mainly enriched in the PI3K-Akt signaling pathway, TNF signaling pathway, IR and other signaling pathways related to T2DM. The results of IR-HepG2 cell model experiments showed that GPAGAP could reduce IR of HepG2 cells induced by high-glucose and high-insulin, and improve glucose consumption of IR-HepG2 cells. GPAGAP could increase the glycogen content, hexokinase (HK) and pyruvate kinase (PK) activities of IR-HepG2 cells, inhibit the accumulation of triglyceride (TG) and total cholesterol (TC) in IR-HepG2 cells, and enhance the activity of superoxide dismutase (SOD) in IR-HepG2 cells, reduce the content of malondialdehyde (MDA) and reactive oxygen species (ROS) in IR-HepG2 cells. The above results suggested that GPAGAP could through multi-target and multi-pathway to improve the glucose metabolism, lipid metabolism and oxidative stress response of IR-HepG2 cells. It has the potential effect of improving insulin resistance in T2DM.

Keywords: antidiabetic peptide; *Andrias davidianus*; collagen hydrolysates; action mechanism prediction; IR-HepG2 cells

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1 Introduction

Diabetes has become a common chronic non-infectious disease that seriously threatens human health[1]. Diabetes is a metabolic disease characterized by a variety of diseases, such as genetics, environment, metabolic disorders, oxidative stress and other factors can cause diabetes, especially type 2 diabetes (T2DM)[2]. Insulin resistance (IR), characterized by insulin hyposensitivity, is the main cause of T2DM[3]. Previous studies had shown that abnormal glucose generation, lipid metabolism and oxidative stress were important pathological basis of IR[4,5], but the underlying molecular mechanism was not completely clear. At present, although clinical drugs (such as biguidine and sulfonylureas) could effectively improve T2DM, these drugs might cause side effects such as abdominal distention, liver damage, diarrhea and different degrees of drug resistance[6]. Therefore, it has become a hot research topic in recent years to find a kind of safe natural medicine with little toxic and side effects that can become a supplement for the treatment of T2DM.

Food-derived bioactive peptides, as natural substances, have been widely studied as hypoglycemic foods or drugs due to their advantages of safety, low side-effect or non-toxicity, and easy absorption[7]. At present, different active peptides were screened from a variety of food raw materials, such as silver carp[8], egg yolk[9], *Andrias davidianus* (meat or skin), etc.[10]. These peptides had inhibitory effects on key enzymes regulating T2DM, indicating that they could improve T2DM. However, it is a complex and time-consuming process to screen active polypeptides with enzyme inhibition from proteolytic hydrolysates due to their lack of targeting, which brought inconvenience to subsequent cell experiments or *in vivo* studies. Therefore, it is very important to find a method of targeted screening for active peptides.

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Network pharmacology and bioinformatics provide new methods for the development, screening, and disease treatment of drugs or pharmaceutical ingredients. The method was based on the whole, collects information on drugs and diseases in various databases, and combines current computer technology to form a network of drugs with diverse components and diseases with complex factors for analysis[11]. It provides ideas for the systematic screening of compounds in mixtures that have the ability to treat specific diseases and their possible mechanisms of action, and lays a theoretical foundation for subsequent research. Therefore, in this study, network pharmacology combined with bioinformatics methods were used to screen a novel diabetes-improving polypeptide from *A. davidianus* skin collagen hydrolyzate and predict the underlying mechanism of its hypoglycemic activity. Then, its effects on cellular glucose metabolism, lipid metabolism and oxidative stress levels were detected by *in vitro* IR-HepG2 cell model experiments. The purpose of this study is to provide new ideas for the screening of food-derived hypoglycemic peptides and the elucidation of their multi-target and multi-way mechanisms to improve T2DM.

2 Materials and methods

2.1 Materials

Fetal bovine serum (FBS) was purchased from MRC Company. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone Company (USA). HepG2 cells were provided by Shaanxi Normal University (China). Insulin was purchased from Sigma Aldrich (USA). Trypsin and Liver Glycogen Kit (BC0345) was purchased from Beijing Solarbio Technology Co., Ltd. (China). Hexokinase (HK) Kit (A077-3-1), Pyruvate Kinase (PK) Kit (A076-2-1), superoxide dismutase (SOD) and malondialdehyde (MDA) determination kits were purchased from Nanjing Jiancheng Institute of Biological Engineering (China). All other reagents were of analytical pure grade.

2.2 Preparation of enzymatic hydrolysates of collagen from *A. davidianus* skin

Alkaline protease (enzyme activity = 5,500 U/g) was added into collagen solution from *A. davidianus* skin at a solid-liquid ratio of 1:30 (mg/mL), temperature of 55 °C and pH 9 for 120 min. Then the reaction was terminated at 95 °C for 15 min. After cooling, the supernatant was collected by centrifugation (5,000 × *g*, 4 °C, 20 min), and freeze-dried for LC-MS/MS analysis.

2.3 LC-MS/MS analysis

The enzymatic hydrolysates of collagen was analyzed by LC-MS system (Thermo Fisher, USA). Chromatographic separation was accomplished on Halo Peptide ES-C_{18} (2.1 mm × 150 mm, 2 μm) at a flow rate of 0.3 mL/min. The column temperature was maintained at 30 °C. The mobile phase was composed of 0.1% formic acid (FA)/H_2O (A) and 0.1% FA/CH_3CN (B) with the following gradient elution program: 0–50 min, 4%–50% B; 50–54 min, 50%–100% B; 54–60 min, 100% B. The injection amount was 10 μL. The MS spectra were acquired with SIM mode in both positive and negative ion modes. The detector voltage, 4.5 kV; ion spray temperature, 300 °C. MS signals were collected by data dependent MS/MS ($ddMS^2$).

2.4 Screening, mechanism prediction and preparation of hypoglycemic peptide

2.4.1 *Screening of hypoglycemic peptide*

According to LC-MS/MS information of enzymatic hydrolysates of *A. davidianus* skin collagen, the molecular structure of peptides was confirmed using PubChem (https://pubchem.ncbi.nlm.nih.gov/) and ChemDraw 19.0 software. SwissADME (http://www.swissadme.ch/) was used to screen peptides with good "drug like" properties[12].

2.4.2 *Targets prediction and component-target network construction*

Target genes linked to the selected peptide were identified using the SwissTarget Prediction database (http://www.Swisstargetprediction.ch/) with the "*Homo sapiens*" species setting (probability ≥ 0.1). with "type 2 diabetes" and "T2DM" as key words, T2DM-related targets were screened out from human genome database GeneCards (https://www.genecards.org/), OMIM database (http://www.omim.org), TTD database (http://bidd.nus.edu.sg/group/cjttd) and DrugBank (https://go.drugbank.com/). The common targets gained by overlapping targets related to the peptide and T2DM targets using the UniProt database (http://www.uniprot.org/) were taken as the potential therapeutic targets of peptide in the treatment of T2DM. The common targets of peptide and T2DM were uploaded to String database (http://stringdb.org/), setting the species as "*Homo sapiens*" and the minimum interaction threshold as "high confidence (0.7)", and protein-protein interaction (PPI) network was obtained for further analysis. Cytoscape 3.8.0 (http://www.cytoscape.org/) was adopted to visualize the results.

2.4.3 *GO and KEGG pathway enrichment analysis*

The common targets were imported into Metascape platform, and $P < 0.01$ was set for enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways[13]. Origin Lab in 2018 and Bioinformatics Tools (http://www.bioinformatics.com.cn/) were used to visualize the results.

2.4.4 *Component-target-pathway network construction*

Cytoscape 3.8.0 was used to analyze and demonstrate the relationships among peptide, targets and pathways to predict the overall pharmacological mechanism of peptides therapy for T2DM.

2.4.5 *Preparation of hypoglycemic peptide*

According to the screening results, the active peptide was artificially synthesized by traditional solid-phase synthesis method (Hefei Keshengjing Peptide Biological Co., Ltd., China), and its purity was greater than 95%, which was used for subsequent cell experiments.

2.5 Cell assay

2.5.1 *Cell culture and model establishment*

HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C, under a 5% CO_2, 95% relative humidity atmosphere. Then, cells were seeded into 96 well cell culture plates at a density of 1.0×10^5 cells/mL and incubated for

24 h. Then the cells were treated with high concentration of glucose (25 mmol/L) and insulin (1×10^{-5} mol/L) for 24 h. The establishment of IR cell model was referred to the method of Yang *et al.*[14]. IR-HepG2 cells were treated in low-sugar (5.5 mmol/L) medium containing different concentrations of active peptides (0, 0.5, 1 and 2 mmol/L) for 24 h to determine the optimal concentration of active peptides. Cells viability was detected by thiazolyl blue (MTT) method.

2.5.2 *Glucose consumption analysis*

The glucose consumption was estimated by the method of Nadia *et al.*[2] with slight modification. Briefly, the cells were divided into 4 groups for further research. (1) normal (insulin-free, 5.5 mmol/L glucose), (2) model (IR), (3) control (0.5 mmol/L metformin alone), (4) peptides alone. The cells were seeded into a 96-well plate (1.0×10^5 cells/mL) with five wells left as blanks. The cells were treated with fresh medium containing different concentrations of peptide (0, 0.5, 1 and 2 mmol/L) for 24 h, then the supernatant was taken and the glucose oxidase (GOD-POD) kit (Nanjing Jicheng Institute of Biological Engineering, A154-1-1) was used to quantify the glucose concentration. Glucose consumption was calculated by the glucose concentrations of blank wells minus glucose concentrations in plated wells.

2.5.3 *Effects of hypoglycemic peptide (GPAGAP) on glucose metabolism in IR-HepG2 cells*

HepG2 cells were inoculated into 6-well cell plates at a density of 10^6/well and placed in an incubator at 37 °C and 5% CO_2 for 12 h. After cell adherence, normal group, model group, control group and peptide group were established according to section 2.5.2 and cultured for 24 h. Then the cells were washed with PBS for 2 times and collected. The contents of glycogen, HK and PK in cells were determined by liver glycogen kit (BC0345), HK kit (A077-3-1) and PK kit (A076-2-1). Total protein content was determined by BCA protein analysis kit (No. E-BC-K318-M), and glycogen content was expressed in proportion to total protein.

2.5.4 *Effects of hypoglycemic peptide (GPAGAP) on lipid metabolism in IR-HepG2 cells*

The cells were grouped and cultured according to method as described in section 2.5.3. Intracellular total cholesterol (TC) and triglyceride (TG) contents were determined with TC and TG kits.

2.5.5 *Effects of hypoglycemic peptide (GPAGAP) on oxidative stress in IR-HepG2 cells*

The cells were grouped and cultured according to method as described in section 2.5.3. Determination of MDA, SOD and reactive oxygen species (ROS) activities were carried out using kits according to manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). The cultured cells were collected and crushed on ice with an ultrasonic breaker. The cleaved cells were centrifuged at 4 °C at 10,000 × *g* for 10 min, and total protein concentration in supernatant was determined using BCA protein analysis kit (Elabscience Biotechnology Co., Ltd., China). The content of MDA and the relative activities of SOD were expressed as a ratio to total protein. ROS content in cells was determined by DCFH DA fluorescence probe, and the results were compared with blank control group.

2.6 Statistical analysis

All experiments were performed in triplicate at least and data were expressed as mean ± standard deviation (SD). SPSS 25.0 software was used to evaluate the significant difference ($P < 0.05$) among the mean values from one-way ANOVA and Duncan's multiple tests.

3 Results

3.1 Screening of antidiabetic peptides from collagen hydrolysates of *A. davidianus* skin

The collagen hydrolysates were identified and amino acid sequencing was performed by LC-MS/MS. According to Lipinski's rule, 76 six-peptide analyzed by LC-MS/MS were screened for drug-like properties. The screening conditions were as follows: (1) relative molecular weight (M_w) ≤ 500, (2) hydrogen bond donor ≤ 5, (3) hydrogen bond acceptor ≤ 10, and (4) lipid water partition coefficient ≤ 4.15. The results showed that a new peptide had good drug-like properties, and its amino acid sequence was Gly-Pro-Ala-Gly-Ala-Pro (GPAGAP, M_w = 468.51 Da). The molecular structure of GPAGAP was shown in Fig. 1.

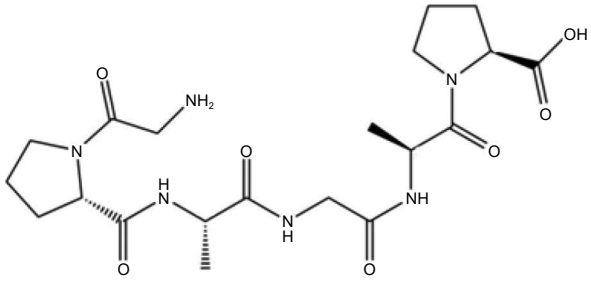

Figure 1 Chemical structure of GPAGAP.

3.2 Searching for common targets and key targets of GPAGAP-T2DM

The 3D structure of GPAGAP was uploaded to SwissTarget Prediction website for peptide target prediction, and 354 related targets were displayed after removing duplicity.

In the GeneCards database, a search result based on the keyword "T2DM" showed 830 relevant targets. A total of 1,059 T2DM related targets were collected by TTD, OMIM and DrugBank databases to supplement related targets and delete repeated targets. A total of 105 targets after the intersection of GPAGAP and T2DM targets were selected as common targets of GPAGAP-T2DM (Fig. 2).

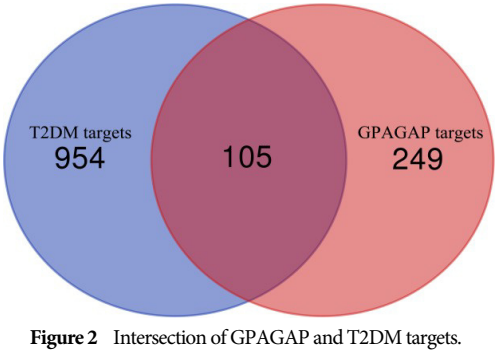

Figure 2 Intersection of GPAGAP and T2DM targets.

Common targets are submitted to STRING11.0 platform to obtain target PPI networks, as shown in Fig. 3. PPI network contains 105 nodes and 1,059 edges, wherein nodes represent different target proteins and edges represent interactions between different target proteins[15]. In PPI complex network, some areas

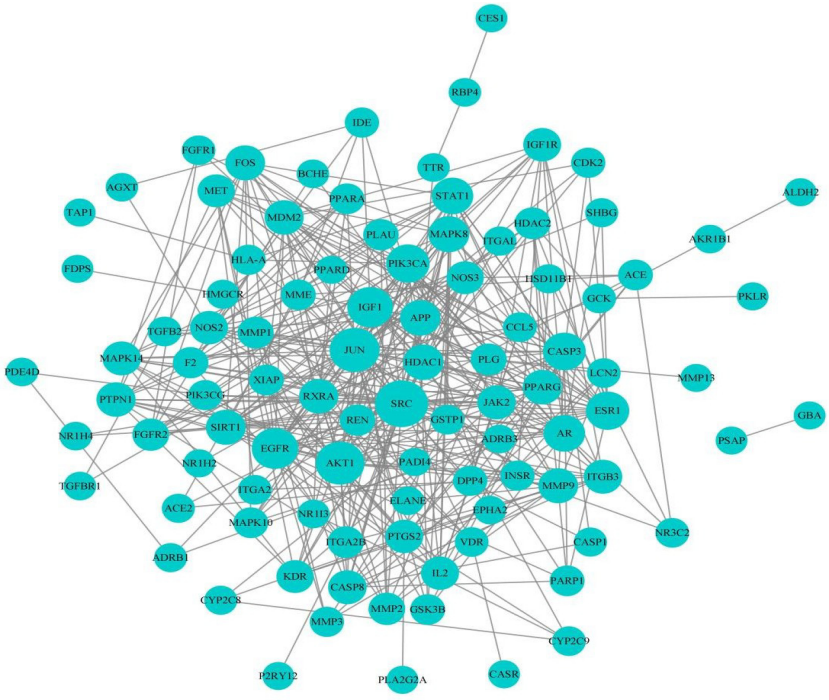

Figure 3 PPI network of 105 common targets of GPAGAP and T2DM.

with high density were called community or module, which were considered to be collections with biological significance and played an important role in the whole PPI network. The PPI network interaction of GPAGAP-T2MD was analyzed by MOCDE plug-in in Cytoscape 3.8.0 through molecular complex detection algorithm, and a total of 31 targets were obtained in 3 modules (Fig. 4). These results suggest that these targets play an important role in improving the occurrence and progression of diabetes mellitus by GPAGAP.

3.3 GO and KEGG enrichment analysis of GPAGAP-T2DM

GO and KEGG pathway enrichment analysis were performed on 105 common targets by Metascape database ($P < 0.01$). A total of 271 records were obtained by GO enrichment analysis, of which biological process (184), molecular function (57) and cell composition (23) accounted for 69.70%, 21.59% and 8.71%, respectively. In the biological process category (Fig. 5A), the target

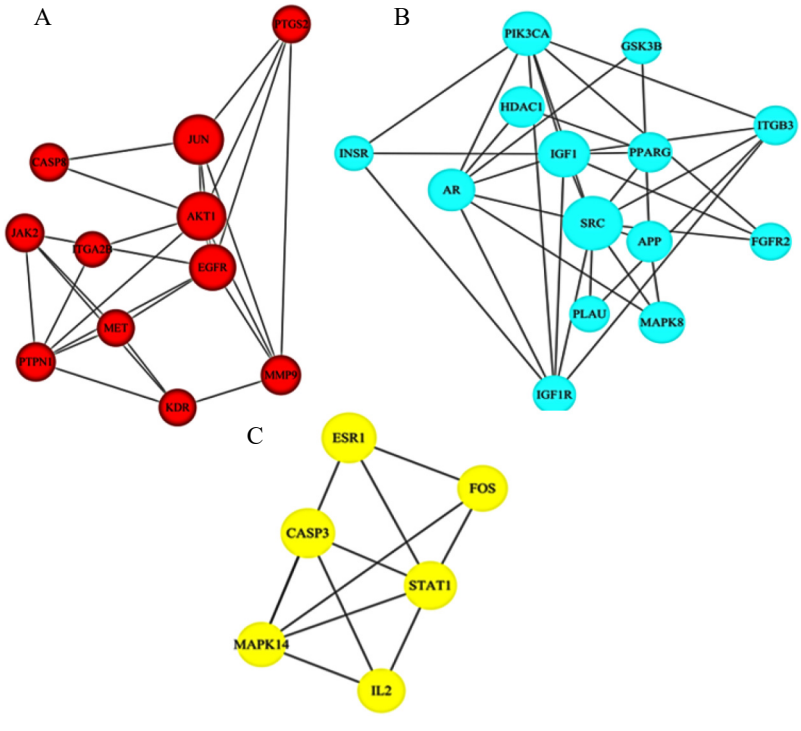

Figure 4 Modules in the GPAGAP-T2DM target PPI network. There are 31 important targets in the three modules (A, B and C) that represent different degrees of connectivity, intermediation, and tightness.

proteins were mainly involved in the regulation of inflammatory responses and the negative regulation of apoptosis; in the molecular function category (Fig. 5B), the target proteins were mainly involved in protein enzyme binding, protein binding, RNA

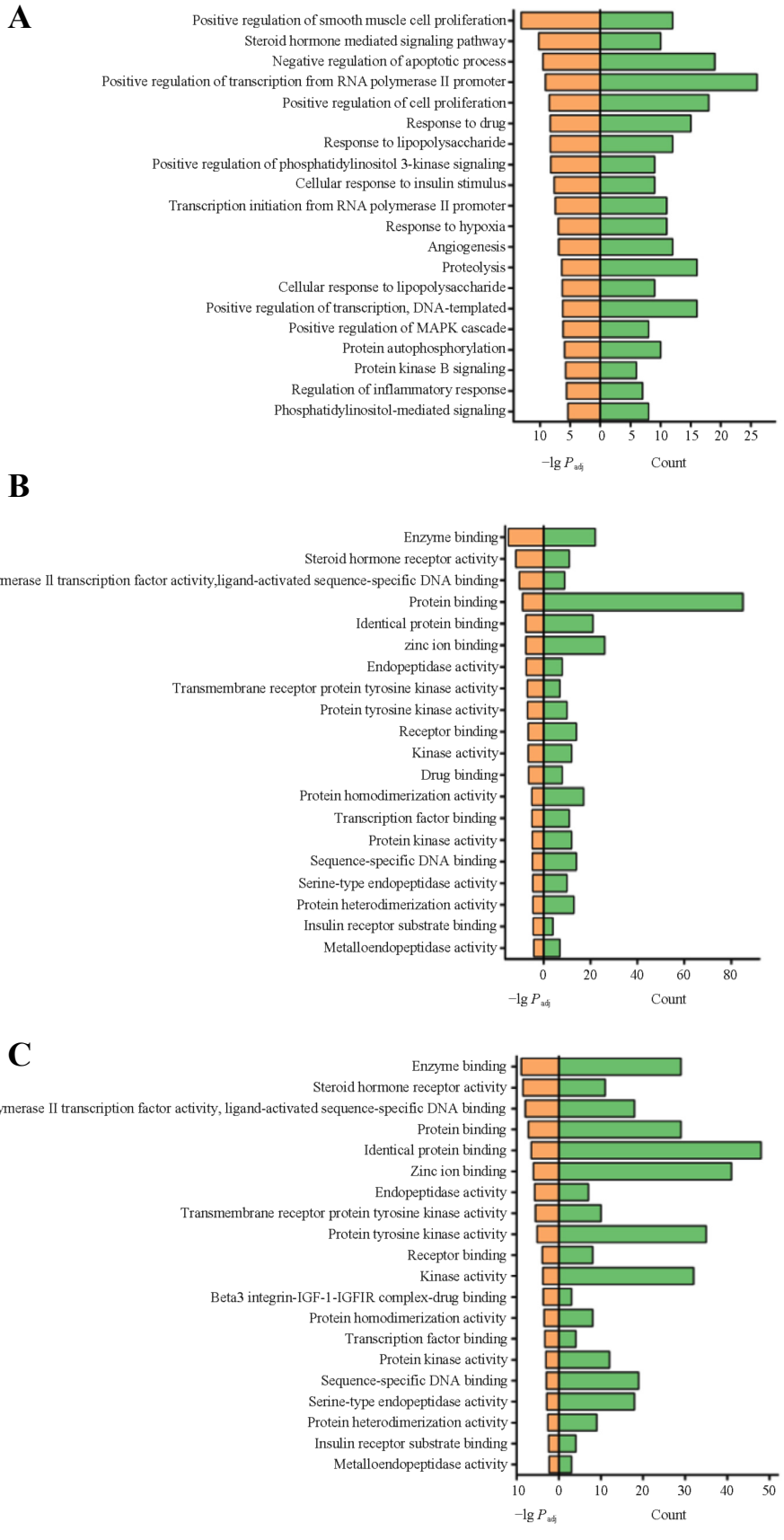

Figure 5 GO enrichment analysis of GPAGAP-T2DM. (A) Biological process, (B) Molecular function, (C) Cellular component.

polymerase II transcription factor activity-ligand activation sequence-specific DNA binding; in the cellular component category (Fig. 5C), the target protein mainly exists in the extracellular matrix and extracellular enzyme binding. GO enrichment analysis results showed that GPAGAP could play a variety of molecular functions in different cellular components through regulating multiple biological processes to achieve anti-diabetes effect. Eighty-five signaling pathways were obtained through KEGG analysis, among which the top 20 pathways related to T2DM mainly included PI3K-Akt signaling pathway, FoxO signaling pathway, IR and TNF signaling pathway (Fig. 6).

3.4 "Peptide-Target-Pathway" network

In order to further elucidate the mechanism of GPAGAP in treating T2DM, a "peptide-targets-pathways" network diagram (Fig. 7) was constructed with 20 pathways, pathway-enriched targets and GPAGAP screened in the above experiment related to

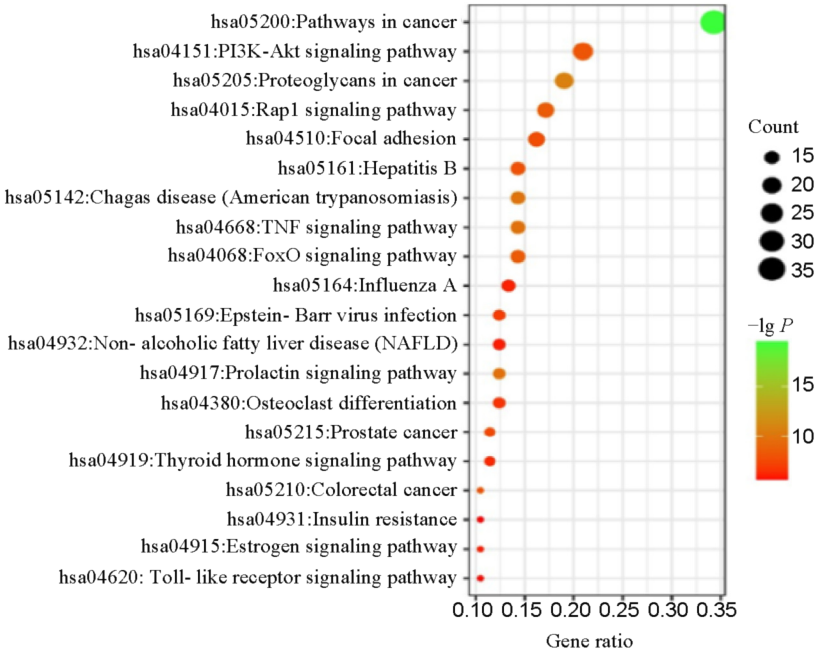

Figure 6 KEGG pathway analysis of GPAGAP-T2DM.

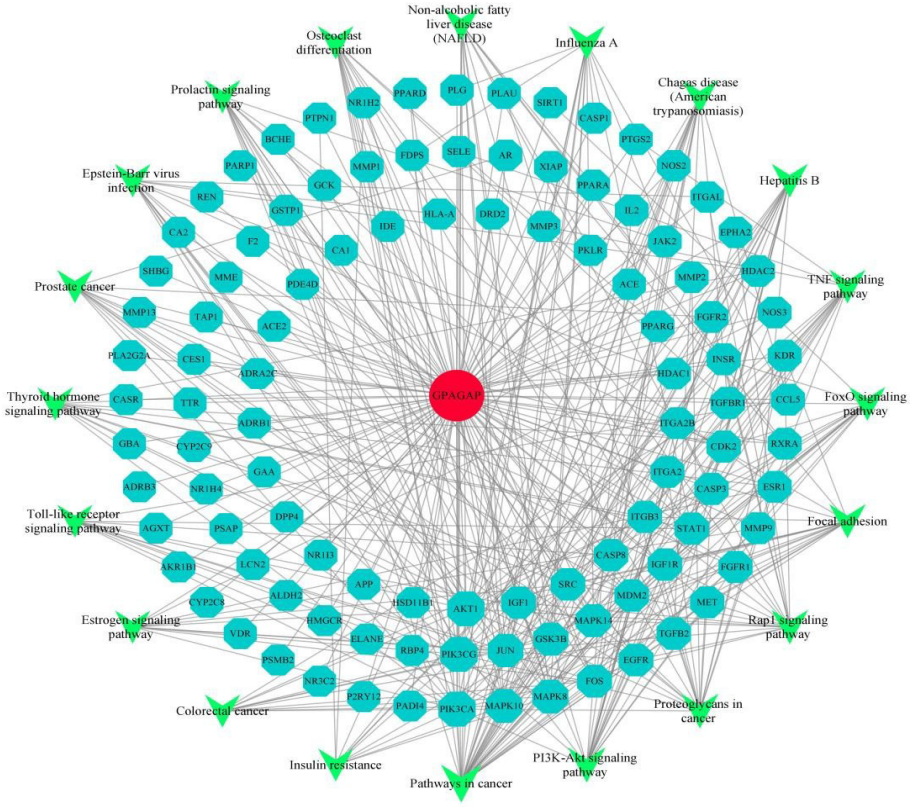

Figure 7 "Peptide-target-pathway" network. The blue nodes represented effect targets, the green ones meant the pathways, and the red one stood for GPAGAP.

T2DM using CytoScape 3.8.0 software. The topological analysis results showed that the degree of PIK3CG (phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit gamma) in the network was 21, the Betweenness was 0.043,13, and the Closeness was 0.572,41. It was predicted that PIK3CG was the main target of GPAGAP in treating T2DM. Serine/threonine kinase proteins (AKT1), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA), mitogen-activated protein kinase 8 (MAPK8), mitogen-activated protein kinase 10 (MAPK10), Jun proto-oncogene (JUN) and mitogen-activated protein kinase 14 (MAPK14) were also relatively important targets. These core targets were mainly enriched in PI3K-Akt signaling pathway, FoxO signaling pathway IR and TNF signaling pathway, indicating that GPAGAP could treat T2DM and its complications through multiple targets and multiple pathways.

3.5 Establishment of IR-HepG2 cell model

IR was a typical feature of T2DM[16]. To evaluate the effect of GPPGPA on T2DM, IR was induced in HepG2 cells by high-glucose (25 mmol/L) and high-insulin, and the IR model was established using glucose consumption as an evaluation index. As shown in Fig. 8A, within the range of insulin concentration of 10^{-9}–10^{-5} mol/L, glucose consumption of HepG2 cells was lower than that of the normal group[17]. The decreases in the rates of glucose consumption were 3.07%, 5.78%, 12.13%, 23.47% and

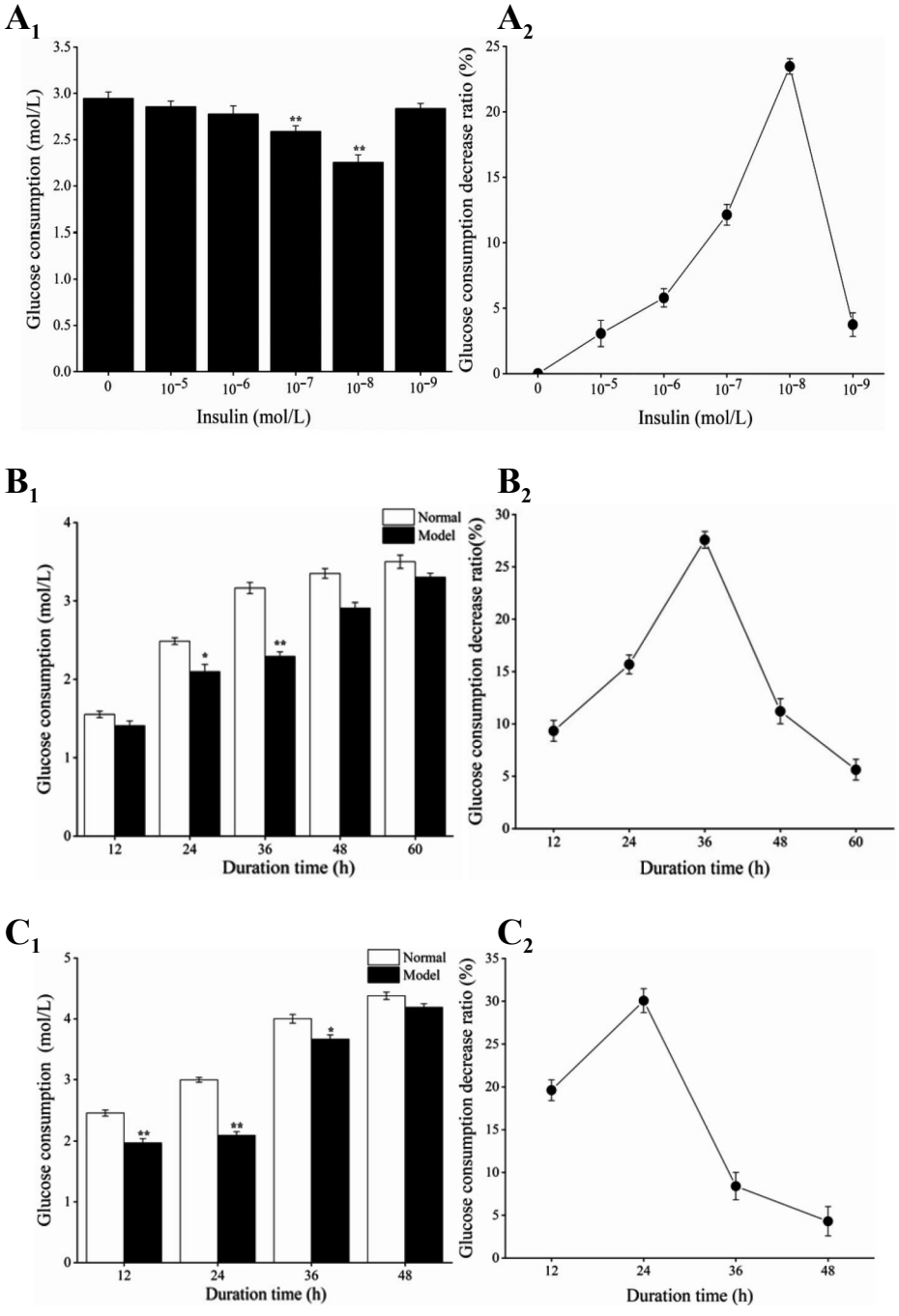

Figure 8 Establishment of IR-HepG2 model induced by high-glucose and high insulin. (A) Effect of insulin concentration on glucose consumption in HepG2 cells, (B) Effect of insulin action time on glucose consumption in HepG2 cells, (C) The stability of IR-HepG2 cell model. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with normal group.

3.74%, respectively. The glucose consumption of 10^{-8} mol/L insulin group was significantly different from that of the normal group ($P < 0.01$), and the decreases in the rates of glucose consumption was the highest. Therefore, 10^{-8} mol/L was the optimal concentration of insulin for inducing HepG2 cells to produce the strongest IR.

As shown in Fig. 8B, when HepG2 cells were treated with 10^{-8} mol/L insulin for 12, 24, 36, 48 and 60 h, glucose consumption decreased by 9.33%, 15.68%, 27.57%, 11.94% and 5.62%, respectively. The decreases in the rates of glucose was the highest at 36 h, and the glucose consumption was significantly different from that of the normal group ($P < 0.01$). Therefore, HepG2 cells induced by 10^{-8} mol/L insulin for 36 h were selected as the IR model cells (IR-HepG2).

The stability of IR model was further tested. After the successful establishment of IR model, model cells were cultured in insulin-free medium with low glucose (5.5 mmol/L) for 12, 24, 36, 48 h. As shown in Fig. 8C, glucose consumption of the model group and the normal group was significantly different at 12, 24 and 36 h, suggesting that the HepG2 cell model induced by high glucose and high insulin could maintain stable IR for 36 h. There was a significant difference in glucose consumption between model group and normal group at 24 h ($P < 0.01$), and the decrease rate of glucose consumption was the highest. Therefore, 24 h was the optimal treatment time for IR-HepG2 model.

3.6 Effect of GPAGAP on glucose consumption of IR-HepG2 cells

IR-HepG2 cells were treated with different concentrations of GPAGAP (0.5, 1 and 2 mmol/L) and metformin (0.5 mmol/L) for 24 h. The results showed that metformin and GPAGAP groups increased glucose consumption (Fig. 9A), cell activity (Fig. 9B) and unit glucose consumption (glucose consumption per unit (GC/CV)) (Fig. 9C) of IR-HepG2 cells. In GPAGAP groups, when the concentration of GPAGAP was 1 mmol/L, glucose consumption, cell activity and unit glucose consumption of IR-HepG2 cells were significantly different from that of model group ($P < 0.01$). This indicated that GPAGAP could effectively reduce IR, and the optimal concentration was 1 mmol/L. Therefore, the concentration of GPAGAP was set to 1 mmol/L in subsequent experiments.

3.7 Effect of GPAGAP on glucose metabolism in IR-HepG2 cells

Glycogen was a multi-branched glucose polysaccharide and a form of human energy storage. It was mainly found in skeletal muscle and liver. Therefore, glycogen synthesis and gluconeogenesis were key factors that control liver glucose output[18]. HK is the first key rate-limiting enzyme in the glycolysis pathway[19], whose activation could increase insulin release and liver glucose utilization, accelerate glucose catabasis and reduce blood glucose. PK is another key enzyme in glycolysis, which catalyzes the final step of glycolysis and transfers the phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), producing a molecule of pyruvate and a molecule of ATP[20]. Therefore, changes in glycogen content and activities of HK and PK could reflect changes in glucose metabolism.

The results showed that glycogen content (Fig. 10A) and

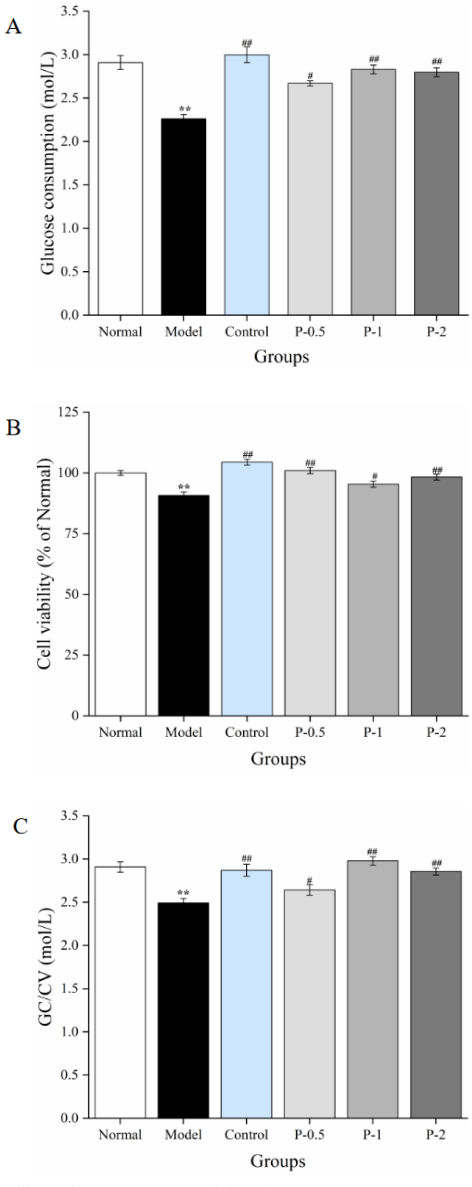

Figure 9 Effect of GPAGAP on cellular glucose consumption. (A) IR-HepG2 cell glucose consumption, (B) IR-HepG2 cell activity, (C) Effect of IR-HepG2 cell glucose consumption per unit. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to normal group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to model group.

activities of HK (Fig. 10B) and activities of PK (Fig. 10C) of IR-HepG2 cells in the model group was significantly lower than that in the normal group HepG2 cells ($P < 0.01$), indicating that IR could restrain the synthesis of glycogen and the activities of HK and PK in the cells. The glycogen content, HK activities and PK activities of metformin (positive control) group and GPAGAP group were significantly increased compared with those of model group IR-HepG2 cells ($P < 0.01$). The results showed that GPAGAP could promote the process of glycogen synthesis and glycolysis, enhance the uptake and utilization of glucose in liver, regulate glucose metabolism and improve IR of cells.

3.8 Effect of GPAGAP on lipid metabolism in IR-HepG2 cells

Excessive accumulation of lipids is a prerequisite for obesity and diabetes[21]. The results showed that compared with the HepG2

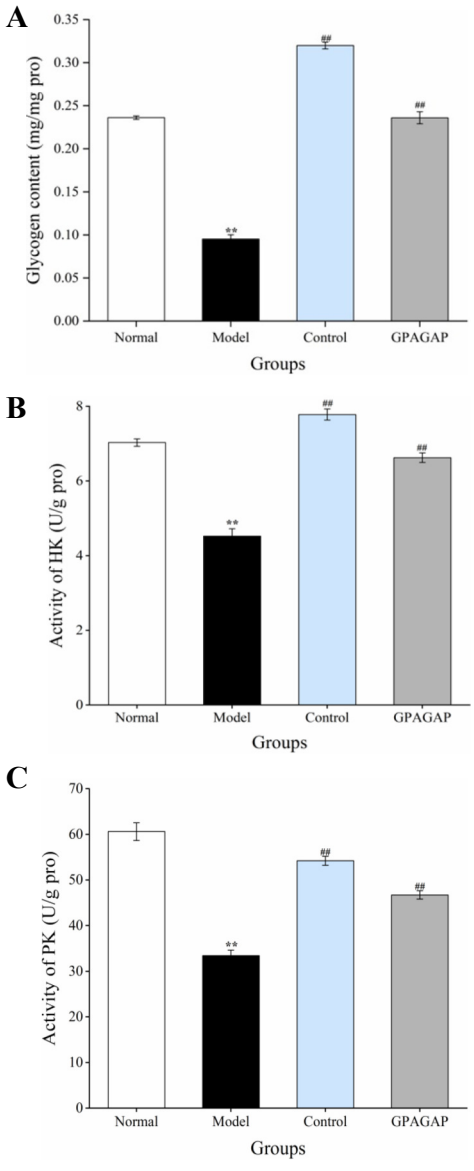

Figure 10 Effect of GPAGAP on Glucose metabolism. (A) Glycogen content, (B) HK activity, (C) PK activity. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to normal group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to model group.

cells of the normal group, the contents of TG (Fig. 11A) and TC (Fig. 11B) in the IR-HepG2 cells of the model group were significantly increased ($P < 0.01$), indicating that TG and TC accumulated in the cells in the IR model and lipid metabolism abnormal. Compared with the model group IR-HepG2, the intracellular TG and TC contents in the positive control group and GPAGAP group were significantly decreased ($P < 0.01$). The results showed that GPAGAP could inhibit the accumulation of TG and TC in IR-HepG2 cells and regulate the lipid metabolism of cells.

3.9 Effect of GPAGAP on oxidative stress in IR-HepG2 cells

Oxidative stress was an important cause of IR[2]. Inhibition of oxidative stress could be used as an adjuvant treatment to lower blood glucose and relieve diabetic complications[22]. The experiment evaluated the effect of GPAGAP on the oxidative stress of IR-HepG2 cells by detecting the level of MDA (Fig. 12A),

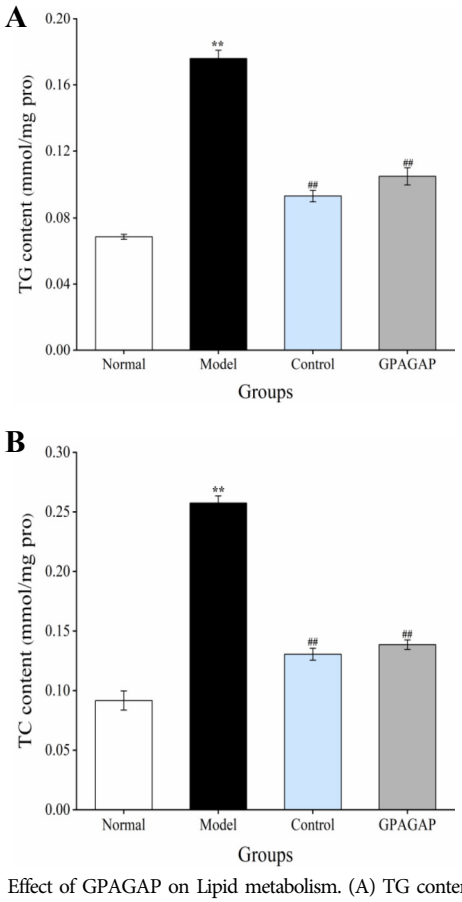

Figure 11 Effect of GPAGAP on Lipid metabolism. (A) TG content, (B) TC content. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to normal group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to model group.

the activity of antioxidant enzymes (SOD) (Fig. 12B) and ROS (Fig. 12C) content. As shown in Fig. 11, compared with normal HepG2 cells, MDA content and ROS content of IR-HepG2 cells in model group were extremely significantly increased ($P < 0.01$), while SOD activity was extremely significantly decreased ($P < 0.01$). The results showed that IR decreased the antioxidant capacity of cells and increased the degree of oxidative stress. Compared with IR-HepG2 group, the significant decrease of MDA content and ROS content and the significant increase of SOD activity in the GPAGAP group indicated that GPAGAP could enhance the antioxidant capacity of IR-HepG2 cells and had a potential protective effect on cellular oxidative stress.

4 Discussion

Many studies had shown that natural active peptides could treat T2DM by lowering blood glucose, but their screening was a time-consuming process and little information had been obtained about their potential mechanisms of action[10]. Therefore, this study fully utilized network pharmacology combined with bioinformatics methods to screen a new peptide GPAGAP from the collagen hydrolysates of *A. davidianus* skin for the treatment of T2DM, predicted and explored its mechanism of action, and preliminarily verified the predicted results by constructing IR-HepG2 cell model. First, the active peptide GPAGAP with good drug-like properties was screened by LC–MS/MS analysis and Lipinski's rule. Using multiple public databases, 105 common targets of GPAGAP and T2DM were mined, and then PPI network was constructed. Further GO and KEGG analysis established the "active peptide-target-pathway" network. The core

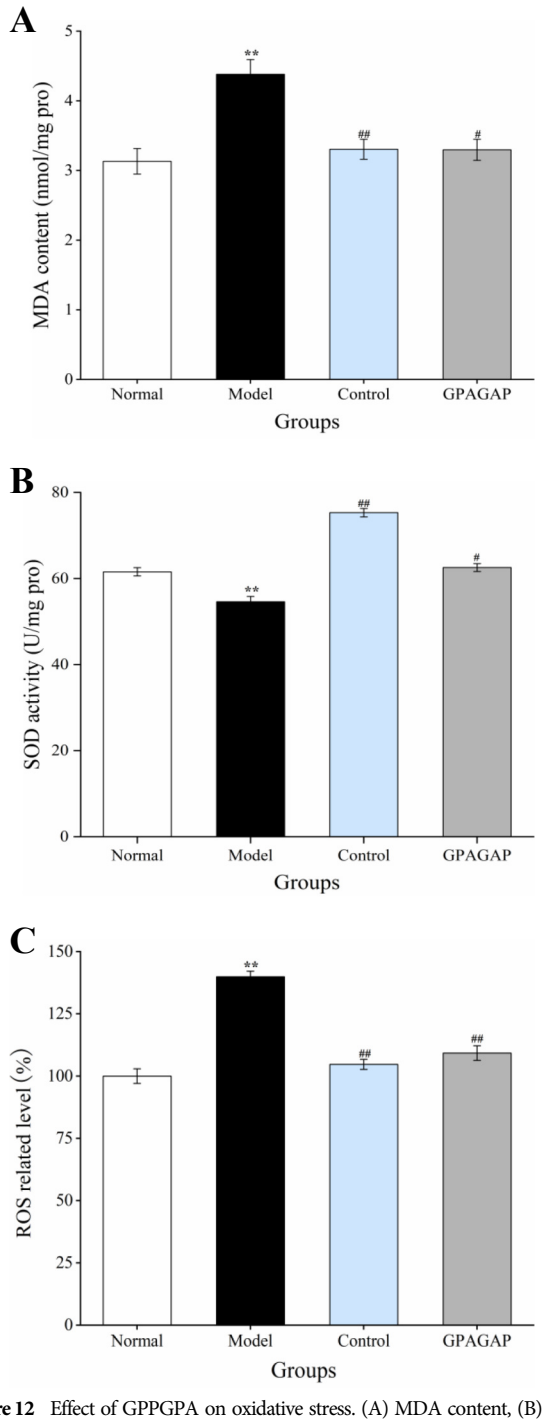

Figure 12 Effect of GPPGPA on oxidative stress. (A) MDA content, (B) SOD activity, (C) ROS related level. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to normal group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared to model group.

targets of PIK3CG, AKT1, PIK3CA, MAPK8 of T2MD improved by GPAGAP were screened. AKT1 was a serine/threonine kinase that promotes cell growth and apoptosis. Down-regulation of *AKT1* mRNA expression could reduce IR in peripheral tissues[23]. Overactivation of AKT1 affected glucose homeostasis through glucagon-mediated mechanisms[24]. PIK3CA was a catalytic subunit of the P13Ks protein family, which was a key protein to maintain the balance of glucose concentration, mainly through reducing glucose uptake to maintain glucose serum level[25]. Meanwhile, P13K could be activated by upstream genes, and the activated P13K could activate AKT and further phosphorylation or inhibit a series of downstream substrates such as apoptosis-related protein changes, thus regulating cell proliferation and apoptosis[26]. Phosphorylation of MAPK8 contributed to the activation of transcription factor activator protein-1 (AP-1), which further initiated the expression of a series of downstream genes and played an important regulatory role in inflammation and diabetes and its complications[11]. JUN, including Jun N-terminal kinase (JNK), Jun B proto-oncogene (JUNB) and Jun C proto-oncogene (JUNC), belonged to the AP-1 transcription factor family and played an important role in inflammatory response[27]. Pan *et al.*[28] reported that inhibition of JNK kinase activity can reduce inflammation and apoptosis induced by high sugar, regulate inflammatory cytokines, promote endocrine metabolism, and participate in the treatment of diabetes and complications. The main targets of GPAGAP in improving T2DM were enriched in signaling pathways such as PI3K-Akt signaling pathway, FoxO signaling pathway, IR and TNF signaling pathway, suggesting that GPAGAP might improve T2DM through multiple targets and multiple pathways.

Many studies had shown that PI3K-Akt signaling pathway, TNF signaling pathway, IR, FoxO signaling pathway and other signaling pathways were related to glucose metabolism, lipid metabolism and oxidative stress levels of organisms. For example, Fuzhu Jiangtang Granules (FJG) could improve antioxidant capacity by reducing MDA level and increasing SOD activity, and the glucose-lowering mechanism was related to P13K-Akt signaling pathway[29]. Casein glycopeptide IPPKKNQDKTE inhibited gluconeogenesis, promoted glycogen synthesis and improved IR by activating IRS/PI3K-Akt signaling pathway[30]. Heme hexapeptide could regulate PI3K-Akt, NRF2-KEAP1 and AMPK signaling pathways to improve IR and promote glucose absorption and glycogen synthesis[31]. Neuropeptide (NPY) could improve IR by reducing the phosphorylation levels of Glycogen synthase kinase 3α (GSK3α), GSK3β, PI3K and Akt[32]. Walnut polypeptides LVRL (Leu-Val-Arg-Leu) and LRYL (Leu-Arg-Tyr-Leu) could protect HepG2 cells from IR and oxidative stress induced by high glucose by activating IRS-1/PI3K/Akt and Nrf2/HO-1 signaling pathways[2]. These reported findings suggested that different natural ingredients might reduce blood glucose through different pathways, thereby improving diabetes. In this study, HepG2 cells were induced by high glucose and insulin to establish IR model successfully, and the effects of GPAGAP on glucose metabolism, lipid metabolism and oxidative stress of IR-HepG2 cells were detected. The results showed that GPAGAP could promote glycogen synthesis and glycolysis, enhance glucose uptake and utilization by liver, and regulate glucose metabolism of IR-HepG2 cells by enhancing glucose consumption and increasing glycogen content, HK and PK activities. GPAGAP can improve lipid metabolism of IR-HepG2 cells by decreasing the content of TG and TC accumulated in IR-HepG2 cells. GPAGAP could enhance the antioxidant capacity of IR-HepG2 cells by decreasing MDA content and ROS content and increasing SOD activity, thus had a potential protective effect on oxidative stress cells. The combined analysis of the prediction results of network pharmacology and the results of cell experiments showed that GPAGAP regulated the glucose metabolism, lipid metabolism and oxidative stress of cells by acting on multiple targets to stimulate different signaling pathways, thereby improving IR in T2MD.

5 Conclusions

In conclusion, based on network pharmacology and

bioinformatics, this study quickly and efficiently screened hypoglycemic peptide GPAGAP from the collagen hydrolysates of *A. davidianus* skin, and constructed a "composition-target pathway" network model to conduct multidimensional and multi-level analysis on the mechanism of GPAGAP in improving T2DM. It provided theoretical support for screening active peptides from proteolytic hydrolyzates and revealing their mechanism of action. In addition, in this study, IR-HepG2 cell experiments were conducted to verify the reliability of the previous screening of GPAGAP and prediction results of action mechanism for improving T2DM from the perspective of improving glucose metabolism, lipid metabolism and oxidative stress, and further provide experimental basis for the action mechanism of GPAGAP in improving T2DM. Although this study verified the potential mechanism of GPAGAP in improving T2DM at the cellular level, the molecular mechanism at the molecular level still requires further experimental studies.

Conflicts of interest

There are no conflicts to declare.

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