Zanthoxylum alkylamides ameliorate protein metabolism disorder in STZ-induced diabetic rats

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Abstract

This study aimed to evaluate the protein metabolism effect of Zanthoxylum alkylamides and to explore the potential mechanism in streptozotocin (STZ)-induced diabetic rats. Diabetic rats were orally treated with 2, 4 and 8 mg per kg bw of alkylamides daily for 28 days. Alkylamides decreased the relative weight of the liver and food intake, significantly increased the relative skeletal muscle weight and significantly decreased the blood urea nitrogen levels. Insulin, insulin-like growth factor 1, total protein (TP) and albumin (ALB), globular proteins and ALB proteins/globulin protein levels in serum significantly increased. TP, RNA content and RNA/DNA ratio significantly increased in the skeletal muscle of diabetic rats. Real-time quantitative polymerase chain reaction results indicated that alkylamides significantly increased the mRNA expression of insulin receptor (InR), IGF1 and insulin-like growth factor 1 receptor (IGF1R) in the liver and skeletal muscle. Moreover, the mRNA and protein expression levels of PI3K, PKB and mTOR significantly increased, whereas those of atrogin-1, muscle ring finger 1 and FOXO in the skeletal muscle significantly decreased. Alkylamides may advance protein synthesis by the PI3K/PKB/mTOR signalling pathway and attenuate the catabolism of protein through the ubiquitin–proteasome pathway. Therefore, it was possible that alkylamides ameliorate protein metabolism disorders in diabetic rats by activating the mTOR pathway.

Introduction

As a global disease, diabetes mellitus seriously endangers human health. It is a glucose, fat and protein metabolism disorder caused by the absolute or relative deficiency in insulin secretion (Michaelides et al. 2016). According to the latest statistics of the International Diabetes Federation, an estimated 415 million adults have diabetes, and 1 of every 11 adults in the world is diagnosed with this disorder. By 2035, the number of diabetics is expected to increase to 592 million, approximately 10% of the total population (IDF, http://www.diabetesatlas.org/). Currently, injection of insulin and oral hypoglycaemic drugs, including sulfonylureas, glinides, biguanides,
α-glucosidase inhibitor and dipeptidyl peptidase-IV inhibitor (Inzucchi 2002, Zhu et al. 2013), is considered the primary treatment for diabetes. However, several problems have emerged, such as drug side effects, drug resistance and drug safety. Therefore, the research and development of natural substances for the safe and efficient treatment of diabetes is important.

Pepper (Zanthoxylum bungeanum Maxim) is a Rutaceae Zanthoxylum (Rutaceae) plant fruit mainly distributed in the Sichuan, Chongqing, Hunan, Shaanxi and Shandong Provinces of China and some Southeast Asian countries. Pepper is commonly used in traditional Chinese medicine and is among the eight main condiments in China. Current research shows that Zanthoxylum alkylamides mainly comprise α-, β- and γ alkylamides, which have antioxidant (Batool et al. 2010, Xia et al. 2011), anticancer (Chou et al. 2011) and hepatoprotective (Guo et al. 2011) effects.

Previous research showed that for rats with a lipid metabolism disorder induced by a high-fat diet, the expression levels of sterol regulatory element-binding protein 2 and 3-hydroxy-3-methylglutaryl-coenzyme reductase in the liver and the ileal bile acid-binding protein and sodium-dependent bile acid transporter in the ileum were significantly reduced by alkylamides. By contrast, the expression levels of transient receptor potential vanilloid subtype 1 (TRPV1) in the liver and ileum were significantly increased by the same kind of reagents. These results revealed that lipid metabolism disorder induced by a high-fat diet was improved by alkylamides (Yu 2015). In addition, fasting blood glucose and plasma fructose levels significantly decreased in diabetic rats, and oral glucose tolerance was improved. Total cholesterol (TC) and triglyceride (TG) levels in the plasma and fat, as well as TC and TG concentrations in the liver, were effectively reduced by alkylamides. These alkylamides prevented the accumulation of fat droplets in the liver of diabetic rats. Alkylamides can also significantly increase the expression of pancreatic–duodenal homeobox 1 (PDX-1), glucose transporter-2 (GLUT2), glucokinase (GK) and TRPV1 in the pancreas of diabetic rats and downregulate the expression of cannabinoid receptor 1 (CB1) in the liver and pancreas (Dossou et al. 2013, You et al. 2015). This explanation indicates that alkylamides can inhibit gluconeogenesis, reduce glycogen output, repair islet function and promote insulin secretion (Oh et al. 2013). However, diabetes is not only characterised by elevated blood glucose and blood lipid levels, but it also causes a protein metabolic disorder (Michaelides et al. 2016). Alkylamides can promote insulin secretion (You et al. 2015), which can promote protein synthesis by activating the PI3K/Akt/mTOR signalling pathway and inhibiting protein decomposition (Lim et al. 2016). However, the effect of alkylamides on protein synthesis and decomposition in diabetic rats has not been reported. Protein synthesis and decomposition have been found to be dependent on the mammalian target of rapamycin (mTOR) pathway. This observation implies that mTOR can be used as a cross-site of protein synthesis and decomposition process. mTOR plays a role in regulating protein metabolism by controlling upstream and downstream molecules (Fogg et al. 2011). The ubiquitin–proteasome pathway (UPP) connects the ubiquitin to the target protein by a three-enzyme cascade. UPP is the most vital and selective protein degradation pathway in mammalian cells, and it mediates the degradation of 80–85% protein in eukaryotes (Chen et al. 2012, Lu et al. 2013). Therefore, streptozotocin (STZ)-induced diabetic rats were used as models to explore the effect of alkylamides on protein metabolism disorder and its mechanism of action. These animal models provided reference for the comprehensive evaluation of the role of alkylamides in diabetic treatment.

Materials and methods

Alkylamide extraction and identification

Following the method of You and coworkers (You et al. 2015), 20g of Z. bungeanum oleoresin and 40g of thin-layer chromatography silica gel (GF-254) activated for 1h at 110°C were stirred and mixed together. A total volume of 200mL of absolute ethyl ether was added to the mixture. The mixture was then sealed and mixed with a magnetic mixer for 2h prior to filtration. The filtrate was discarded afterward. The upper layer was dried and methanol five times its volume was added. The mixture was then sealed and extracted for 6h in a water bath at 55°C prior to filtration. The filtrate was discarded and the extract was collected. The extract was steam-dried by a rotary evaporator. Up to 50mL of ether was added at a boiling range of 30–60°C. Then, reflux extraction was performed for 10h in a water bath at 50°C. The supernatant was frozen and crystallised in a −20°C refrigerator.

Purity analysis of alkylamides was performed by a high-performance liquid phase (Agilent 1260). Exactly 0.05mg alkylamides dried by nitrogen was dissolved in methanol at a volume of 5mL and filtered through a 0.45μm microporous membrane. The samples were applied to a C18 column (4.6mm × 250mm, 5μm, Agilent). Mobile phase A had 50% water and mobile
phase B had 50% methanol. The flow rate was 1 mL/min, sample volume was 10 µL, column temperature was 40°C and ultraviolet detection wavelength was 254 nm.

Diabetes induction and experimental design

This study was conducted in strict compliance with the recommendations of the State Scientific and Technological Commission and with the Guide for Sichuan Laboratory Animal Management Committee (in Chinese). The protocol was approved by Chengdu Qiankun Animal Pharmaceutical Co. Ltd. (Permit Number, SYX (Chua) 2014-190). Eight-week-old male Sprague–Dawley rats were acquired from Chongqing Tengxin Laboratory Animals, Inc. (Chongqing, China; animal licence SCXK 2012-0008). The rats were housed individually in stainless steel metabolism cages (25 cm × 15 cm × 15 cm) in a temperature- and humidity-controlled room maintained at 25 ± 2°C with 12h/12h light/darkness cycles. The rats were allowed free access to a standard diet (Chongqing Tengxin Laboratory Animal, Inc., Chongqing, China) and water ad libitum. The rats were acclimatised to the laboratory environment for one week.

After subjected to overnight fasting, the rats were intraperitoneonally injected with 60 mg per kg bw of STZ (Sigma Chemicals) dissolved in 0.1 mol/L of citrate buffer (pH 4.5) (Qiao et al. 2014). The rats in the normal control group were treated with the same volume of citrate buffer. Three days after STZ was injected, the fasting blood glucose (FBG) of the rats was measured from the tail tip. The rats with FBG levels above 11.1 mmol/L were used as diabetic rats for further study (Baranwal et al. 2015). Fifty rats were randomly divided into five groups, with ten rats in each group. These groups were divided into DM-HD (8 mg per kg bw), DM-MD (4mg per kg bw), DM-LD (2 mg per kg bw), normal group and diabetic model groups. The dose of 0.1 mL (soybean oil)/100 g (body weight) for the normal and diabetic model groups was administrated by gavage, and it was the same dose of soybean oil without alkylamide. The experimental rats were administered with normal feeding and drinking. Daily feed intake and weight of rats were recorded, and FBG was measured every two weeks. Test cycle was set to 28 days. Food supply was blocked for 12h on the last day of the experimental period. The rats were then injected with ether anaesthesia and decapitated. Their blood was extracted and collected in an anticoagulant vessel (Shandong Aosite Medical Instrument Factory, Shandong, China) and then centrifuged at 2683 g 4°C for 15 min. The supernatant was preserved in −20°C. Liver, kidney and gastrocnemius were preserved at −80°C.

Protein synthesis and protein degradation

Protein synthesis was determined by the incorporation of 14-C-phenylalanine (0.05 µCi/mL) (China Institute of Atomic Energy, Beijing, China) into the skeletal muscle in unit time (Voltarelli & de Mello 2008). Weighed fresh skeletal muscle was placed in 5 mL DMEM (Hyclone, Utah, USA), incubated for 30 min with oxygen at 37°C and then incubated for 1 h in DMEM containing 14-C-phenylalanine. The skeletal muscle was then washed three times with phosphate-buffered saline and homogenised. Protein was precipitated with 10% TCA; the supernatant was discarded. Protein was then dissolved with 0.5 mol/L NaOH, and 90% protein was added to the liquid scintillation count. About 10% of protein was used to determine protein concentrations by the RCA method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Skeletal muscle catabolism was assessed by the tyrosine release rate assay of the culture medium (Li et al. 2016). Left skeletal muscle was weighed and immersed in Basal–Krebs–Ringer buffer (1.2 mmol/L NaCl, 4.8 mmol/L KCl, 25 mmol/L NaHCO3, 2.5 mmol/L CaCl2, 1.2 mmol/L KH2PO4, 1.2 mmol/L MgSO4, 5.5 mmol/L glucose, 1.0 g/L bovine serum albumin, 5 U/mL insulin and 5 mmol/L cyclohexamide at pH 7.4). The tissue was then incubated for 2 h with oxygen at 37°C. The culture medium was precipitated with TCA. Supernatant (0.5 mL) was placed in a 2 mL centrifuge tube, and then 1.0 mL 5% TCA was added and mixed with the supernatant. Subsequently, 0.75 mL nitric acid and 0.75 mL 1-nitroso-2-napthol were added and mixed. The supernatant was incubated for 30 min at 55°C and extracted by ethylene dichloride; 200 µL of supernatant was used to measure fluorescence spectrometry (Ex. 450 nm, Em. 550 nm). The final concentration of the samples was calculated by the l-tyrosine standard curve.

Physiological and biochemical analyses

Nucleic acid content determination was conducted with reference to Leonard (Johnson & Chandler 1973). The FBG and fructosamine levels in the plasma were determined using a commercially available kit (Sichuan Maker Biotechnology Co., Ltd, Sichuan, China) on a Hitachi 7020 automatic biochemistry analyser.
RNA extraction and reverse transcription PCR (RT-PCR)

All tissues were homogenised in TRIzol Reagent (Invitrogen), and total RNA was extracted according to the RNA Extraction Kit (OMEGA, USA). The quality of extracted RNA was assessed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Optical purity and density were 1.8 < 260/280 nm < 2.0. Reverse transcription was performed using MLV-RT (Promega).

The rat housekeeping gene β-actin was used as an internal control. Primers for the real-time qRT-PCR were designed by primer 5.0 (Table 1). Real-time qRT-PCR was performed in a CFX96 Touch Real-Time System (Bio-Rad) using SYBR Green Supermix (Bio-Rad). Amplification was conducted in a 10 µL reaction mixture containing 1 µL cDNA (100 ng/µL), 4.2 µL DEPC water, 0.3 µL forward or reverse primer (20 µmol/L) and 4.2 µL SYBR Green Supermix (Bio-Rad) in each well of a 96-well plate. The reaction procedure was 94°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 40 s. Melting curve analysis was performed to confirm the specific amplification. Each expression assay was repeated three times. The CFX Manager Bio-Rad 3 software was used to process real-time quantitative PCR data after amplification. Relative expression levels of the target gene (X) were calculated in relation to the transcription levels of the actin reference gene (R) as 2-ΔΔt, where ΔCt=RtX–RtR. SPSS 20.0 was used to analyse the variance and significance of the results.

Western blot analysis

Skeletal muscle samples were collected from each group and homogenised in an ice-cold lysed buffer containing 2 µg/mL of protease inhibitor (Bi-Yuntian, Beijing, China). The suspensions were centrifuged at 14,000 g at 4°C for 15 min. The supernatant was collected to determine protein content through the BCA method. The proteins were separated by 10% SDS-PAGE and then transferred to a 0.22 µm PVDF membrane. The membranes were blocked with 5% defatted milk powder at 37°C for 2 h and then incubated with anti-mTOR, anti-P-mTOR (Ser2448), anti-PKB, anti-P-PKB (Ser473), anti-PI3K (p110), anti-FOXO1A, anti-FOXO3A, anti-FOXO4A, anti-atrogin-1, anti-MURF1 and anti-tubulin (Abcam) antibodies overnight at 4°C. Afterward, the membranes were washed with TBST and incubated with the corresponding species-specific secondary antibodies (Bioss, Beijing, China) at 37°C for 1 h. After the membrane was washed with TBST, the proteins were visualised through ECL (Bio-Rad) under a chemiluminescence imaging apparatus (Bio-Rad).

Statistical analysis

Results were expressed as mean±standard error and analysed by one-way analysis of variance. Student’s t test was used to detect the differences in the mean between the normal group and the diabetic rat group. The differences were considered significant when P<0.05. Data were processed using SPSS 20.0 and Origin 9.0.

Table 1 Primer sequence and product size.

| Gene     | Forward primer             | Reverse primer            | Product size |
|----------|-----------------------------|---------------------------|--------------|
| mTOR     | ACCAAGGCGCTGGGACCTCTTA     | GGCTGGTTGGGGGTATGTT       | 156          |
| PI3      | CTCAGGGGAAACGTGGACACAC     | TGGTCAACAGGGCTCTTGTG      | 184          |
| PKB      | ACTTCATCCAGACACGAGC        | CCGTACACGCTGTCTTCTT       | 176          |
| Atrogin-1| CATCAGGGAAAGTGGGTATGTT     | GCTCTCCCCAAATGCGATA       | 84           |
| MuRF1    | ATCGGCTGTTGTGGACGAC        | TGGAAAGATGTCGCGACCA       | 355          |
| FOXO1    | TAAGCGCAAATCCAGCAT         | TGGGAGAGAGAAGTACAGTA      | 143          |
| FOXO3A   | CGGCTCATTGGTTTCATGAT       | TCTTGCACTCCATTGGTC        | 163          |
| FOXO4A   | CTGGTTTCTGTTGGTCTGTC       | TAAGCTGCTGCTTGTGCC        | 93           |
| IG1F     | GGCCATCTGGTCTGCTCACCTTT   | CAGCAATTAGAGGACGGCTGCCAC  | 91           |
| IG1FR    | CACCTGGGAAGACCGGCATCATAA  | ATCTGCCCAGCTGGTGCGATG     | 130          |
| InR      | TGCGCTTGTGGTCCAAAGCGTA    | GGTTCTGTTGTCCAAAGCGTA    | 110          |
| β-Actin  | TGCTTACACTGGCATTGAT        | CGAAGTCATGGCACAATAGCA     | 233          |

(= Actin High-Technologies Corp., Tokyo, Japan). Plasma insulin and the insulin-like growth factor (IGF1) were measured by RIA with the Bi-Insulin RIA kit (ERIA Diagnostics Pasteur, Marnesla Coquette, France). Blood urea nitrogen (BUN) was measured by urea enzymatic assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer’s protocols. Protein concentrations were calculated using BCA assay kits (Pierce, USA).
Results

HPLC analysis of alkylamides

The extracted alkylamides were analysed by HPLC, and the retention times of 9.570, 9.758 and 10.153 min were obtained. The relative contents of hydroxy-α-sanshool, hydroxy-β-sanshool and hydroxy-γ-sanshool were 26.48, 39.83 and 29.19%, respectively (Fig. 1), consistent with those in previous studies (Dossou et al. 2013, Yu 2015).

Effect of alkylamides on the body weight and relative weight of organs

After 28 days, the weight gain of the diabetic group was significantly lower than that of the normal control group, and the weight of the alkylamide-treated group increased significantly. Compared with that of the normal group, feed intake of the STZ-induced diabetic rats and relative liver weights significantly increased, and the relative skeletal muscle weights significantly decreased. Alkylamides can significantly reduce feed intake and relative liver weights and significantly elevate the relative skeletal muscle weights (Table 2). Moreover, compared with those in the diabetic model, TP, RNA content and RNA/DNA ratio were significantly increased by alkylamides in the skeletal muscle of diabetic rats, but no significant difference was found in DNA content (Table 2).

Plasma parameters

The BUN level of the STZ-induced diabetic rats was markedly increased compared with that of the normal group (P < 0.05). Alkylamides could significantly reduce the BUN level. The BUN levels of diabetic rats administered intragastrically with high (DM-HD), middle (DM-MD) and low (DM-LD) doses of alkylamides were reduced by 25.81, 35.26 and 14.57%, respectively (Table 3). The Cr content of diabetic rats markedly increased compared with that of the normal group, and it significantly reduced compared with that of STZ-induced diabetic rats by alkylamides (Table 3).

The TP, albumin (ALB) and globulin (GPs) levels in the serum reflect the state of protein absorption and metabolism. The TP, ALB and ALB/GPs levels of the STZ-induced diabetic rats markedly decreased compared with those of the normal group. Alkylamides can significantly enhance TP, ALB and ALB/GPs levels in the serum of diabetic rats (Table 3).

Compared with the normal group, the serum insulin content of the model group was significantly reduced by 156.49% (P < 0.05). With alkylamide treatment for 28 days, the serum insulin levels of diabetic rats increased by 74.71, 55.40 and 31.45% in DM-HD, DM-MD and DM-LD (P < 0.05), respectively. IGF1 was also significantly elevated, with DM-HD (8 mg per kg bw) and DM-MD (4 mg per kg bw) having the most significant increase (Table 3). In addition, alkylamides significantly reduced FBG and fructosamine levels in the serum of diabetic rats (Fig. 2A and B).

Effects of alkylamides on muscle protein synthesis and protein degradation

Compared with the normal findings, protein synthesis was significantly reduced and protein catabolism was significantly increased in the diabetes model (P < 0.05) (Fig. 3A and B). With alkylamide treatment, protein synthesis significantly increased, and the increase in high and medium doses was significantly higher than that in the low dose (Fig. 3A). Interestingly, protein catabolism was significantly reduced (Fig. 3B).

Effect of alkylamides on the expression of genes InR, IGF1 and IGF1R

Compared with that of the normal group, the mRNA expressions of the insulin receptor (InR) in the liver and the skeletal muscle of the model group were significantly reduced by 89.86% and 225.00% (P < 0.05), respectively. With alkylamide treatment for 28 days, the mRNA expressions of InR in the liver of diabetic rats were elevated by 63.04, 77.54 and 56.52% (P < 0.05) and those in the skeletal muscle were elevated by 208.33, 216.67...
and 158.33% (P < 0.05) in DM-HD, DM-MD and DM-LD, respectively (Fig. 4A).

Compared with that of the normal group, the mRNA expression of IGF1 and IGF1R in the liver and skeletal muscle of the diabetic rats markedly decreased, but it increased remarkably compared with that of the STZ-induced diabetic rats after 28 days of alkyamide treatments (Fig. 4B and C). The mRNA expressions of IGF1R in the liver of DM-HD, DM-MD and DM-LD groups did not show any significant difference (Fig. 4B).

### Skeletal muscle expression of genes related to protein synthesis

Compared with those in the normal control, mTOR mRNA and protein levels were reduced by 147.42% and 28.82% (P < 0.05), respectively; p-mTOR (Ser2448) protein levels were upregulated by 5.05% (P > 0.05); PKB mRNA and protein levels were reduced by 81.96% and 104.71% (P < 0.05), respectively; p-PKB (Ser473) protein levels were upregulated by 76.5% (P > 0.05); PI3K mRNA levels were reduced by 114.98% and PI3K (p110) protein levels by 103.66% (P < 0.05). Compared with those in the diabetic model, mTOR mRNA levels were elevated by 47.96, 47.71 and 25.55% (P < 0.05); mTOR protein levels were upregulated by 11.83, 25.76 and 17.46% (P < 0.05) and p-mTOR (Ser2448) protein levels were upregulated by 52.39, 100.21 and 53.78% (P < 0.05). PKB mRNA levels were elevated by 18.18, 18.06 and 10.61% (P < 0.05); PKB protein levels were upregulated by 43.72, 94.58 and 31.91% (P < 0.05) and p-PKB (Ser473) protein levels were upregulated by 36.18, 78.92 and 37.43% (P < 0.05). PI3K mRNA levels were elevated by 47.96, 47.71 and 25.54% (P < 0.05); and PI3K (p110) protein levels were upregulated by 26.47, 48.03 and 24.97% (P < 0.05) (Fig. 5A, B and C).

### Table 2 Growth performance and muscle characteristics.

|                  | NC       | DM       | DM-HD    | DM-MD    | DM-LD    |
|------------------|----------|----------|----------|----------|----------|
| Initial weight (g) | 203.47 ± 8.35<sup>a</sup> | 205.45 ± 6.08<sup>a</sup> | 198.50 ± 8.50<sup>a</sup> | 208.25 ± 7.74<sup>a</sup> | 208.88 ± 6.66<sup>a</sup> |
| Growth rate (g/4 W) | 90.67 ± 7.06 | 15.38 ± 3.70<sup>a</sup> | 36.78 ± 3.91<sup>c</sup> | 42.00 ± 4.46<sup>b</sup> | 24.57 ± 3.35<sup>b</sup> |
| Feed intake (g/4 W) | 556.70 ± 24.15 | 103.76 ± 25.82<sup>a</sup> | 800.09 ± 30.77<sup>b</sup> | 852.79 ± 21.29<sup>b</sup> | 926.59 ± 26.62<sup>b</sup> |
| Skeletal muscle (%) | 3.18 ± 0.29 | 2.19 ± 0.47<sup>a</sup> | 2.79 ± 0.23<sup>b</sup> | 2.97 ± 0.59<sup>b</sup> | 2.58 ± 0.45<sup>c</sup> |
| Liver (%)         | 2.91 ± 0.48 | 4.50 ± 0.23<sup>a</sup> | 3.25 ± 0.48<sup>b</sup> | 3.46 ± 0.19<sup>b</sup> | 3.76 ± 0.29<sup>c</sup> |
| TP (mg/g)         | 5.53 ± 0.14 | 3.94 ± 0.20<sup>a</sup> | 4.61 ± 0.30<sup>b</sup> | 4.54 ± 0.77<sup>b</sup> | 4.21 ± 0.29<sup>c</sup> |
| RNA (mg/g)        | 0.38 ± 0.001 | 0.33 ± 0.002<sup>a</sup> | 0.36 ± 0.001<sup>b</sup> | 0.36 ± 0.001<sup>b</sup> | 0.34 ± 0.001<sup>c</sup> |
| DNA (mg/g)        | 0.31 ± 0.005 | 0.31 ± 0.005 | 0.31 ± 0.003 | 0.31 ± 0.009 | 0.31 ± 0.011 |
| RNA/DNA ratio     | 1.20 ± 0.02 | 1.05 ± 0.01<sup>a</sup> | 1.15 ± 0.01<sup>b</sup> | 1.14 ± 0.03<sup>b</sup> | 1.10 ± 0.04<sup>c</sup> |

Values are the means ± s.e.m. (n = 10). Means with different superscript letters are significantly different among the diabetic rats (**P < 0.05**); *means are significantly different (**P < 0.05**) from the means in the control group.

### Table 3 Plasma parameters.

|         | NC       | DM       | DM-HD    | DM-MD    | DM-LD    |
|---------|----------|----------|----------|----------|----------|
| BUN (mmol/L) | 4.95 ± 0.28 | 9.36 ± 0.57<sup>a</sup> | 7.44 ± 0.46<sup>b</sup> | 6.92 ± 0.39<sup>b</sup> | 8.17 ± 0.52<sup>c</sup> |
| Cr (µmol/L)  | 35.36 ± 1.93 | 46.69 ± 1.18<sup>a</sup> | 34.76 ± 1.38<sup>b</sup> | 36.94 ± 1.85<sup>b</sup> | 41.65 ± 1.73<sup>c</sup> |
| Insulin (U/mL) | 37.73 ± 1.28 | 14.71 ± 1.53<sup>a</sup> | 25.70 ± 1.42<sup>b</sup> | 22.86 ± 1.55<sup>b</sup> | 20.28 ± 1.35<sup>c</sup> |
| IGF1 (ng/mL) | 48.14 ± 2.54 | 22.44 ± 2.45<sup>a</sup> | 35.23 ± 2.53<sup>b</sup> | 40.77 ± 2.09<sup>c</sup> | 32.35 ± 2.32<sup>d</sup> |
| TP (g/L)     | 64.77 ± 2.21 | 42.60 ± 2.13<sup>a</sup> | 56.68 ± 1.59<sup>b</sup> | 54.18 ± 1.46<sup>b</sup> | 48.69 ± 2.23<sup>c</sup> |
| ALB (g/L)    | 39.93 ± 1.06 | 16.86 ± 1.33<sup>a</sup> | 28.18 ± 1.44<sup>b</sup> | 27.23 ± 1.40<sup>b</sup> | 20.30 ± 2.10<sup>c</sup> |
| GPS (g/L)    | 24.84 ± 1.21 | 25.74 ± 0.85 | 28.51 ± 2.19<sup>b</sup> | 26.95 ± 1.01 | 28.39 ± 0.43 |
| ALB/GPs      | 1.61 ± 0.04 | 0.65 ± 0.03<sup>a</sup> | 0.99 ± 0.12<sup>b</sup> | 1.01 ± 0.07<sup>b</sup> | 0.71 ± 0.08<sup>a</sup> |

Conditions were the same as those defined in Table 2. Values are the means ± s.e.m. (n = 10). Means with different superscript letters are significantly different among the diabetic rats (**P < 0.05**); *means are significantly different (**P < 0.05**) from the means in the control group.
levels were increased by 68.60% and 36.07%; FOXOA3 mRNA and protein levels were increased by 46.94% and 27.34% and FOXOA4 mRNA and protein levels were increased by 16.13% and 61.45%, respectively (Fig. 6A, B and C). Compared with the diabetic model, groups that received alkylamide treatment for 28 days downregulated atrogin-1 mRNA levels by 58.20, 71.32 and 53.73% and atrogin-1 protein expression levels were upregulated by 57.27, 54.37 and 63.86% in DM-HD, DM-MD and DM-LD (P<0.05), respectively. MURF1 mRNA levels were downregulated by 36.56, 47.13 and 15.36%, and MURF1 protein expression levels were upregulated by 108.16, 44.14 and 20.66% in DM-HD, DM-MD and DM-LD (P<0.05), respectively. FOXOA1 mRNA levels were downregulated by 66.78, 62.78 and 41.79%, and FOXOA1 protein expression levels were upregulated by 118.15, 52.25 and 24.08% in DM-HD, DM-MD and DM-LD (P<0.05), respectively. FOXOA3 mRNA levels were downregulated by 70.60, 54.83 and 33.94%, and FOXOA3 protein expression levels were upregulated by 43.31, 59.97 and 45.48% in DM-HD, DM-MD and DM-LD (P<0.05), respectively. FOXOA4 mRNA levels were downregulated by 16.00, 13.87 and 12.83%, and FOXOA4 protein expression levels were upregulated by 86.91, 121.39 and 96.85% in DM-HD, DM-MD and DM-LD (P<0.05), respectively (Fig. 6A, B and C).

Discussion

Diabetes mellitus is a metabolic disorder. Although many thorough studies have been conducted on type 2 diabetes mellitus (T2DM), the incidence of type 1 diabetes (T1DM), which has extremely serious complications, is increasing yearly (Tierney et al. 2012). Therefore, in recent years, the pathogenesis of T1DM has been examined intensely in research. STZ is widely used in the construction of the diabetes model experiment because it can specifically destruct pancreatic beta cells, which cause T1DM (Bortolin et al. 2015, Carlos et al. 2015, Lin & Sun 2015). In this experiment, the FBG levels of the normal control group were maintained within the normal range (4–6 mmol/L) throughout the feeding period. The diabetic group always achieved an FBG greater than 11 mmol/L, thus showing that the model was constructed successfully and stably. The FBG and fructosamine levels of the rats in the alkylamide-treated groups significantly decreased after two weeks of the experiment (Fig. 2A and B). This finding is consistent with that in previous studies (You et al. 2015).

To a certain extent, as an important biochemical index of animals, TP reflects the level of protein deposition (Friesen et al. 1992). The BUN level is a reflection of protein metabolism status and can be used as an indicator of nitrogen utilisation and protein deposition. Decreased BUN can increase nitrogen deposition and protein synthesis (Chikhou et al. 1993, Newell 1999, Wang et al. 2011, Zhang et al. 2012). Results showed that protein contents in the serum and skeletal muscle significantly increased and that the BUN content was significantly decreased by alkylamides in diabetic rats (Tables 2 and 3). These findings reveal the increase in protein deposition...
Alkylamides ameliorate protein metabolism disorder

In diabetic rats, the number of organelles in cells is constant, and DNA content is not sensitive to the change in environmental conditions (Dorch et al. 1983). The RNA/DNA ratio was not affected by the number and cell size in the sample; thus, estimating the growth state is more accurate than the single RNA content (Laimek et al. 2008). Results show that TP content, RNA content and RNA/DNA ratio of skeletal muscle were increased by alkylamides in diabetic rats. Conversely, DNA content did not significantly change and the apparent relative muscle skeletal weight increased (Tables 2 and 3). At the same time, alkylamides accelerated skeletal muscle protein synthesis in diabetic rats in the in vitro experiment (Fig. 2A). These results imply that alkylamides can accelerate protein synthesis and promote skeletal muscle protein deposition in diabetic rats.

Diabetes not only increases blood glucose and lipid levels but also causes protein metabolism disorder (Michaelides et al. 2016). Consumptive disease usually decreases total serum protein and ALB (Hu et al. 2004). The protein consumption of diabetic rats was higher than that of the normal control group with alkylamide treatment, and the TP and ALB consumption was reduced (Table 3). ALB/GPs <1 commonly exist in nephrotic syndrome (Zhon 2015). ALB/GPs >1.0 in the normal control, ALB/GPs <1.0 in the diabetic model and the BUN and Cr in the diabetic model were significantly higher than those in the normal group. These values show that the diabetic rats suffered from a complicated kidney disease caused by protein metabolism disorders. With alkylamide treatment, BUN and Cr content significantly decreased in the serum, and the ratio of ALB/GPs was higher than that of the model group (Table 3). In vitro skeletal muscle culture also indicated that alkylamides could significantly reduce muscle protein catabolism (Fig. 2B). This finding reveals that alkylamides can regulate protein metabolism. The mechanism may be that alkylamides repair damaged islet cells and tissues and promote the secretion of insulin (You et al. 2015). With alkylamide treatment, plasma insulin and IGF1 level of the alkylamide-treated group were significantly elevated (Table 3). Insulin can promote protein synthesis and inhibit protein decomposition (Michaelides et al. 2013). Increased insulin and IGF1 contents in the serum can enhance the protein metabolism of the body. The parameter results indicate that alkylamides can ameliorate protein metabolism disturbance in STZ-induced diabetic rats. In addition, IGF1 can stimulate skeletal muscle development through the autocrine and paracrine pathways (Sacheck et al. 2004). Insulin and IGF1 functions are performed mainly by binding to their respective receptor InR and IGF1R (Brisson & Barton 2012, Shen et al. 2015). The mRNA expression of InR, IGF1 and IGF1R was significantly increased by alkylamides in the liver and skeletal muscle of the diabetic rats (Fig. 4A, B and C). Alkylamides may ameliorate protein metabolism disturbance through the mediation of insulin and IGF1.

Figure 3
Protein synthesis and protein degradation. (A) Protein synthesis was measured from the rate of incorporation of L-[U-14C]phenylalanine into isolated, incubated skeletal muscle. (B) Protein degradation was measured as the rate of tyrosine released from isolated skeletal muscle. Values are the means±s.e.m. (n=10). Conditions were the same as those defined in Fig. 2. Means with different superscript letters are significantly different among the diabetic rats (P<0.05); *means are significantly different (P<0.05) from the means in the control group.

of diabetic rats. Messenger RNA and transporter RNA are involved in protein synthesis. Once the protein synthesis rate is accelerated, RNA content increases. Thus, RNA content is a sensitive index for measuring the biological growth rate. The number of organelles in cells is constant, and DNA content is not sensitive to the change in environmental conditions (Dorch et al. 1983). The RNA/DNA ratio was not affected by the number and cell size in the sample; thus, estimating the growth state is more accurate than the single RNA content (Laimek et al. 2008). Results show that TP content, RNA content and RNA/DNA ratio of skeletal muscle were increased by

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In the past few years, studies were conducted on insulin and IGF1 signalling pathways leading to translation initiation, which regulates the changes in protein synthesis. mTOR can be activated by amino acids and insulin stimulation (Wang & Proud 2010, Liu et al. 2012). The main stage of the entire process of cellular protein synthesis is the translation of proteins, and the translation initiation process determines the translation speed of mRNA. mTOR plays a key role in process of IGF1-, insulin- and amino acid-regulating translation initiation. In the regulatory pathways, IGF1 binds with IGF1R to form the IGF1R/IR complex; multi-tyrosine residues of phosphorylated IGF1R/IR are activated; activated IGF1R/IR recruits the p85 subunit of PI3K through the phosphorylated substrate protein and transmits a signal to the P110 subunit to trigger the activation of PI3K; activated PI3K catalyses the tertiary phosphorylation of PI-4-P, PI-4 and 5-P2 to create PI3, 4-P2 and PI3, 4 and 5-P3; and the downstream molecules PKB convene to the cytomembrane. PI-3, 4 and 5-P3 can activate phosphatidylcholine-dependent kinase-1 (PDK-1), thus inducing the 308 Thr phosphorylation of the PKB in the presence of PDK-2. The 473 Ser phosphorylation of the PKB (phosphorylated PKB) activates mTOR through the activation of Rheb (the Ras homolog enriched in the brain), which is the upstream factor of mTOR (Fan et al. 2004, Kang & Cho 2015, Meena & Kanwar 2015, Sun et al. 2016). Moreover, amino acid can be used as a signal molecule to directly act on the mTOR for activation (Hara et al. 1998, Kimball & Jefferson 2006, Dukes et al. 2015, Trevizani et al. 2015). Activated mTOR improves protein synthesis through the direct phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and ribosome protein S6 kinase 1 (S6K1). The phosphorylation of 4EBP1 inhibits the binding to the cap-binding protein, making it and the cap structure dependent on the translation initiation required by the eukaryotic translation initiation factor complex formation. The S6K1 activation of various effector proteins increases the mRNA expression in the process of translation initiation and elongation (Karlsson et al. 2013, 2015, Morita et al. 2013). The mRNA and protein expression levels of mTOR, PKB and PI3K in the skeletal muscle of the diabetic rats decreased remarkably compared with those of the normal group (Fig. 5A, B and C). Alkylamides significantly increased the mRNA expressions of mTOR, PKB and PI3K of DM-HD and DM-MD, which were better than those of DM-LD (Fig. 5C). Furthermore, the protein expressions of PI3K (p110), phosphorylated mTOR (ser2448) and phosphorylated...
PKB (ser437) of the diabetic rats increased as shown by Western blot (Fig. 5A and B). The results indicated that alkylamides could reduce the risk of protein synthesis reduction in STZ-induced diabetics. The signal pathway was Ins/IGF1 → PI3K → PKB → mTOR.

Figure 5
Protein synthesis protein (A and B) and gene (C) expressions in the skeletal muscle of diabetic rats treated with alkylamides. Values are the means ± S.E.M. (n = 10). Conditions were the same as those defined in Fig. 2. Means with different superscript letters are significantly different among the diabetic rats (P < 0.05); *means are significantly different (P < 0.05) from the means in the control group. mTOR, mammalian target of rapamycin; p-mTOR (Ser2448), the 2448 ser phosphorylation of the mTOR; PKB, protein kinase B; p-PKB (Ser473), The 473 Ser phosphorylation of the PKB; PI3K, phosphatidylinositol 3-kinase.

UPP was the most vital and selective protein degradation pathway in mammalian cells (Nielsen et al. 1994, Chen et al. 2012). Under the joint actions of ubiquitin (Ub)-activating enzyme (E1), Ub-carrier protein (E2) and Ub-ligase (E3), ubiquitin binded with the target protein, ubiquitinated proteins were degraded into amino acids and a few small oligopeptides by 26S proteasome (Lu et al. 2013). The connection speed of Ub binding with the target protein was considered a rate-limiting step of UPP pathway, which was mediated by muscle atrophy F-box (atrogin-1/MAFbx) and MURF1. The activation of FOXO proteins by dephosphorylation was transferred from the cytoplasm into the nucleus to promote the transcription of atrogin-1/MAFbx and MURF1 (Brownawell et al. 2001, Zhang et al. 2002, Stitt et al. 2004, Stefanetti et al. 2015).

This experiment showed that the mRNA expressions of atrogin-1, MURF1, FOXO1A, FOXO3A and FOXO4A of the diabetic rats markedly increased compared with those of the normal group. Consequently, the insufficient secretion of insulin in the diabetic rats not only caused carbohydrate and lipid metabolism disorders but also led to protein metabolism disorders, including decreased protein synthesis and strengthened protein catabolism. In the alkylamide treatments, the mRNA and protein expression levels of atrogin-1 and MURF1 significantly decreased compared with those of the diabetic rats (Fig. 6A, B and C).

The main FOXO transcription factors consist of FOXO1A, FOXO3A and FOXO4A, which act as the upstream signals of muscle-specific E3 ligase, atrogin-1 and MURF1 (Pond et al. 2014). The mRNA and protein expression levels of FOXO1A, FOXO3A and FOXO4A exhibit similar responses to alkylamide treatments. Therefore, the correlation between the levels of FOXO activation and the transcription of its downstream elements (atrogin-1/MAFbx and MURF1) can be clarified (Fig. 6A, B and C) (Tesseraud et al. 2009). In view of the molecular signal transduction pathway, mTOR and MURF1/MAFbx are regulated by the upstream factor PKB. In the case of muscle atrophy, the declining activity of PI3K decreases the phosphorylation level of PKB. The inactivation of mTOR decreased protein synthesis. Conversely, FOXO entered the nucleus and initiated the transcription of MURF1 and MAFbx, thus increasing the decomposition of protein. Ultimately, this leads to the loss and atrophy of skeletal muscle, whereas the mechanism of skeletal muscle hypertrophy was opposite (Sandri et al. 2004, Kimball & Jefferson 2006, Shenkman et al. 2015). Therefore, mTOR plays a vital role in the process of protein synthesis and decomposition.
Conclusion

The alkylamide regulation mechanism of protein metabolism in diabetic rats increases the secretion of insulin, enhances protein synthesis by upregulating the mRNA and protein expression levels of PI3K, PKB and mTOR in skeletal muscles and reduces protein catabolism by downregulating the mRNA and protein expression levels of atrogin-1/MAFbx, MURF1 and FOXO in skeletal muscles. This study illustrates that alkylamides may increase protein synthesis through the Ins/IGF1→PI3K→PKB→mTOR pathway and reduce protein catabolism through the UPP pathway in skeletal muscles in STZ diabetic rats. Unfortunately, the mTOR pathway is affected by amino acid and energy levels; thus, this topic should be explored further. As hydroxy-α-sanshoo, hydroxy-β-sanshool and hydroxy-γ-sanshool mixtures were used in the experiments, more studies should be conducted to isolate their individual components and examine their efficacies separately. The optimal dosage and treatment effects of alkylamides as well as the effect of alkylamides on the treatment of T2DM should be further investigated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T Y Ren and J Q Kan conceived and designed the study; T Y Ren and Y P Zhu were involved in the study concept and design and in drafting the manuscript; X J Xia, Y B Ding and Jing Guo collected the data.

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