Research Paper

Jinlida granule inhibits palmitic acid induced-intracellular lipid accumulation and enhances autophagy in NIT-1 pancreatic β cells through AMPK activation

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Article info

Article history:
Received 20 August 2014
Received in revised form 6 November 2014
Accepted 1 December 2014
Available online 13 December 2014

Keywords:
Jinlida granule
Type 2 diabetes
Lipid metabolism
Autophagy
AMPK activated protein kinase
NIT-1 cells

Abstract

Ethnopharmacological relevance: Jinlida granule (JLDG), composed of seventeen Chinese medical herbs, is a widely used Chinese herbal prescription for treating diabetes mellitus. However, the mechanism underlying this effect remains unclear. To determine the main components in JLDG and to explore the effect of JLDG on autophagy and lipid accumulation in NIT-1 pancreatic β cells exposed to palmitic acid (PA) through AMPK activated protein kinase (AMPK) signaling pathway.

Materials and methods: JLDG was prepared and the main components contained in the granules were identified by ultra performance liquid chromatography (UPLC) fingerprint. Intracellular lipid accumulation in NIT-1 cells was induced by culturing with medium containing PA. Intracellular lipid droplets were observed by Oil Red O staining and triglyceride (TG) content was measured by colorimetric assay. The formation of autophagosomes was observed under transmission electron microscope. The expression of AMPK and phospho-AMPK (pAMPK) proteins as well as its downstream fatty acid metabolism-related proteins (fatty acid synthase, FAS; acetyl-coA carboxylase, ACC; carnitine acyltransferase 1, CPT-1) and autophagy-related genes (mammal target of rapamycin, mTOR; tuberous sclerosis complex 1, TSC1; microtubule-associated protein 1 light chain 3, LC3-II) were determined by Western blot. The expression of sterol regulating element binding protein 1c (SREBP-1c) mRNA was examined by real time PCR (RT-PCR).

Results: Our data showed that JLDG could significantly reduce PA-induced intracellular lipid accumulation in NIT-1 pancreatic β cells. This effect was associated with increased protein expression of pAMPK and AMPK in NIT-1 cells. Treatment with JLDG also decreased the expression of AMPK downstream lipogenic genes (SREBP-1c mRNA, FAS and ACC proteins) whereas increased the expression of fatty acid oxidation gene (CPT-1 protein). Additionally, JLDG-treated cells displayed a markedly increase in the number of autophagosomes which was accompanied by the down-regulation of mTOR and the up-regulation of TSC1 and LC3-II proteins expression. However, when AMPK phosphorylation was inhibited by Compound C, JLDG supplementation did not exhibit any effect on the expression of these AMPK downstream molecules in NIT-1 cells.

Conclusions: The results suggest that JLDG could reduce intracellular lipid accumulation and enhance the autophagy in NIT-1 pancreatic β cells cultured with PA. The mechanism is possibly mediated by AMPK activation.

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Abbreviations: JLDG, Jinlida granule; TCM, traditional Chinese medicine; ACC, acetyl-coA carboxylase; FAS, fatty acid synthase; CPT-1, carnitine acyl transferase 1; AMPK, AMPK activated protein kinase; pAMPK, phospho-AMPK activated protein kinase; SREBP-1c, sterol regulating element binding protein 1c; mTOR, mammal target of rapamycin; TSC1, tuberous sclerosis complex 1; LC3-II, microtubule-associated protein 1 light chain 3; PA, palmitate; T2DM, type 2 diabetes mellitus; RT-PCR, real time PCR; TG, triglyceride; UPLC, ultra performance liquid chromatography; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside

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http://dx.doi.org/10.1016/j.jep.2014.12.005
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1. Introduction

The prevalence of diabetes mellitus (DM) has continued to increase globally, especially in China. According to the latest report, the prevalence of diabetes was estimated to be 11.6%, representing a total of 113.9 million Chinese adults with diabetes (Xu et al., 2013).

Type 2 diabetes mellitus (T2DM) is the most common form which accounts for 90–95% of those with diabetes. In the onset and progression of T2DM, β cell dysfunction caused by elevated levels of glucose and/or free fatty acids (FFAs) play an important role (Marchetti et al., 2010). Chronic exposure to FFAs leads to lipid overload of β cells, impaired insulin secretion and apoptotic cell death (Zhou and Grill, 1994; Shimabukuro et al., 1998; Piro et al., 2002). Therefore the strategy for protecting pancreatic β cells against lipid overload may have a potential for the prevention and treatment of diabetes.

Autophagy is a lysosomal degradative pathway that protects cells from death under stressful conditions. This process consists of several sequential steps—sequestoration, transport to the lysosome, degradation, and utilization of degradation products (Mizushima, 2007). Through the autophagic process, damaged organelles and proteins are degraded or recycled, which is shown as an adaptive pro-survival response (Ryter and Choi, 2013). Recently, intracellular lipid droplets (LDs) have been also identified as the substrate for autophagy. Autophagy mobilizes lipids from LDs for metabolism through a process termed lipophagy, thereby preventing intracellular lipid overload (Singh et al., 2009; Dong and Czaja, 2011). Defective autophagy has been found to be related to lipid metabolic disorders such as fatty liver and obesity (Singh and Cuervo, 2012). Furthermore, when autophagy is impaired, the architecture and function of beta-cells could not be maintained (Fujitani et al., 2009).

However, the relation between autophagy and lipid metabolism in pancreatic β cells under the condition of lipid overload has been rarely reported.

DM is referred to as “Xiao Ke” disease in Traditional Chinese Medicine (TCM). “Xiao” means emaciation and “Ke” means thirst. But these two typical symptoms may not be obvious in patients with diabetes. Conversely, some patients have no symptoms except for obesity, which is a condition of excess body fat. According to the theory of TCM, excess body fat is described as excess phlegm and moisture caused by deficiency of spleen. Thus, tonifying spleen is considered as the substrate for autophagy. It contains seventeen herbs including Ginseng for tonifying spleen. It contains seventeen herbs including Semei Litchi (Litchi chinensis Franch.), Rhizoma Anemarrhenae (Anemarrhena asphodeloides Bge.), Herba Epimedii (Epimedium brevicornum Maxim.), Radix Salviae Miltiorrhizae (Salvia miltiorrhiza Bge.), Radix Puerariae (Pueraria lobata (Willd.) Ohwi.), Semen Litchi (Litchi chinensis Sonn.), and Cortex Lycii (Lycium chinense Mill.). In modern Chinese medicine practice, JLDG is widely used to treat diabetes mellitus. Clinical trials have also demonstrated that homeostasis model assessment of β-cell function (HOMA-β) score is improved after oral intake of JLDG in diabetic patients (Gao et al., 2010; Yu and Yang, 2013). However, the mechanism underlying this protection of β-cells remains unclear. Recently, few researches have shown that JLDG exerted good protection of β-cells as well as other organs through multi-target activity in diabetic rats (Shi et al., 2012; Wu et al., 2012; Li and Zhao, 2013).

In the present study, we investigated the effect of JLDG on palmitic acid (PA)-induced lipid accumulation and autophagy in NIT-1 pancreatic β cells.

2. Material and methods

2.1. Herbal materials and the preparation of JLDG

JLDG was supplied by Yiling Pharmaceutical Co. Ltd. (Shijiazhuang, Hebei, China). Seventeen kinds of herbs contained in JLDG were all purchased from Meiwei Traditional Chinese Medicine Company in Hebei Province (Anguo, China). The voucher specimen was deposited at the herbarium of the Pharmacy Faculty at the Beijing University of Chinese Medicine and authenticated by the Department of Pharmacognosy, Beijing University of Chinese Medicine (Beijing, China). Seventeen herbs were crushed into crude grains and extracted by refluxing with boiling water. Then the solution obtained was subsided by ethanol and the extract was concentrated and vacuum-dried to granules. Finally, per gram dry granule obtained is equivalent to 6.69 g raw medical herbs. For the present experiment, suspension solution of the granules was made in 0.5% of carboxymethyl cellulose solution before use.

2.2. Chemicals and reagents

Bovine serum albumin (BSA), palmitate (PA) and rapamycin (Rapa) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Pioglitazone (PIO) hydrochloride tablets were purchased from Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). 5-Aminomidazole-4-carboxamide 1β-β-ribofuranoside (AICAR) and Compound C were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) and fetal bovine serum (FBS) were obtained from Hyclone Laboratories Inc. (Logan, UT, USA). Penicillin, streptomycin, trypsin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit were obtained from Guge biological Technology Co. (Wuhan, Hubei, China). Triglyceride colorimetric assay kit was purchased from Shanghai Mind Bioengineering Co, Ltd (Shanghai, China). Bicinchoninic acid (BCA) protein assay kit and enhanced chemiluminescence (ECL) reagent were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-mouse fatty acid synthase (FAS), AMP activated protein kinase (AMPK), acetyl-coA carboxylase (ACC) and carnitine acyl transferase 1 (CPT-1) antibodies were from Abcam Company (Hongkong, China). Rabbit anti-mouse phospho-AMPK activated protein kinase (pAMPK) was obtained from Millipore Corporation (Billerica, MA, USA). Rabbit anti-mouse mammal target of rapamycin (mTOR), microtubule-associated protein 1 light chain 3 (LC3-II) and tuberous sclerosis complex 1 (TSC1) were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trizol reagent, PrimeScript RT reagent Kit and SYBR Premix Ex Taq were purchased from Takara Bio Inc. (Dalian, Liaoning, China).

2.3. Preparation of JLDG-containing serum

The rat dose of JLDG was obtained by the conversion of the human dose to rat equivalent dose based on body surface areas. Male SD rats (200–250 g) were obtained from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Rats were housed under specific pathogen-free conditions and fed a standard rodent laboratory diet from SLAC Laboratory Animal Co., Ltd for two weeks. Then rats were randomly divided into four groups. Rats in the three JLDG groups were orally administrated low (0.75 g/kg), intermediate (1.5 g/kg) and high (3.0 g/kg) doses of JLDG, respectively. Rats in PIO-treated group were orally administrated PIO (25 mg/kg). The corresponding therapy was
administered twice a day for three days. After 1 h of the last intragastric administration, all rats were anesthetized by intraperitoneal injection of sodium pentobarbital and gathered blood through abdominal aorta under sterile condition. Serum obtained was then sterilized by filtering through a 0.22 mm filter and stored at −20 °C till needed for use. All procedures involving animals complied with the Institutional Animal Care and Use Committees of Huazhong University of Science and technology.

2.4. Determinations of the main chemical constituents in JLDG by UPLC analysis

Ultra performance liquid chromatography (UPLC) fingerprint was performed to identify the main chemical constituents in JLDG (Jiang et al., 2013). Tanshinol sodium, puerarin, salvianolic acid B, epimendin B, epimendin C, icerarin, ginsenoside Rb1, ginsenoside Rg, ginsenoside Rb2, purchased from National Institutes for Food and Drug Control (Beijing, China), were taken as reference standards. UPLC was performed on a Acquity UPLC system (Waters Corporation, Milford, MA, USA) equipped with an Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm). The mobile phase included acetonitrile (B) and 0.1% phosphate (D) at a flow speed of 0.3 mL/min in the condition of column temperature 40 °C. The gradient elution was as follows: 0–1 min, 4–4%B, 1–3 min, 4–7.5%B, 3–12 min, 7.5–17.5%B, 12–13 min, 17–20%B, 13–20 min, 20–32%B, 20–21 min, 32–40%B, 21–25 min, 40–56%B, 25–29 min, 56–90%B, 29–35 min, 90–95%B. There were ten batches of JLDG supplied by Yiling Pharmaceutical Co. Ltd to detect the similarity. The similarity of fingerprints was analyzed by professional software named Similarity Evaluation System for Chromatogram Fingerprint of Traditional Chinese Medicine (version of 2004A).

2.5. Cell culture, viability assay and intervention

The mouse NIT-1 pancreatic β cells were provided by Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin and maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Mediums containing different drug concentrations were evaluated by MTT assay according to manufacturer’s protocol. Under the conditions of this study, 10% intermediate dose JLDG serum and 10% PIO serum had negligible effect on cell viability (data not shown).

Approximately, 1 × 10^3/ml cells were transferred into 96 well-culture plates. After cells had adhered, the medium was replaced by serum containing different drugs. The cell viability in the serum containing different concentration of drugs was evaluated by MTT assay. The purity and concentration of total RNA were measured by a Nucleic Acid/Protein Analyzer (Thermo, Rockford, USA). Then 2 μg of total RNA was reverse-transcribed using a PrimeScript RT reagent Kit in a total reaction volume of 20 μl. cDNA was synthesized in a
Mastercycler gradient PCR apparatus (Eppendorf Company, Hamburg, Germany). Then 2.0 μl of cDNA was amplified in a 20 μl PCR reaction containing 6.8 μl ddH2O, 0.4 μl forward primer, 0.4 μl reverse primer, 0.4 μl ROX Reference Dye (50 x) and 10.0 μl SYBR premix EX TaqTM (TaKaRa Company, Dalian, China) with an Applied Biosystems StepOne Real-Time PCR System (StepOne, Foster City, USA). The reaction

Fig. 1. UPLC fingerprints of jinlida granule from ten batches.

Fig. 2. UPLC fingerprint of JLDG (a) and reference standards (b). Peak number and identity: 4: tanshinol; 17: puerarin; 34: salvianolic acid B; 37: epimendin B; 38: epimendin C; 39: icarin; 41: ginsenoside Rb1; 42: ginsenoside Rc; 44: ginsenoside Rb2.
included stage1, 95 °C for 30 s; stage2, 95 °C for 5 s; stage 3, 60 °C for 30 s. The primer sequences were as follows: SREBP-1c: forward, 5'-CCGAGATGTGCGAACTGGA-3'; reverse, 5'-GAAGTCACTGTCTTGGTTGTT-GATG-3'; β-actin: forward, 5'-CATCCGTAAGACCTCTATGCAAC-3'; reverse, 5'-ATGGAGCCACCGATCCACA-3'. 2^ΔΔCT was used to calculate the relative fold-changes of each group.

2.10. Statistical analysis

All results were presented as mean ± standard deviation (SD) and analyzed using SPSS 19.0 software. One-way analysis of variance (ANOVA) was used to determine the statistical significance. Based on data assumed equal variances or not assumed equal variances, a LSD or Dunnett’s T3 test was used, respectively. Meanwhile, descriptive and variance homogeneity test were also followed. P < 0.05 was considered statistically significant.

3. Results

3.1. UPLC profile of JLDG

UPLC fingerprint of JLDG from ten batches was shown in Fig. 1. The result showed that the similarity index of ten batches was up to 0.99 (data not shown). Fifty common peaks were detected (Fig. 2a) and nine of them were identified by comparing the retention time and peak height with reference standard (Fig. 2b). The main constituent of JLDG were as follows: tanshinol, puerarin, salvianolic acid B, epimendin B, epimendin C, icariin, ginsenoside Rb1, ginsenoside Rc and ginsenoside Rb2.

3.2. The effect of JLDG decreased intracellular lipid accumulation in NIT-1 cells

To determine the effect of JLDG on lipid accumulation in NIT-1 cells, Oil Red O staining and methods of colorimetric assay were used. As shown in Fig. 3, intracellular lipid droplets in JLDG group were significantly decreased compared with those in model group. Meanwhile, intracellular TG content was also remarkably reduced (P < 0.05) in the cells of JLDG group (Fig. 4). This reduction of intracellular lipid accumulation was also found after the intervention with AICAR (P < 0.05), which are known activators of AMPK. However, the AMPK inhibitor Compound C blocked the effect of JLDG on decreasing PA-induced intracellular lipid accumulation in NIT-1 cells (P < 0.05).

3.3. The effect of JLDG on the expressions of AMPK and its downstream fatty acid metabolism-related genes in NIT-1 pancreatic β cells

Since AMPK activation requires phosphorylation of threonine residue (T172) in the catalytic α subunit (Bright et al., 2009), we examined the protein expression of pAMPK and AMPK in NIT-1 cells. As shown in Fig. 5A and B, in our study, treatment with JLDG exhibited a significant increase in the protein expression of pAMPK and AMPK compared with the model group (P < 0.01). With regard to the ratio of pAMPK to AMPK (Fig. 5C), it also presented a fold change (P < 0.01) of at least 1.5, indicating a significant activation of AMPK after JLDG supplementation. Consistent with AMPK activation, the expression of AMPK downstream lipogenic genes (SREBP-1c mRNA, FAS and ACC proteins) was reduced (P < 0.05; P < 0.01) whereas the expression of fatty acid oxidation gene (CPT-1 protein)
Fig. 5. The effect of JLDG on the expressions of AMPK and its downstream fatty acid metabolism-related genes in NIT-1 pancreatic β cells. JLDG supplementation inhibited the expression of lipogenic genes and enhanced the expression of fatty-acid oxidase gene through AMPK activation. Representative gels and protein levels for AMPK (A), pAMPK (B), FAS (D), ACC (E) and CPT-1 (F) measured by Western blot in NIT-1 pancreatic β cells. pAMPK/AMPK ratio (C) and SREBP-1c (G) mRNA levels in NIT-1 cells from control, model, JLDG, AICAR, Compound C and PIO groups. Each bar represents means ± SD from three wells. *P < 0.05 and **P < 0.01, significantly different from control. ΔP < 0.05 and ΔΔP < 0.01, significantly different from model group. #P < 0.05 and ##P < 0.01, significantly different from JLDG group.
was enhanced \((P < 0.01)\) (Fig. 5D to G). However, when AMPK phosphorylation was inhibited by Compound C, JLDG administration did not exhibit any effect on the expression of these AMPK downstream molecules in NIT-1 cells \((P < 0.05; P < 0.01)\). These findings indicate that the effect of JLDG on decreasing intracellular TG content may be due to the activation of AMPK.

### 3.4. The effect of JLDG on PA-induced autophagy and the expression of autophagy-related genes in NIT-1 pancreatic β cells

To verify if enhanced autophagy is involved in the effect of JLD on PA-induced lipid accumulation in NIT-1 cells, the formation of autophagosomes was observed and the expression of autophagy-related genes were examined. As shown in Fig. 6, PA supplementation increased the number of intracellular autophagosomes in NIT-1 cells of model group, accompanied by over-expression \((P < 0.01)\) of LC3-II proteins (Fig. 7C), a well-known marker of autophagy. It may serves as an adaptive response under conditions of lipid overload. The cellular autophagy inducer Rapa also markedly enhanced the autophagic vacuoles formation in NIT-1 cells. Compared with the model group, JLDG-treated cells exhibited an obvious increase in the number of intracellular autophagosomes. This increase was associated with decreased expression of mTOR protein \((P < 0.05)\) (Fig. 7A) and increased expression of TSC1 and LC3-IIproteins \((P < 0.05; P < 0.01)\) (Fig. 7B and C). Similar results were also obtained when cells were cultured with AMPK activators (PIO and AICAR) and autophagy inducer (Rapa) \((P < 0.01)\). However, supplementation with AMPK inhibitor, Compound C blocked the enhancing effect of JLDG on the autophagy in NIT-1 β cells \((P < 0.01)\). Together, these results suggest that JLDG may enhance PA-induced autophagy in β cells via AMPK activation.

### 4. Discussion

JLDG is a Chinese herbal prescription widely used to treat diabetes mellitus in the clinical practice of traditional Chinese medicine. However, the mechanism underlying its anti-hyperglycemic effect is still unknown. In the present study, by using serum pharmacology, we found that JLDG could enhance the autophagy and reduce fat deposition in pancreatic β cells exposed to elevated concentration of FFAs. We also found that both of these actions were inhibited by Compound C, an AMPK inhibitor. Since increased autophagy is considered as a protective mechanism against fatty acid-induced lipotoxicity (Mei et al., 2011), we suppose that the anti-diabetic effect of JLDG might be associated with its protection of pancreatic β cells via AMPK activation.

In type 2 diabetes mellitus, a progressive decline of β-cell function and mass is a common feature of the disease (Fonseca, 2009). It has been demonstrated that many factors are related to the reduction of β cell function and mass such as hyperglycemia, elevated FFAs, islet amyloid, cytokines and insulin resistance (Cernea and Dobreanu, 2013). Among these, FFAs have attracted much attention (Shao et al., 2013). Generally, β cells undergo metabolic adaptations including lipid accumulation and insulin release under conditions of high FFAs levels (Vernier et al., 2012). However, when lipid influx exceeds its capacity for lipid storage, lipotoxicity may occur. Elevated FFAs may trigger endoplasmic reticulum (ER) stress followed by the activation of autophagy (Kharroubi et al., 2004; Ogata et al., 2006). Ultrastructural study has also proved that the exposition of β cells to PA, but not to high glucose, is associated with the formation of lipid droplets and autophagolysomes, together with enlarged and prevalently perinuclear cysternae, indicating a remarkable ER stress (Martino et al., 2012). Here, activated autophagy has been proposed as a cell survival...
response to lipid overload. On one side, stimulation of autophagy could reduce ER stress associated with the attenuation of apoptosis in β cells (Bachar-Wikstrom et al., 2013). On the other side, autophagy could degrade lipid droplets through a process termed lipophagy (Dong and Czaja, 2011). Thus, activation of autophagy may become a new therapeutic approach for maintenance of normal β cell function and survival.

AMPK is known to be a key regulator of cellular lipid metabolism (Hardie, 2008), and is considered as a positive regulator that stimulates autophagy in response to lipid overload. Moreover, AMPK is a nutrient and energy sensor that maintains cellular energy homeostasis (Hardie et al., 2012). The inhibition of AMPK is always associated with the development of fat deposition disorders such as obesity and diabetes. It has been long recognized that AMPK activation could regulate lipid metabolism by stimulating fatty acid oxidation and inhibiting lipogenesis (Hardie, 2004; O’Neill et al., 2013). Therefore the antilipotoxic effect of AMPK activation represents a new therapeutic target for the treatment of obesity and type 2 diabetes. Until now, numerous AMPK activators have been reported. Natural activator (e.g. berberine, resveratrol and capsaicin) and chemical synthesis activator (e.g. AICAR, PIO and metformin) have been proved to be involved in lipid metabolism (Srivastava et al., 2012). However, their effect in the regulation of autophagy has been poorly reported. In the present study, we found that JLDG, as a new AMPK activator, not only inhibited intracellular fat deposition but also stimulated autophagy in NIT-1 cells. On the one hand, JLDG could directly enhance the lipolysis by up-regulation of CPT-1 and inhibit the lipogenesis by down-regulation of SREBP-1C, ACC and FAS. On the other hand, JLDG could decrease the expression of mTOR protein and increase the expression of TSC1 and LC3-II proteins, leading to the enhancement of autophagy. Based on the concept of lipophagy which connects autophagy and lipid metabolism the stimulation of autophagy by JLDG might also contribute to the reduction of fat deposition within β cells. However, these effects of JLDG can be eliminated in the presence of AMPK inhibitor Compound C. Collectively, it is obvious that Chinese herbal medicine JLDG, via AMPK activation, can directly or indirectly attenuate intracellular lipid overload as discussed above.

However, some limitations of our work should be mentioned. In the present study, we did not investigate the effect of JLDG on PA-induced insulin release and apoptosis in NIT-1 cells. Therefore the effect of JLDG on maintaining β-cell function and mass should be further confirmed. Actually, some studies take opposing views on intracellular fat deposition. They even think that the ability of accumulating triglyceride might be able to protect NIT-1 pancreatic β cells from PA-induced lipotoxicity (Cnop et al., 2001; Choi et al., 2011) Furthermore, an over stimulated autophagy might also...
lead to non-apoptotic cell death (Tsujimoto and Shimizu, 2005). Hence, more studies are needed in order to explore the mechanisms underlying the anti-diabetic effect of JLDG.

5. Conclusion

JLDG containing serum can reduce intracellular lipid accumulation and enhance the autophagy in NIH-3T3 pancreatic β cells cultured with PA. The mechanism is possibly mediated by AMPK activation.

Acknowledgments

This work was supported by the grant from national major technological plan of major drug innovation in the twelfth five years program (2011ZX09401-020) and grants from National Natural Science Foundation of China (Nos. 81173370, 81273683, 81373871).

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