RESEARCH

APS could potentially activate hepatic insulin signaling in HFD-induced IR mice

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Abstract

Astragalus polysaccharide (APS) is the main component of *Astragalus membranaceus*, an anti-diabetic herb being used for thousands of years in Traditional Chinese medicine (TCM). In this study, we aimed to evaluate the impact of APS on hepatic insulin signaling, autophagy and ER stress response in high-fat-diet (HFD)-induced insulin resistance (IR) mice. APS was intra-gastrically administrated and metformin was used as a control medicine. Apart from monitoring the changes in the important parameters of IR progression, the gene and protein expression of the key factors marking the state of hepatic ER stress and autophagic flux were examined. We found that, largely comparable to the metformin regime, APS treatment resulted in an overall improvement of IR, as indicated by better control of body weight and blood glucose/lipid levels, recovery of liver functions and regained insulin sensitivity. In particular, the excessive and pro-apoptotic ER stress response and inhibition of autophagy, as a result of prolonged HFD exposure, were significantly corrected by APS administration, indicating a switch of the cellular fate in favor of cell survival. Using the HepG2/IR cell model, we demonstrated that APS modulated the insulin-initiated phosphorylation cascades in a similar manner to metformin. This study provides a rationale for exploiting the insulin-sensitizing potential of APS, which has a therapeutic performance almost equivalent to metformin, to enrich our options in the treatment of IR.

Key Words

- ▶ Astragalus polysaccharide
- ▶ insulin resistance
- ▶ endoplasmic reticulum stress
- ▶ autophagy
- ▶ liver
- ▶ traditional Chinese medicine

*Journal of Molecular
Endocrinology*
(2019) **63**, 77–91

Introduction

In societies beset by excessive energy intake and sedentary lifestyles, the rising prevalence of insulin resistance (IR) poses as the driving force to propel the pandemic of metabolic syndromes, type 2 diabetes mellitus (T2DM) and many other diseases (Samuel & Shulman 2012, Soltis *et al.* 2017). Targeted management of IR *per se* is still intangible because of the multiplicity of the mechanisms responsible for its development. Metformin has been used successfully to alleviate IR in diabetes. However, concerns about potential side effects of metformin have been raised despite its effects in suppression of endogenous glucose output and restoration of peripheral insulin sensitivity (McCreight *et al.* 2016). Evidence of the effectiveness and safety of the present pharmaceutical approaches to improve the systemic IR are still too lean to satisfy the requirement for the prevention and treatment of IR (Rogowicz-Frontczak *et al.* 2017).

As the main detoxifying organ of the body, the liver plays a central role in metabolic functions (Bechmann *et al.* 2012). Abnormalities in the reciprocal regulation between autophagy and endoplasmic reticulum (ER) stress in the liver have been associated with overfeeding, aberrant lipid metabolism and IR (Kwanten *et al.* 2014), with the involvement of mTOR signaling (Codogno & Meijer 2010).

ER is a principal organelle responsible for energy management (Hotamisligil 2010) and the correct folding and maturation of nascent proteins. Pathological disturbance leading to accumulation of misfolded proteins within the ER, termed as ER stress, triggers the revolutionarily adapted unfolded protein response (UPR) to restore ER proteostasis. Failure in ER stress rectification facilitates the development of fatty liver (Malhi & Kaufman 2011), inflammation (Cao *et al.* 2016), apoptosis, autophagy disorder (Li *et al.* 2015) and IR. Therefore, amelioration of ER stress stands as a crucial point to avert IR.

In the 6-week-old male C57BL/6J mice, feeding with high-fat (60% calories) diet resulted in the development of IR at the end of 13 weeks (Sah *et al.* 2016), accompanied by induction of ER stress and reduced hepatic autophagy (Liu *et al.* 2009), which emerge as an important mediator of the IR onset (Flamment *et al.* 2012). As an integral part of cellular strategies of UPR to purge the toxic accumulation of misfolded proteins and to provide amino acid recycling, autophagy is initiated by low-grade ER stress to preserve normal cellular homeostasis. But under sustained ER stress such as IR, over-activated mTORC1 or other kinases represses autophagy action (Kim & Guan 2015).

Astragalus polysaccharide (APS), the main bioactive component of the Chinese herb *Astragalus membranaceus* (Huangqi), is extracted from the dried roots of Huangqi (Park *et al.* 2014). The long history of the medicinal use of APS in traditional Chinese medicine (TCM) has demonstrated its various effects in the treatment of obesity, hepatic steatosis (Huang *et al.* 2017), immunomodulation (Zhou *et al.* 2017), hepatoprotection (Yan *et al.* 2009, Liu *et al.* 2015) and anti-aging (Liu *et al.* 2017). In the light of previous studies showing that APS could attenuate IR (Mao *et al.* 2009), reduce hepatic ER stress and restore glucose homeostasis in diabetic KKAY mouse (Mao *et al.* 2007) to regulate liver metabolism, we hypothesized that APS may promote autophagy and prevent ER stress in the liver that, in turn, influences hepatic and systemic glucose metabolism to achieve liver protection.

Materials and methods

Animals and diet intervention

All experimental procedures involving animals were approved by the Animal Ethics Committee of Dalian Medical University. Five-week-old male C57BL/6 mice (purchased from Vital River Laboratory Animal Technology Co., Ltd., Beijing, China, SCXK (Jing) 2012-0001) were allowed to adapt to their surroundings for 1 week with normal feeding before HFD feeding for 8 weeks to develop IR and the normal control group was given regular chow (RC) (Supplementary data and Supplementary Fig. 1, see section on supplementary data given at the end of this article). All applicable institutional and/or national guidelines for the care and use of animals were followed.

For an additional 12 weeks, the RC group was given a daily oral gavage with distilled water, while the HFD mice were randomly divided into three groups ($n=10$ per group): HFD group (a daily oral gavage of distilled water), positive control group gavaged with metformin 300mg/kg every day (HFD+MET), HFD+APS group gavaged with APS 800mg/kg every day. The mice were maintained in specific pathogen-free (SPF) conditions of constant temperature ($23\pm2^{\circ}\text{C}$) and humidity ($50\%\pm10\%$), with 12h/12h light/darkness cycle. Purified water and food were provided *ad libitum* (except as noted).

Cell culture and treatment

The human hepatoma HepG2 cell line was obtained from Dalian Medical University. The cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin and

treated with or without 0.25 mM palmitate (PA) for 24 h to establish a cellular model of IR. APS or metformin was added to the cell culture medium for another 24 h. Before harvesting, the cells were stimulated by 100 nM insulin for 20 min.

Diets, drugs and materials

The HFD (60% calories from fat, D12492) and RC (10% calories from fat, D12450J) were purchased from Beijing Keaoxieli Co., Ltd, China. APS (purity $\geq 98\%$) was purchased from Shanghai Yuanye Bio-technology Co., Ltd, China and metformin was purchased from TCI Shanghai Co., Ltd, China. SYBR and reagent kits for real-time PCR were purchased from Takara Bio Inc.. Antibodies against GAPDH, AMPK, GRP78, ATF6, BCL-2 and BAX were from Proteintech Group Inc., China. Antibodies against GLUT2, IRS1, p-IRS1, p-AMPK, ACC, p-ACC, AKT, p-AKT, mTOR, p-mTOR, 4EBP1, p-4EBP1, P70S6K, p-P70S6K, GSK3 β , p-GSK3 β , ATG5, ATG3, LC3, BECLIN-1 and β -ACTIN were acquired from Abcam. RIPA was from Biotek Corporation, Beijing, China. Kits for blood biochemistry were from Jiancheng Bioengineering Institute (Nanjing, China). Kits for G6pase and PEPCK were from Solarbio Life Sciences (Beijing, China). Insulin was from Wanbang Biochemical Pharmaceutical Co., Jiangsu, China.

Glucose tolerance tests

Glucose tolerance test (GTT) was performed after 8 weeks on the diets and again after 10 weeks of gavage. Following an overnight fasting for about 16 h, the mice were subjected to intraperitoneal (i.p.) glucose injection (2 g/kg body weight). Glucose values were measured at 0 (fasting blood), 15, 30, 60 and 120 min, using blood obtained from tail cut.

Insulin tolerance tests

Insulin tolerance test (ITT) was performed after 9 weeks on the diets and again after 11 weeks of gavage. Four-hour-fasted mice were administrated with insulin (i.p. 0.75 IU/body weight) for the determination of glucose levels at 0, 15, 30, and 60 min, using blood obtained from tail cut.

Determination of blood glucose concentration

Blood glucose concentration was monitored from cut tail over the gavage period at 2-week interval, the first

drop of blood was discarded. Fasting blood glucose was determined following a 4-h fasting in mice.

Blood sampling and tissue collections

After 10 weeks of gavage, blood was collected from orbital venous plexus for biochemical determinations. At the end of 12 weeks of gavage, the blood of the 4-h-fasted mice was collected again during killing. The blood samples were stored at 4°C overnight prior to centrifugation at 1300 *g* for 10 min to obtain the serum which was stored at -80°C for further analysis. The liver of each mouse was resected and weighed on ice immediately, before being divided into small portions for -80°C conservation or formalin fixation.

Measurements for lipid, insulin, cytokine, PEPCK and G6pase

Levels of total cholesterol (T-CHO), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), cytokines, insulin and the activities of alanine (ALT) and aspartate (AST) transaminases were measured with commercial kits from Jiancheng Bioengineering Institute. PEPCK and G6pase in liver tissue were measured by ultraviolet spectrophotometry with kits from Solarbio.

Total RNA extraction and mRNA quantification by qRT-PCR

Total RNA obtained from liver was prepared with TRIzol reagent from 100 mg frozen liver tissues and dissolved in RNase-free water. Total RNA removed of genomic DNA by DNA eraser buffer was reverse transcribed to cDNA. Quantitative PCR was performed with Applied Biosystems 7900 real-time PCR using the SYBR method in a total reaction volume of 10 μL containing 5 μL SYBR Premix DimerErase (2 $\times$) and 1 μL template. The primer sequences used in qRT-PCR were shown in Table 1.

Western blotting

Total proteins of the liver tissues and HepG2 were extracted using RIPA agents and quantified with BCA protein assay kit. Equal amount of proteins from tissue homogenates were subjected to SDS-PAGE separation and then transferred to PVDF membranes. The protein expression levels of AMPK, p-AMPK, ACC, p-ACC, IRS1, p-IRS1, AKT, p-AKT, GSK3 β , p-GSK3 β , mTOR, p-mTOR, 4EBP1, p-4EBP1, P70S6K, p-P70S6K, GRP78, ATF6, ATG5, ATG3, BECLIN-1

Table 1 Primer sequences of qRT-PCR.

Gene	Primer 1	Primer 2
<i>Glut2</i>	TCAGAAGACAAGATCACCGGA	GCTGGTGTGACTGTAAGTGGG
<i>Pepck</i>	ACACCCTGGGGCTCTAATCT	CCGTCCCTCATACATCTGGC
<i>G6pase</i>	TCGCGCTTGGATTCTACCTG	GCCGCTCACACCATCTCTTA
<i>Tnf-α</i>	CCAAAGGGATGAGAAGTTCC	CTTGGTGGTTTGCTACGAC
<i>Grp78</i>	ACCTATTCTCGTTCGGTGT	CGATGAGGCGCTTGGCAT
<i>Atf6</i>	GGGTTCGGATATCGCTGT	CTTCAGCTGCTCATTCTCTG
<i>Atg3</i>	ACACGGTGAAGGGAAAGGC	TGGTGGAATAAGTGATCTCCAG
<i>Beclin-1</i>	ATGGAGGGGTCTAAGGCGTC	TCCTCTCTGAGTTAGCCTCT
<i>Gapdh</i>	GGAGAAACCTGCCAAGTATG	GGAGTTGCTGTTGAAGTCGC
<i>β-actin</i>	GACCCAGATCATGTTTGAGAC	ACAATGCCTGTGGTACGAC

and LC3 were detected with specific antibodies. β -ACTIN and GAPDH were used as loading control. The densities of immunoblot bands were acquired with Quantity One software (Bio-Rad).

Histological and morphological examination

The liver tissues were prepared sequentially through fixation in 10% formalin solution, paraffin embedding and a 4 μ m sectioning procedure before hematoxylin and eosin staining (H&E). Liver morphological states were observed by an optical microscope.

Oil red O staining

The liver tissues were fixed in 10% formalin solution followed by sucrose gradient dehydration for 2 days. Successive sections were cut at 10 μ m by freezing microtome. Slides were processed through Oil red O staining. The slides were observed immediately by an optical microscope.

Immunohistochemistry

Paraffin-embedded tissues sectioned at 4 μ m thickness mounted on poly-L-lysine-coated slides were deparaffinized in xylene and rehydrated in a graded ethanol series to phosphate-buffered saline (PBS) to detect the expression of BCL-2 and BAX, as well as GLUT2 in liver. DAB was used as the chromogen to visualize the positive expression. The slides were observed using a digital camera and a bright field microscope to collect images.

Statistical analysis

All data were presented as mean $\pm$ S.D. except that the data of qRT-PCR were presented as mean $\pm$ S.E.M. Statistical analysis of group differences was carried out

using a one-way ANOVA test or two-tailed Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

APS reduced body weight and liver weight, attenuated insulin resistance in HFD-induced IR mice

The body weight of all groups was measured every week during the entire experimental period. As shown in Fig. 1A, mice fed with HFD had significantly more rapid weight gain than RC-fed group, which was hampered respectively in mice with APS or metformin from the beginning of the gavage at week 6 and week 4. At the end of the experiment, the mean body weight (Fig. 1B) and liver weight (Fig. 1C) in APS-treated groups were reduced by 9.8 and 11.7%, respectively, compared to untreated HFD mice. GTT and ITT were compared in mice with different treatment after 10 and 11 weeks of gavage, respectively. HFD-induced IR was significantly improved by APS supplementation, as indicated by reduced GTT (Fig. 1D) and ITT (Fig. 1F), and further confirmed by AUC of GTT (Fig. 1E) and ITT (Fig. 1G), to the extent quite comparable to metformin treatment. Interestingly, the blood glucose was efficiently decreased after 2-h-gavage in mice treated with metformin for only 2 weeks, while no effect was seen in APS group at the same time point (Fig. 2A). But similar noticeable blood glucose decline was achieved 8 weeks after treatment with either metformin or APS (Fig. 2B). Both APS and metformin treatment resulted in lowered random (Fig. 2C) or fasting (Fig. 2D) blood glucose, fed (Fig. 2E) or fasting (Fig. 2F) insulin levels, compared to the HFD group, after more than 10 weeks of treatment. These findings indicated that the attenuation of IR states was achieved by APS supplementation to a degree similar to metformin, albeit with some delay.

Figure 1

Effects of APS on the phenotypes related to HFD-induced insulin resistance in mice. (A) The growth curve of mice body weights during the experiment. (B) Body weights at the end of the experiment. (C) Liver weights at the end of the experiment. (D) GTT in mice after 10 weeks of treatment. (E) The area under curve (AUC) of GTT calculated from the original graph. (F) ITT in mice after 11 weeks of treatment. (G) AUC of ITT calculated from the original graph. ($n = 10$ for each group). $*P < 0.05$.

APS improved biochemical parameters and modulated serum cytokine profile in HFD-induced IR mice

As displayed in Table 2, the elevated serum levels of LDL, TG and T-CHO by HFD feeding were corrected by APS by 57.9, 53.4 and 39.1% respectively, close to the results achieved by metformin treatment (60.6, 55.4 and 46.6% respectively). Accordingly, the lowered HDL concentration caused by HFD exposure was significantly increased by either APS (74.5%) or metformin (104.5%). CHO and TG content in liver tissue were reduced with APS or metformin treatment compared to the liver in HFD mice (Table 3).

Figure 2

Effects of APS on blood glucose and insulin levels. (A) Blood glucose from cut tail after 2-h gavage of each group, 2 weeks in treatment ($n = 7$ for each group). (B) Blood glucose from cut tail after 2-h gavage of each group, 8 weeks in treatment ($n = 7$ for each group). (C) Random blood glucose from cut tail, 10 weeks in treatment ($n = 7$ for each group). (D) Fasting blood glucose from cut tail, 11 weeks in treatment ($n = 7$ for each group). (E) Fed serum insulin values ($n = 5$ for each group). (F) Fasting serum insulin values ($n = 5$ for each group). $*P < 0.05$.

The liver function damaged by HFD feeding, shown by higher ALT and AST levels, was recovered by APS or metformin. In addition, HFD-induced chronic, systemic, mild inflammation in mice was markedly reversed by APS, indicated by the decline of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β , LEPTIN and the elevation of ADIPONECTIN in serum (Table 4).

Liver histomorphology and IRS-1/AMPK/ACC signaling were improved by APS supplementation in HFD-induced IR mice

The morphological manifestations of the liver damage of HFD, revealed by H&E staining, include structural disorders of hepatic lobules and increased intracellular fat vacuoles. An amelioration of these pathologic alterations in hepatic

Table 2 Blood lipid biochemistry test.

Biochemical parameters	RC	HFD	HFD + MET	HFD + APS
LDL (mmol/L)	1.34 ± 0.12	6.38 ± 0.48 ^a	2.51 ± 0.16 ^a	2.68 ± 0.23 ^a
HDL (mmol/L)	1.30 ± 0.08	0.57 ± 0.06 ^a	1.17 ± 0.08 ^a	1.00 ± 0.08 ^b
TG (mmol/L)	1.01 ± 0.06	2.75 ± 0.12 ^a	1.23 ± 0.06 ^b	1.28 ± 0.07 ^a
T-CHO (mmol/L)	2.13 ± 0.16	5.09 ± 0.17 ^a	2.72 ± 0.18 ^b	3.10 ± 0.12 ^b
ALT (μkat/L)	0.39 ± 0.03	1.55 ± 0.09 ^a	0.60 ± 0.03 ^a	0.63 ± 0.04 ^a
AST (μkat/L)	0.84 ± 0.08	1.86 ± 0.11 ^a	1.25 ± 0.13 ^b	1.33 ± 0.08 ^b

($\bar{x} \pm s$) ($n = 5$ for each group, ^a $P < 0.01$, ^b $P < 0.05$).

lobule structure was observed in APS- or metformin-treated group. The HFD-induced lipid accumulation in the liver was decreased by APS or metformin treatment (Fig. 3A). qRT-PCR analysis of *Glut2* gene (Fig. 3B) and immunohistochemistry examination of GLUT2 protein (Fig. 3C, brown stain) in liver demonstrated a decreased GLUT2 expression in HFD mice compared to RC group, which was recovered in HFD+MET and HFD+APS groups. The main event in IR occurrence is the impeded insulin action cascade of tyrosine phosphorylation of the insulin receptor substrate-1/AMP-activated protein kinase/acetyl CoA carboxylate (IRS-1/AMPK/ACC), as shown by Western blot analysis (Fig. 3D). Similar to metformin, APS treatment had the capacity to increase IRS-1, AMPK and ACC phosphorylation, shown by the significantly increased ratio of p-IRS-1/IRS-1, p-AMPK/AMPK and p-ACC/ACC, in comparison with that of the untreated HFD mice.

APS had regulatory effects on mTOR/4EBP1 and AKT/PI3K pathways in IR mice

In diet-induced obesity or IR models, the nutrient-sensing loop formed by mTOR/4EBP1/S6K1 and IRS1/AKT/PI3K pathways is disrupted, which contributes substantially to the impaired insulin signaling. Excessive intake of energy or insulin resistance causes overexpression of mTOR which activates S6K1 phosphorylation; as a feedback, mTOR/S6K1 signaling pathway negatively regulates insulin sensitivity by increasing IRS-1^{Ser636/639} phosphorylation level, thus inhibiting insulin signal transmission and inducing IR. In the present study, we found that the enhanced phosphorylation of mTOR, 4EBP1 and S6K1, resulted from HFD feeding, were significantly blunted

by either APS or metformin, shown by the decreased ratio of p-mTOR/mTOR, p-4EBP1/4EBP1 and p-S6K1/S6K1 (Fig. 4A). It suggested an inhibitory effect of APS on the mTOR/4EBP1 signaling.

AKT/PI3K pathway activation could promote glucose uptake and glucose homeostasis in the liver. Protein analysis demonstrated that the ratio of p-AKT/AKT, p-GSK3β/GSK3β and p-PI3K/PI3K were downregulated in HFD group, compared to the RC group, and elevated by APS or metformin treatment (Fig. 4B). Moreover, the downstream molecules of Akt, such as PEPCK and G6pase were analyzed. qRT-PCR analysis of *Pepck* and *G6pase* genes in liver demonstrated a decreased *Pepck* and *G6pase* mRNA in HFD mice compared to the RC group, which was recovered with metformin or APS treatment (Fig. 4C). The enzyme activities of PEPCK and G6pase were assayed by UV spectrophotometry and both were inhibited by HFD treatment but significantly restored by APS or metformin therapy (Fig. 4D). This could, at least in part, be responsible for the improved glucose metabolism in the treated mice.

APS alleviated the ER stress and steered Bcl-2/BAX expression toward cell survival in the liver of IR mice

Liver expression of *Tnf-α* (Fig. 5A), *Grp78* (Fig. 5B) and *Atf6* (Fig. 5C) genes, representing pro-inflammatory reactions, ER stress initiation and sensing respectively, were determined by qRT-PCR. A counter-action of APS or metformin treatment against the increase of these important ER stress markers by HFD was observed. A similar effect was seen in protein expressions of GRP78 and ATF6 (Fig. 5D).

Table 3 TG and CHO in liver tissue.

Biochemical Parameters	RC	HFD	HFD + MET	HFD + APS
TG (μmol/g)	14.39 ± 1.21	43.65 ± 4.35 ^a	22.88 ± 2.17 ^a	25.20 ± 2.02 ^b
CHO (μmol/g)	53.70 ± 4.32	87.98 ± 7.74 ^a	60.54 ± 6.35 ^b	65.42 ± 6.43 ^b

($\bar{x} \pm s$) ($n = 5$ for each group, ^a $P < 0.01$, ^b $P < 0.05$).

Table 4 Serum inflammatory cytokine profile.

Cytokines	RC	HFD	HFD + MET	HFD + APS
TNF- α (pg/mL)	23.9 $\pm$ 1.83	261.8 $\pm$ 20.04 ^a	61.4 $\pm$ 4.66 ^a	48.4 $\pm$ 3.56 ^a
IL-6 (pg/mL)	23.5 $\pm$ 1.61	119.2 $\pm$ 7.22 ^a	61.7 $\pm$ 2.84 ^b	40.3 $\pm$ 2.55 ^a
IL-1 β (pg/mL)	12.6 $\pm$ 1.79	71.9 $\pm$ 4.46 ^a	24.5 $\pm$ 1.92 ^a	20.5 $\pm$ 1.26 ^a
LEPTIN (pg/mL)	6.5 $\pm$ 0.49	17.6 $\pm$ 0.99 ^a	10.0 $\pm$ 0.81 ^b	9.3 $\pm$ 0.88 ^b
ADIPONECTIN (μ g/mL)	11.4 $\pm$ 0.78	6.8 $\pm$ 0.66 ^b	9.42 $\pm$ 0.62 ^b	10.3 $\pm$ 0.84 ^b

($\bar{x} \pm s$) ($n = 5$ for each group, ^a $P < 0.01$, ^b $P < 0.05$).

When the ER stress response is overwhelmed by the aggregation of misfolded proteins, the cell death machinery is switched on by the disassociation of ER stress receptors from GRP78, leading to suppression of the pro-survival protein BCL-2 and the commitment of the pro-apoptotic protein BAX (Kim & Park 2017), as seen in the immunohistochemistry analysis of GRP78, BCL-2 and BAX proteins in liver tissue of HFD-fed mice (Fig. 5E). This lethal ER stress response was successfully relieved by APS or metformin treatment (Fig. 5E).

APS promoted autophagy via regulating ATGs, BECLIN-1 and LC3-II/LC3-I in the liver of IR mice

The occurrence of catabolic autophagy upon micro-environmental stress stimuli serves as a cellular survival response, rather than apoptosis. In HFD mice, an inhibited liver autophagy was indicated by lower mRNA expressions of *Atg3* and *Beclin-1* than in RC mice, and both were significantly increased by APS or metformin (Fig. 6A and B). Western blot results showed that the levels of ATG3, ATG12 and BECLIN-1 proteins decreased in HFD group compared to RC group, but dramatically increased in the HFD+APS and HFD+MET groups, implying that the inhibition of hepatic autophagy in HFD mice was reversed by APS or metformin. The autophagy flux was measured by the ratio of LC3-II/LC3-I. During the induction of the autophagosomal degradation, LC3-I is converted to its lipidated and active form LC3-II. Thus, the LC3-II/LC3-I ratio is used as an indicative sign of the autophagy status in cells. The increased proportion of LC3-II is correlated with a higher degree of autophagy, which was indeed evidently displayed in HFD+APS or HFD+MET group, compared to HFD mice without drugs, shown by immunoblot analysis (Fig. 6C). ATG3 and BECLIN-1 were inhibited in HFD group, metformin and APS treatment had the capacity to increase ATG3 and BECLIN-1, shown by immunohistochemistry analysis, which were consistent with the trend of autophagy shown by Western blot (Fig. 6D).

APS promoted insulin signal transduction in HepG2 cells

In order to understand whether APS directly affected the insulin signaling pathway, a cellular IR model was established by culturing HepG2 cells with FFA. As shown in Fig. 7A, APS evidently increased the levels of p-AMPK and p-PI3K in the IR cells but not the cells without FFA pre-treatment. The results illustrated that APS would directly affect insulin signaling pathway.

AMPK and PI3K were also detected, as well as mTOR, to study the different effects between APS and metformin in HepG2 cells with IR. As expected, upon FFA stimulation, a failed AMPK and PI3K phosphorylation and overactivation of mTOR were observed in the IR HepG2 cells, unlike the FFA-free control cells. However, the declined ratio of p-AMPK/AMPK and p-PI3K/PI3K in the IR cells were significantly elevated by APS or metformin treatment, while the enhanced phosphorylation of mTOR resulted from FFA were significantly inhibited by either APS or metformin (Fig. 7B). It suggested that APS and metformin reactivated insulin signaling in HepG2 cells via similar mechanisms. However, metformin exhibited more pronounced effects on these proteins than APS.

Discussion and conclusion

The anti-diabetic potency of APS has been well acknowledged. In this study, we report for the first time that APS leverages ER stress and autophagy action to attain hepatic insulin sensitizing.

The liver is the major organ responsible for glucose metabolism and insulin utilization, which has significant impact on postprandial glycaemia and occurred hepatic IR within 7 days of HFD (Turner *et al.* 2013). Several studies have linked obesity with the development of IR with hepatic ER stress (Kim *et al.* 2015). ER stress appears to act directly as a negative modulator of the insulin signaling pathway (Yilmaz 2017), but also indirectly by promoting lipid accumulation (Wang *et al.* 2018).

Figure 3

APS reversed histomorphology of HFD-induced fatty liver and promoted the insulin signaling in the liver. (A) H&E and Oil red O staining results of liver. (B) Relative mRNA expression of *Glut2* in liver. The qRT-PCR was performed in quadruplicates ($n = 4$ for each group). (C) Western blot and densitometric analysis of total and phosphorylation levels of GLUT2 in the liver ($n = 4$ for each group). (D) Representative images of GLUT2 immunohistochemistry staining in liver ($n = 3$ for each group). (E) Western blot and densitometric analysis of total and phosphorylation levels of IRS-1, AMPK and ACC in the liver ($n = 4$ for each group). * $P < 0.05$.

Figure 4

APS had regulatory effects on mTOR/4EBP1 and AKT/PI3K pathways in mice with insulin resistance. (A) Western blot and densitometric analysis of total and phosphorylation levels of mTOR, 4EBP1 and S6K1 in liver ($n = 4$ for each group). (B) Western blot and densitometric analysis of total and phosphorylation levels of AKT, GSK3 β and PI3K in the liver ($n = 4$ for each group). (C) Relative mRNA expression of *Pepck* and *G6pase* in liver. (D) Enzyme activity analysis of PEPCK and G6pase in liver by UV spectrophotometer. * $P < 0.05$.

Figure 5

APS alleviated the ER stress and regulated BCL-2/BAX in the liver of insulin resistance mice. The mRNA expressions of *Tnf-α* (A), *Grp78* (B) and *Atf6* (C) in liver analyzed by qRT-PCR. Data were normalized by *Gapdh* and expressed as fold changes ($n = 3$ for each group). $*P < 0.05$. (D) Western blots results and densitometric analysis of GRP78 and ATF6 ($n = 4$ for each group). $*P < 0.05$. (E) Immunohistochemistry staining of GRP78, BCL-2 and BAX in the liver.

Figure 6

APS promoted autophagy via regulating ATGs, BECLIN-1 and LC3 in the liver of HFD-induced insulin resistance mice. qRT-PCR analysis of mRNA expressions of *Atg3* (A) and *Beclin-1* (B) in the liver. Data were normalized by *Gapdh* and expressed as fold changes ($n=3$ for each group). $*P<0.05$. (C) Western blots results of ATG3, ATG12, BECLIN-1 and LC3I, LC3II. Expressions of ATG3, ATG12, BECLIN-1 and LC3II/LC3I were also evaluated by densitometric calculations ($n=4$ for each group). $*P<0.05$. (D) Immunohistochemistry staining of ATG3 and BECLIN-1 in the liver.

A

B

Figure 7

APS promoted insulin signaling pathway transduction in HepG2 cells with or without FFA. (A) Western blot and densitometric analysis of total and phosphorylation levels of AMPK and PI3K in HepG2 by treated with APS with or without FFA ($n = 4$ for each group). (B) Western blot and densitometric analysis of total and phosphorylation levels of AMPK, mTOR and PI3K in HepG2 with or without FFA and the effects of metformin and APS ($n = 4$ for each group). * $P < 0.05$.

In response to chronic ER stress, an adaptive signaling pathway known as UPR is triggered initially to restore ER proteostasis. However, excessive UPR can be accountable

for inflammation, inflammasome activation and ER stress, apoptosis, autophagy and death of hepatocytes (Senft & Ronai 2015). Similar to metformin, APS treatment reduced

ATF6 and GRP78 proteins, indicating a prevention of ER stress inception. The same group pointed out that the effect of APS to re-establish glucose homeostasis through PTP1B was expanded from only in skeletal muscle to the liver when the dosage was increased from 400 to 700 mg/kg/day and the duration of the therapy extended from 5 to 8 weeks, while remained non-cytotoxic (Wu *et al.* 2005, Wang *et al.* 2009). An optimal anti-diabetic effect was indeed achieved by combining a low dose of APS (200 mg/kg/day) with *Crataegus* flavonoids to synergistically restore islet function (Cui *et al.* 2016). Similarly, we adopted a dose regime of 800 mg/kg/day for 12 weeks in the present study. It is reasonable to deduce that the hypoglycemic effect of APS was attributed to multi-tissue targeting.

In an overlapped manner, stimulation of the other two ER stress transducers, IRE1 α and PERK, also results in activation of CHOP, CASPASE-12 and JNK signaling. JNK-mediated serine phosphorylation of IRS1 blocked IRS1 tyrosine phosphorylation (Salvado *et al.* 2015) and thereby impairs insulin signaling and downstream PI3K pathway, leads to inhibited anti-apoptotic actions of BCL-2 family and therefore cell death (Doyle *et al.* 2011, Hacker 2014). Upon APS therapy, we observed a significant increase of IRS1 tyrosine phosphorylation and PI3K activity, and changes in BCL-2/BAX protein expression in favor of cell survival, which suggested a relieving of cell damage and execution.

Under sustained ER stress, phosphorylation of 4EBP1 and S6K1 by over-activated mTORC1 or other kinases, such as GSK3 β , represses autophagy action, as confirmed in our HFD mouse model. APS successfully enhanced p-AKT, p-GSK3 β and p-PI3K and reduced phosphorylation of 4EBP1 and S6K1, which redirected the cell into autophagy. The promoted hepatic autophagy by APS treatment was proved by increased expression of ATGs and BECLIN-1 at both mRNA and protein levels, as well as the flip of the declined LC3-II/LC3-I ratio, indicating a higher rate of autophagy flux and conversion of autophagosomes. Importantly, stimulation of autophagy can overcome pathologically augmented ER stress and UPR, therefore protect liver cells and promote hepatic metabolic function, thus the systematic IR would be alleviated. In line with our data, other studies also found that APS restores glucose homeostasis through normalizing liver ER functioning, as shown in its regulation of XBP1 (*XhoI* site-binding protein 1, an ER stress indicator) and the hepatic GSK3 β activity in a diabetic KK Δ y mouse model (Mao *et al.* 2007).

Nutrient overload is the major cause of fatty liver, sub-acute level of inflammation and the improved

hepatic insulin signaling, in turn, protects liver tissue from the onset of the nonalcoholic fatty disease (NAFLD) (Utzschneider & Kahn 2006). In our experiment, we provided evidence that APS treatment not only suppressed serum TNF- α concentrations but also altered the liver cytokine profile by inhibiting *Tnf- α* mRNA expression.

AMPK is the master energy sensor regulating the dynamic balance of ATP/AMP level and plays a decisive role in lipoglyco-metabolism; furthermore, AMPK activation suppresses hepatic lipid accumulation in HFD-fed mice (Li *et al.* 2014). In this study, we found that APS administration activated AMPK, and subsequently mitigated the nutrient-induced ER stress and restored autophagy, which may steer cellular fate from anabolic to a catabolic ATP-producing pathway. A crosstalk between AMPK and AKT pathways was also proposed that may contribute to the APS-potentialized insulin signaling *in vivo* and *in vitro* (Zou *et al.* 2009).

Despite its broad use in diabetes, occurrence of adverse responses of metformin in a substantial proportion of patients calls for more drug alternatives. The rich resources provided by the herbal TCM is certainly a highly desirable avenue (Xiao & Luo 2018). In the present work, the preservation of insulin sensitivity achieved by APS prescription completely matched the performance of metformin. Compared to metformin, APS has similar effects on insulin signaling pathway, but maybe low side effects. They only differed in that the reduction of serum glucose concentration was more rapid with metformin than with APS, as a significant lowering of blood glucose was detected only 2 weeks of metformin treatment, while the hypoglycemic impact of APS was measured after 8 weeks of treatment. This may reflect the intrinsic difference in the chemical nature of the drugs that APS is a polysaccharide and metformin is essentially a biguanide product.

En route to a safe and efficient employment of APS to prevent IR progress clinically, the following questions still need to be settled: 1. Whether APS is superior to metformin in certain groups of patient, especially in metformin-intolerant people; 2. Whether and what side effects may arise from APS therapy; 3. Whether the activity of APS to avert the loss of insulin sensitivity can be synergistically or additively augmented via co-treatment with other drugs such as metformin. Furthermore, the quality of polysaccharide may be different from batch to batch. By cleverly integrating herbal components harboring various chemical traits, we could not only effectively facilitate multi-targeting of IR, but also minimize any unwanted off-target outcome derived from a high dose of a single drug.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-19-0035>.

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.

Funding

This work was supported by the Ministry of Science and Technology of China (Grant No. 2014DFA32120), the National Science Foundation of China (Grant No. 81471000, 31871163) and the Natural Science Foundation of Liaoning (Grant No. 2014023042) to Y W, the National Science Foundation of China (Grant No. 81600668) to L R.

Acknowledgments

The authors thank our technical assistants for their help in acquiring the data and skillful technical assistance. The authors would like to thank the staff of the animal housing facility located at Dalian Medical University for their excellent technical support.

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Received in final form 3 May 2019

Accepted 28 May 2019

Accepted Preprint published online 28 May 2019