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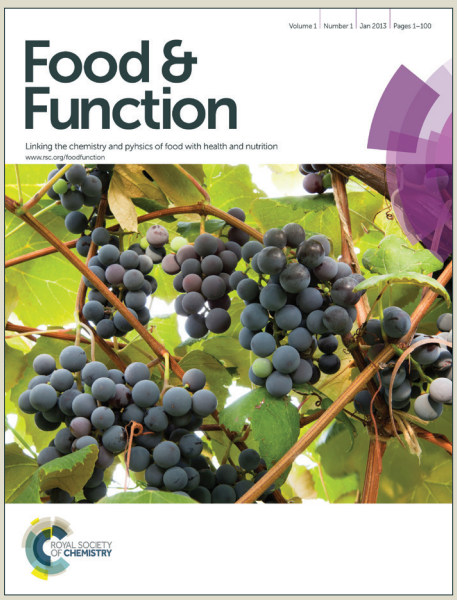

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Rice bran protein hydrolysates prevented interleukin-6- and high glucose-induced insulin resistance in HepG2 cells

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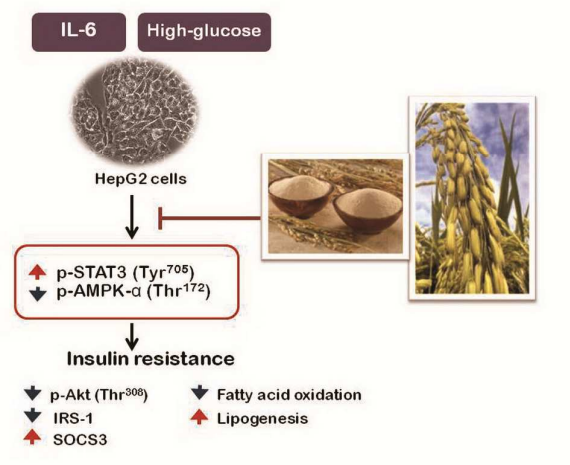

Highlight of the work

Rice bran protein hydrolysates prevent insulin resistance by suppression of IL-6- and high glucose-mediated impairment of insulin signaling

ABSTRACT:

Rice bran, a byproduct of rice milling process, contains various nutrients and biologically active compounds. Rice bran protein hydrolysates have various pharmacological activities such as antidiabetic and antidyslipidemic effects. However, there are limited studies about the mechanisms of rice bran protein hydrolysates (RBP) on insulin resistance and lipid metabolism. RBP used in this study were prepared from Thai Jasmine rice. When HepG2 cells were treated with IL-6, the IRS-1 expression and Akt phosphorylation were suppressed. This effect of IL-6 was prevented by RBP in association with inhibition of STAT3 phosphorylation and SOCS3 expression. RBP could increase the phospho-AMPK levels and inhibited IL-6- or high glucose -induced suppression of AMPK and Akt activation. High glucose-induced dysregulation of the expression of lipogenic genes including SREBP-1c, FASN and CPT-1 was normalized by RBP treatment. Moreover, impaired glucose utilization in insulin resistant HepG2 cells was significantly alleviated by concurrent treatment with RBP. Our results suggested that RBP suppresses inflammatory cytokine signaling and activates AMPK and, thereby these effects may underlie the insulin sensitizing effect.

KEY WORDS: Insulin resistance, Rice bran protein hydrolysates, IL-6, AMPK, STAT3 signaling.

ABBREVATIONS: RBP, Rice bran protein hydrolysates; HepG2, human hepatocellular liver carcinoma cells, IL-6, Inteleukin-6; INF-γ, Interferon-γ; TNF-α, Tumor necrosis factor-α; STAT3, Signal transducer and activator of transcription 3; SOCS3, Suppressor of cytokines signaling 3; JAK, Janus Kinase; AMPK, 5' AMP-activated protein kinase; IRS-1, Insulin substrate receptor-1; SREBP-1C, sterol regulatory element-binding protein 1c; FASN, Fatty acid synthase; CPT-1, Carnitine palmitoyltransferase-1; qRT-PCR, Quantitative real-time polymerase chain reaction; GLUT4, Glucose transporter 4; E/S, Enzyme/substrate; HG, high-glucose.

Introduction

Rice bran is a byproduct from the rice milling process derived from the outer layer of the rice grain. It contains a number of nutrients and biological active compounds.[1] Among these phytochemicals, steryl ferulates, inositols, plant sterol, tocols, flavonoids, and particularly γ-oryzanol have been intensively studied because of their crucial roles in physiological and pathological processes.[1-2] Antidiabetic, antidyslipidemic, antihypertensive, anti-allergic, anti-inflammatory and anticarcinogenic activities of rice bran have been demonstrated both in *in vivo* and *in vitro* experiments.[2-5]

Rice bran protein is an important constituent accounting for 10-15% by weight of rice bran and it consists of 37% water-soluble, 31% salt-soluble, 2% alcohol-soluble, and 27% alkali-soluble storage proteins.[1] Proteolytic modification can improve functional properties of rice bran protein, e.g. water solubility and increase of applicability of the proteins. [6] Thus protein hydrolysates of rice bran are suitable preparation for ultimate use. Rice bran protein hydrolysates (RBP) have been employed to provide nutrients for individuals who have difficulties in the digestion of intact proteins. Their unique properties as hypoallergenicity [1] and possessing pharmacological activities, such as antidiabetic, antidyslipidemic and antihypertensive activities, [2,5] are important as food supplements and new pharmaceutical applications.

Insulin resistance is a state of target cells/tissues with decreased responsiveness to normal circulating levels of insulin, and is a hallmark of type 2 diabetes. Insulin resistance is, thus, a central component in metabolic syndrome and cardiovascular diseases.[7] Insulin resistance is associated with low grade inflammation provoked by immune cells where macrophages infiltrate into expanding adipose tissues of obese subject with consequent elevation of circulating inflammatory cytokines and free fatty acids.[7-8] It is reported that insulin resistance has a strong correlation with the elevation of local and circulating proinflammatory cytokines; TNF-α, IL-1, INF-γ and IL-6.[9-10] Of these proinflammatory cytokines, IL-6 has the strongest correlation with insulin resistance and type 2 diabetes.[9,11] IL-6 signals through the phosphorylation of signal transducer and activator of transcription 3 (STAT3), a down-stream signal molecule of IL-6 receptor and subsequent induction

of suppressor of cytokine signaling 3 (SOCS3), a negative regulator of cytokine signaling. [12] In turn, SOCS3 has been suggested to an important mediator of insulin resistance. [13-14]

Prolonged hyperglycaemia is also a major contributor to insulin resistance. [15] The mechanism by which hyperglycemia causes insulin resistance is associated with an increase in advance glycation end products (AGEs) resulting in oxidative stress, and that contributes to the impaired activation of Akt. [16] It is well established that AMP-activated protein kinase (AMPK) is a main cellular regulator of energy homeostasis at the cellular level [17], and involves in the enhancement of insulin sensitivity. [17-18] The activation of AMPK leads to the inhibition of lipogenesis via the suppression of SREBP-1c in the liver [19], inhibits lipid synthesis, via FASN [20], increases glucose uptake [21] and increases fatty acid oxidation in the skeletal muscles, especially promotes the expression of CPT-1. In contrast, the AMPK activity is suppressed in disorders associated with insulin resistance.[18]

There is limited information about rice bran protein on the amelioration of insulin resistance and lipid metabolism. The present study was to investigate the effects of RBP prepared from Thai Jasmine rice, which is famous and consumed worldwide, on the regulation of insulin signaling in HepG2 cells under the insulin resistant state induced by IL-6 or hyperglycemia. Our results provide the evidences that RBP is effective in preventing the IL-6- or high glucose-mediated induction of insulin resistance in HepG2 cells.

Results and discussion

RBP prevented IL-6-induced degradation of IRS-1 and suppression of Akt phosphorylation

Chronic release of inflammatory cytokines is causally linked with obesity and insulin resistance. [9] Especially, IL-6 has been shown to play critical role in the development of insulin resistance in HepG2 cells. [10] To evaluate the effect of RBP on IL-6 induced inhibition of insulin signaling, HepG2 cells were treated with IL-6 and the expression of IRS-1 and the activation of Akt were determined. It was apparent that insulin stimulated markedly IRS-1 expression and Thr^{308}-Akt phosphorylation, and those insulin actions were suppressed by IL-6 treatment (Fig. 1A and Fig. 1B,

respectively). Pretreatment with RBP prevented IL-6-induced down-regulation of IRS-1 and restored phosphorylation state of Akt (Fig. 1A and B). RBP itself was able to induce IRS-1 expression. These indicate that RBP confers the protection against the effects of IL-6 on insulin signaling in HepG2 cells. The protection on insulin signaling may be from the inhibition of IL-6 signaling pathway and partly from increased expression of IRS-1.

RBP ameliorated IL-6 induced STAT3 activation and SOCS-3 expression

A mechanism of IL-6-induced insulin resistance has been proposed to involve JAK-STAT3-SOCS3 pathway, where IL-6 induces the phosphorylation of STAT3 with subsequent induction of expression of SOCS3.[13,22] SOCS3 subsequently inhibits insulin signaling by blockade of IRS activation and enhances IRS-1/IRS-2 degradation.[13-14] To explore the effect of RBP on IL-6-induced insulin resistance in HepG2 cells, the cells were pretreated with RBP for overnight and then stimulated with IL-6. IL-6 treatment caused drastic increase of phosphorylation of STAT3, however this effect was inhibited by the pretreatment with RBP in a dose-dependent manner (Fig. 2A). The expression of SOCS3 mRNA was remarkably increased by IL-6, and the increase was diminished in a dose-dependent manner by RBP pretreatment (Fig. 2B). The basal expression (control) of phospho-STAT3 and SOCS3 was very low. The results indicated that RBP inhibited SOCS3 expression in association with the suppression of its upstream STAT3 phosphorylation.

RBP stimulated AMPK phosphorylation and prevented IL-6 – evoked suppression of phosphorylation

AMPK is a critical signaling molecule in the maintenance of cellular energy homeostasis. [18] Activation of hepatic AMPK leads to increased fatty acid oxidation, inhibition of hepatic gluconeogenesis and suppression of IL-6 signaling [18,23] resulting in alleviation of insulin resistance. Our present study was to explore whether RBP could activate AMPK, HepG2 cells were pretreated with RBP and then with IL-6, and the degree of phosphorylation of Thr^{172}-AMPK-α was analysed. As was expected, IL-6 treatment caused a decrease in phospho-AMPK without changing the total-AMPK. Pretreatment with RBP prevented the IL-6-induced decrease of phospho-AMPK and

phospho-AMPK/AMPK ratios (Fig. 3). Similarly, metformin, a known AMPK activator, can alleviate IL-6-induced suppression of AMPK phosphorylation. Moreover, RBP alone strongly induced AMPK phosphorylation. It is probable that RBP induces phosporylation of AMPK and phospho-AMPK subsequently inhibits IL-6-stimulated phosphorylation of JAK, STAT3 and their downstream target genes including SOCS3 [23], resulting in repression of IL-6-induced insulin resistance.

High glucose-induced suppression of AMPK and Akt was prevented by RBP

Apart from proinflammatory cytokines that can induce insulin resistance, exposure to high glucose can induce insulin resistance in HepG2 cells.[24] To explore whether high glucose medium (hyperglycemic condition) induced impairment of AMPK signaling could be prevented by RBP. HepG2 cells were cultured in high glucose (30 mmol L^{-1}) DMEM, and phospho-AMPK was analyzed by Western immunoblot. As shown in Figure 4, exposure of HepG2 cells to high glucose decreased phosphorylation of AMPK and Akt when compared with HepG2 cultured in normal glucose medium (Fig.4A & B). Treatment with RBP prevented the suppression of phosphorylation of AMPK and Akt in high glucose culture condition (Fig. 4A & B). This is consistent with a recent study which has shown that the reactivation of AMPK by a flavones glycoside, naringin, in hyperglycemic-induced insulin-resistant liver cells *in vivo* and *in vitro* is associated with insulin responsiveness [25].

To confirm the effect of RBP on AMPK, cells were pretreated with compound C, a potent AMPK inhibitor. Compound C increased high glucose-induced suppression of AMPK phosphorylation (Fig. 4A, last lane). Treatment with RBP partly restore the depressed phosphorylation state of AMPK (Fig. 4A, lane 5 vs lane 6). Similarly, RBP also had a trend to alleviate compound C-induced suppression of Akt phosphorylation (Fig.4B).

RBP prevented high glucose-induced derangement in lipogenic gene expression

Lipid overload is causally related to insulin resistance.[7] AMPK plays important role in inhibition of lipid synthesis genes; SREBP-1c and FASN, and promotion of the expression of lipid oxidation gene, CPT-1.[18] These lipogenic effects of AMPK underlie the insulin sensitizing effect. [24] Since high glucose medium induced insulin resistance characterized by the abnormal expression of

lipogenic genes, the study was to examine whether RBP can attenuate the deranged expression of these genes. For this purpose, HepG2 cells were grown in high glucose DMEM with various concentrations of RBP for 24 h. The expression of SREBP-1c, FASN and CPT1 were measured by RT-qPCR. The results showed that SREBP-1c and FASN expression in the cells grown in high glucose condition were significantly higher than those in the cells cultured in normal glucose condition. Treatment with RBP attenuated the expression of those lipogenic genes (Fig. 5A and B). On the other hand, expression of CPT-1 was suppressed in high glucose condition, but the treatment of RBP restored the CPT-1 expression to the basal level (Fig. 5C).

Moreover, compound C, a potent AMPK inhibitor, caused a slightly enhanced high glucose-induced upregulation of SREBP-1c and down-regulation of CPT-1 mRNA expression. However, RBP treatment apparently restored the levels of those lipogenic gene expression, i.e. a tendency of decreased expression of SREBP-1c and FASN (Fig. 5A and B, lane 6-7), and reversed the suppressed expression of CPT-1 (Fig. 5C, lane 6-7).

These results are consistent with the recent study[5] using rice bran enzymatic extract ameliorated the dyslipidemic state, i.e. hypertriglyceridemia, hypercholesterolemia and elevated non-esterified fatty acids. Our study suggests that the mechanism of RBP in the modulation of lipid homeostasis genes via the activation of AMPK may be the underlying insulin sensitizing effect.

RBP improved glucose utilization of HepG2 cells

Insulin resistance always exhibits the impaired glucose utilization. To evaluate the ultimate effect of RBP on the glucose utilization of HepG2 cells, the cells were cultured with or without RBP in high glucose medium for induction of insulin resistance and glucose levels at varied incubation time were analyzed. In normal glucose (Control) medium condition (5.5 mmol L^{-1}), HepG2 consumed glucose in the rate that sharply increased following the incubation time. At the end of incubation period (32 h), glucose in medium was depleted to < 10 mg dL^{-1} (0.55 mmol L^{-1}; the lower limit of detection). In contrast, in high glucose medium, HepG2 showed a lower rate of glucose disappearance, particularly at the last period of 24-32 h, indicating an impaired glucose utilization of HepG2 cells (Table 1). When HepG2 cells were concurrently treated with RBP (400-1600 μg mL^{-1})

or metformin (Met; 2.5 mmol L^{-1}), the glucose utilization was improved, particularly at high concentrations of RBP (Table 1). Our study support recent reports using rice bran enzymatic extract resulting in the reduction of HOMA-IR in obese Zucker rats implying an attenuation of insulin resistance.[5] Our results demonstrated that there was improvement in glucose utilization of high glucose-induced insulin resistant HepG2 cells by the treatment of RBP.

The components in RBP responsible for the insulin sensitizing effect are not characterized in this study. Although the main composition in RBP is protein hydrolysates, it is still possible that RBP contains small amount of compounds including flavonoids, and gamma-oryzanol which are known to ameliorate insulin resistance.[26] The peptide fragments from enzymatic hydrolysis of rice bran protein containing certain amino acid sequence at amino terminal have been shown to possess dipeptidyl peptidase IV inhibitory activity [27] which may be responsible for the insulin sensitizing effect. Further study may be needed to characterize the active components from RBP.

Table 1 The effect of RBP on glucose utilization of HepG2 cells in high glucose-medium at varied incubation time.

Time (h)	0	18	24	32
Control (normal)	96.3 ± 0.9	77 ± 1.3	62.75 ± 1.1	<10
HG control	458.5* ± 1.5^{a}	435.3 ± 2.5$^{\dagger b,\ddagger a}$	421 ± 1.5^{c,a}	421.5 ± 1.2^{c,a}
HG-RBP400	458.5 ± 1.5 ^{a}	426.3 ± 2.3^{b,b}	417.3 ± 1.1^{c,b}	415.8 ± 1.5^{c,b}
HG-RBP800	458.5 ± 1.5 ^{a}	416.3 ± 1.3^{b,c}	402.3 ± 0.9^{c,c}	402.3 ± 1.3^{c,c}
HG-RBP1600	458.5 ± 1.5 ^{a}	414.3 ± 1.4^{b,c}	397.0 ± 1.4^{c,c}	388.5 ± 1.3^{d,d}
HG-Met	458.5± 1.5 ^{a}	393.3 ± 0.9^{b,d}	377.8 ± 1.3^{c,d}	372.0 ± 1.4^{d,e}

*Data are expressed as mean ± SE, from 4 determinations, and analyzed by two-way ANOVA. †Comparisons for the period of incubation times within the same treatment, ‡comparisons for the treatments within the same period of incubation time. Values treated with the same letter are not significantly different, $p<0.05$

Experimental

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), non-essential amino acids and TRIzol® reagent were purchased from Gibco (Life Technologies, Eugene, OR, USA). Fetal bovine serum (FBS) was obtained from Hyclone laboratories (South Logan, UT, USA). iScript Reverse Transcription Supermix for qPCR and SsoFast EvaGreen Supermix were obtained from BIO-RAD (Hercules, CA, USA). RIPA lysis buffer and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant interleukin-6 was obtained from Prospect-Bio (Israel). Halt Phosphatase Inhibitor Cocktail was purchased from Thermo Scientific (Rockford, IL, USA.). Goat polyclonal antibody against β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies: rabbit monoclonal anti-phospho-AMPKα (Thr^{172}), anti-AMPK, anti-phospho-Akt (Thr^{308}), anti-Akt, anti-phospho-STAT3 (Tyr^{705}), and anti-IRS-1 antibodies and mouse monoclonal anti-STAT3 antibody were purchased from Cell Signaling (Danvers, MA, USA). The ECL™ prime Western blotting chemiluminescence system and polyvinylidene difluoride (PVDF) membranes were purchased from Amersham Biosciences (GE Healthcare Life Science, Piscataway, NJ, USA).

Preparation of Rice bran protein hydrolysates

Rice bran from jasmine rice (Hom Mali 105) defatted with cold-press extraction, was supplied from the Community Organic Produces Enterprise, Lopburi, Thailand. Rice bran protein hydrolysates (RBP) were prepared according to the method of Timachai. [28] In brief, rice bran was suspended in distilled water and adjusted to pH 11.0 for alkaline solubilization. The slurry was centrifuged at 5000 g for 30 min, and the supernatant was obtained and adjusted to pH 4.5 for PI precipitation. The precipitate was suspended in distilled water at pH 7.0 and subjected to proteolysis using a commercial enzyme, Protease G6 (Genencor International, USA). The proteolysis was

performed using 3% enzyme per substrate with pH 8.0 at 55°C for 4 hr. Then the enzyme was inactivated at 85°C for 15 min. After centrifugation, the protein hydrolysates were freeze-dried to obtain RBP powder. The yield of crude RBP powder was 8.8% by weight from the defatted rice bran. The crude RBP was suspended in distilled water and subjected to ultrafiltration using a membrane with molecular weight cut-off 50 kDa. The filtrate containing the low molecular weight RBP peptides was freeze-dried and stored in air-tight container kept at -20°C. The protein content, crude fat, moisture and total phenolic content were analyzed according the established methods [29], Association of Official Agricultural Chemists (AOAC) 1999.[30] The composition of RBP were; protein content (in RBP low molecular weight): 46.6g/100 g powder, crude fat: 16.32%, moisture: 2.01% and total phenolic content: 39.1 mg of gallic acid per g extract.

Cell culture and treatments

The HepG2 cells (American Type Culture Collection (ATCC), Rockville, MD, USA) were maintained in DMEM media with 1 g L^{-1} glucose (5.5 mmol L^{-1} glucose), L-glutamine, supplemented with 10% fetal bovine serum (FBS), 1% MEM non-essential amino acids, 12.5 mmol L^{-1} HEPES, 100 U mL^{-1} penicillin, and 50 μg mL^{-1} gentamicin. The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C and the medium was renewed every 2 days. HepG2 cells were cultured in complete medium until they reached 70% confluence. The cells were used for assays after an overnight starvation in the serum free-medium.

In experiments with IL-6, HepG2 cells were cultured in a 6-well plate at a density of 3.0 $x10^5$ cells per well in DMEM medium. After cell attachment, cultured cells were incubated with different concentrations of RBP (400, 800 and 1,600 μg mL^{-1}) for 18 h in serum-free medium prior treatment with IL-6 (20 ng mL^{-1}) for 30 min. In the study of the effect of IL-6 on Akt and IRS, cultured cells were stimulated with insulin (100 nmol L^{-1}) for 10 min in the presence of IL-6. Then whole cell lysates were extracted with RIPA buffer [150 mmol L^{-1} NaCL, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol L^{-1} Tris, pH 8.0] supplemented with protease and phosphatase inhibitors. In high glucose-induced insulin resistance experiments, the cells were starved for 18 h, and then cultured in the medium containing serum with either normal (5.5 mmol L^{-1}) or high

(30 mmol L^{-1}) glucose in the presence of varied concentrations of RBP (400- 1,600 μg mL^{-1}) or compound C for 24 h. The whole cell lysates were extracted for further analysis. In other set of experiment, total RNA was extracted and expression of the lipid homeostasis genes; SREBP-1c, FASN and CPT-1 was analyzed by reverse-transcription and real-time-PCR technique.

Western Blot Analysis

After treatment, cultured cells were washed with PBS and lysed with RIPA buffer containing protease and phosphatase inhibitors for 15 minutes on ice. The whole cell lysates were centrifuged at 12,000 rpm for 30 min. Supernatant was collected and stored at -80°C until used. The protein concentration was determined using the Bradford's reagent. Twenty micrograms of protein sample was subjected to 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked for 2 h at 4°C with 5% (w/v) bovine serum albumin (BSA) in PBS containing 0.1% (v/v) Tween-20. The PVDF membranes were then incubated overnight at 4°C with primary antibodies in PBS containing 5% BSA and 0.1% Tween-20 at the following concentrations: anti-phospho-AMPKα (1:500), anti-AMPK (1:1,000), anti-phospho-Akt (1:500), anti-Akt (1:1,000), anti-phospho-STAT3 (1:1,000), anti-STAT3 (1:1,000) and anti-IRS-1 (1:1,000), and a goat polyclonal antibody against β-actin (1:2,500). The primary antibody was removed and the blots were extensively washed with PBS/Tween-20. The blots were then incubated for 2 h at 4 °C with 1:2,500 dilution of respective horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, anti-mouse or anti-goat IgG). After removal of the secondary antibody and extensive washings with PBS buffer, the blots were incubated with ECL substrate solution (ECL™ Prime Western Blotting Detection Reagent). The bands of the specific Tyr^{705}-STAT3, STAT3, Thr^{172}-AMPK, AMPK, Thr^{308}-Akt, Akt, IRS-1 and β-actin were visualized and captured by ImageQuant LAS4000 mini (GE Healthcare Life Science). The expression of proteins of interest was normalized with β-actin as a loading control.

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

Total RNA was extracted from cultured cells by TRIZol® reagents according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 2 µg of total RNA using iScript Reverse Transcription Supermix® at 25°C for 5 min, 42°C for 30 min, with a final step of 5 min at 85°C in a C1000 Thermal cycler (Bio-RAD). qPCR analysis was performed starting with 4 µL of cDNA template, in a final volume of 15 µL PCR reaction, with 0.5 µM of each primer, PCR supermix 7.5 µL and sterile water (2 µl) performed in a LightCycler®480 Real-Time PCR instrument (Roche Applied Science). Samples were incubated in the light cycler apparatus for an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation step at 95°C for 15 sec and extension step at 60°C for 30 sec. The specific primers used are described in **Table 2.** The results for the expression of specific mRNAs were expressed relative to β-actin. Relative fold change for target mRNA was calculated using the standard curve method. Amplification of specific transcripts was confirmed by melting curves profiles generated at the end of each run.

Glucose utilization assay

Cultured medium from cell cultures was taken at indicated time intervals, centrifuged to obtain the cell free-supernatant and stored at -20°C. Glucose levels in medium were analyzed by Glucometer, ACCU-CHEK® (Roche diagnostics, UT, USA).

Table 2 Nucleotide sequences of primers used for PCR *(Homo sapiens)*

Gene (Human)	Forward primer	Reverse Primer	PCR product
β-actin (NM_001101.3)	5′-TGCCATCCTAAAAGCCAC-3′	5′-TCAACTGGTCTCAAGTCAGTG-3′	290 bp
SREBP-1c (NM_001005291.2)	5′-GCCATGGATTGCACTTT-3′	5′-CAAGAGAGGAGCTCAATG-3′	181 bp
FASN (NM_004104.4)	5′-CATCCAGATAGGCCTCATAGAC-3′	5′- CTCCATGAAGTAGGAGTGGAAG-3′	391 bp
CPT-1 (NM_001031847.2)	5′-GATTTTGCTGTCGGTCTTGG-3′	5′-CTCTTGCTGCCTGAATGTGA-3′	193 bp
SOCS3 (NM_003955.4	5'-CAAGGACGGAGACTTCGATT-3'	5'- AACTTGCTGTGGGTGACCAT-3'	137 bp

Statistical analysis

All values are expressed as the mean ± standard error of the mean (SE). All experiments were analyzed by an analysis of variance (ANOVA) with post-hoc test by Student-Newman-Keuls. A p-value <0.05 was considered significant.

Conclusion

RBP showed the beneficial effect on insulin resistant state, where the state is predominantly manifested in type 2 diabetes and metabolic syndrome. Our results clearly showed that RBP possess insulin sensitizing effect in two types of in vitro models of insulin resistance induced by proinflammatory cytokine, IL-6 or by high glucose-exposure. The beneficial effect of rice bran has been reported in animal and human studies, we demonstrated here the mechanisms of action of RBP are the associated suppression of IL-6 activation with down-regulation of SOCS3 expression and regain insulin signaling. The RBP-mediated insulin sensitizing effect may also be due to the induction of AMPK phosphorylation and modulation of the abnormal lipogenic gene expression. Our study supports the notion that consumption of RBP might be beneficial for prevention and amelioration of metabolic syndrome and type 2 diabetes.

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Conflict of interest

The authors declare that there are no conflicts of interest.

References

1 C. Fabian and Y. H. Ju, *Crit Rev Food Sci Nutr*,2011 **51,** 816-827.

2 M. S. Islam, R. Nagasaka, K. Ohara, T. Hosoya, H. Ozaki, H. Ushio and M. Hori, *Curr Top Med Chem*,2011 **11,** 1847-1853.

3 Y. Hoshino, N. Hirashima, M. Nakanishi and T. Furuno, *Inflamm Res*,2010 **59,** 615-625.

4 S. P. Choi, S. P. Kim, M. Y. Kang, S. H. Nam and M. Friedman, *J Agric Food Chem*,2010 **58,** 10007-10015.

5 M. L. Justo, R. Rodriguez-Rodriguez, C. M. Claro, M. Alvarez de Sotomayor, J. Parrado and M. D. Herrera, *Eur J Nutr*,2013 **52,** 789-797.

6 K. Bandyopadhyay, G. Misra and S. Ghosh, *J Oleo Sci*,2008 **57,** 47-52.

7 L. Lionetti, M. P. Mollica, A. Lombardi, G. Cavaliere, G. Gifuni and A. Barletta, *Nutr Metab Cardiovasc Dis*,2009 **19,** 146-152.

8 J. M. Olefsky and C. K. Glass, *Annu Rev Physiol*,2010 **72,** 219-246.

9 P. A. Kern, S. Ranganathan, C. Li, L. Wood and G. Ranganathan, *Am J Physiol Endocrinol Metab*,2001 **280,** E745-751.

10 J. J. Senn, P. J. Klover, I. A. Nowak and R. A. Mooney, *Diabetes*,2002 **51,** 3391-3399.

11 J. C. Pickup, G. D. Chusney, S. M. Thomas and D. Burt, *Life Sci*,2000 **67,** 291-300.

12 D. L. Krebs and D. J. Hilton, *J Cell Sci*,2000 **113 (Pt 16),** 2813-2819.

13 J. J. Senn, P. J. Klover, I. A. Nowak, T. A. Zimmers, L. G. Koniaris, R. W. Furlanetto and R. A. Mooney, *J Biol Chem*,2003 **278,** 13740-13746.

14 L. Rui, M. Yuan, D. Frantz, S. Shoelson and M. F. White, *J Biol Chem*,2002 **277,** 42394-42398.

15 P. J. Klover and R. A. Mooney, *Int J Biochem Cell Biol*,2004 **36,** 753-758.

16 E. Tomas, Y. S. Lin, Z. Dagher, A. Saha, Z. Luo, Y. Ido and N. B. Ruderman, *Ann N Y Acad Sci*,2002 **967,** 43-51.

17 Y. C. Long and J. R. Zierath, *J Clin Invest*,2006 **116,** 1776-1783.

18 B. Viollet, B. Guigas, J. Leclerc, S. Hebrard, L. Lantier, R. Mounier, F. Andreelli and M. Foretz, *Acta Physiol (Oxf)*,2009 **196,** 81-98.

19 H. Shimano, N. Yahagi, M. Amemiya-Kudo, A. H. Hasty, J. Osuga, Y. Tamura, F. Shionoiri, Y. Iizuka, K. Ohashi, K. Harada, T. Gotoda, S. Ishibashi and N. Yamada, *J Biol Chem*,1999 **274,** 35832-35839.

20 M. Foretz, D. Carling, C. Guichard, P. Ferre and F. Foufelle, *J Biol Chem*,1998 **273,** 14767-14771.

21 T. Hayashi, M. F. Hirshman, E. J. Kurth, W. W. Winder and L. J. Goodyear, *Diabetes*,1998 **47,** 1369-1373.

22 J. H. Kim, J. E. Kim, H. Y. Liu, W. Cao and J. Chen, *J Biol Chem*,2008 **283,** 708-715.

23 A. Nerstedt, E. Cansby, M. Amrutkar, U. Smith and M. Mahlapuu, *Mol Cell Endocrinol*,2013 **375,** 68-78.

24 M. Zang, A. Zuccollo, X. Hou, D. Nagata, K. Walsh, H. Herscovitz, P. Brecher, N. B. Ruderman and R. A. Cohen, *J Biol Chem*,2004 **279,** 47898-47905.

25 P. Pu, D. M. Gao, S. Mohamed, J. Chen, J. Zhang, X. Y. Zhou, N. J. Zhou, J. Xie and H. Jiang, *Arch Biochem Biophys*,2012 **518,** 61-70.

26 H. H. Cheng, C. Y. Ma, T. W. Chou, Y. Y. Chen and M. H. Lai, *Int J Vitam Nutr Res*,2010 **80,** 45-53.

27 T. Hatanaka, Y. Inoue, J. Arima, Y. Kumagai, H. Usuki, K. Kawakami, M. Kimura and T. Mukaihara, *Food Chem*,2012 **134,** 797-802.

28 S. Timachai, M.Sc. Thesis, Khon Kaen University, 2011

29 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J Biol Chem*,1951 **193,** 265-275.

30 E. Gonzalez de Mejia, Y. S. Song, M. V. Ramirez-Mares and H. Kobayashi, *J Agric Food Chem*,2005 **53,** 1966-1973.

Fig. 1 RBP inhibits IL-6-induced suppression of insulin signaling. HepG2 cells were pretreated with various concentrations of RBP (400-1600 μg mL^{-1}) in serum-free DMEM for 18 h prior stimulation with 100 nmol L^{-1} insulin for 10 min with or without 20 ng mL^{-1} IL-6 for 30 min. Total whole cell lysates were subjected to Western blot analysis for IRS-1 (A) and phospho-(Thr^{308})-Akt and total Akt (B) using β-acin as an internal control. Data are expressed as mean ± SE of three independent experiments. Bars treated with the same letters are not significantly different, $p>0.05$.

Fig. 2 RBP prevents IL-6-induced STAT3 phosphorylation and SOCS-3 expression in HepG2 cells. HepG2 cells were pretreated with various concentrations of RBP (400-1600 μg mL^{-1}) in serum-free DMEM for 18 h prior stimulation with 20 ng mL^{-1} IL-6 for 30 min. Total whole cell lysates were extracted and subjected to Western blot analysis for detection of phospho-(Tyr^{705})-STAT3 and total STAT3 with specific antibodies and β-actin as an internal control (A). In another set of experiment, cultured cells were treated with RBP as the above prior stimulation with 20 ng mL^{-1} IL-6 for 1 h, and total RNA was isolated and relative expression of SOCS3 mRNA was determined by RT-qPCR (B) using β-actin as an internal control. Data are expressed as mean ± SE of three independent experiments. Bars treated with the same letters are not significantly different, $p>0.05$.

Fig. 3 RBP activated AMPK in IL-6-evoked inhibition of AMPK phosphorylation. HepG2 cells were grown in serum-free DMEM in various concentrations of RBP (400, 800 and 1600 μg mL^{-1}) or metformin (2.5 mmol L^{-1}) for 18 h. Then cultured cells were stimulated with IL-6 (20 ng mL^{-1}) for 30 min. Total whole cell lysates were collected and analysed by Western blot using antibodies specific for phospho-(Thr^{172}) AMPK-α and total AMPK-α. Data are expressed as mean ± SE of three independent experiments. Bars treated with the same letters are not significantly different, $p>0.05$.

Fig. 4 Protective effect of RBP on high glucose-induced suppression of AMPK and Akt phosphorylation. HepG2 were cultured in serum-free medium for 18 h, then changed to normal (5.5

mmol L^{-1}) or high (30 mmol L^{-1}) glucose DMEM containing 10% FBS in the absence or presence of RBP at indicated concentrations for an additional 24 h. Compound C (10 μmol L^{-1}) was added to the cultured cells 1 h prior incubation with RBP. Cell lysates were collected and analysed by Western blot using antibodies specific for phospho-(Thr^{172}) AMPK-α and total AMPK-α (A.), phospho-(Thr^{308})-Akt and total Akt (B.) and β-actin as an internal control. Data are expressed as mean ± SE of three independent experiments. Bars treated with the same letters are not significantly different, $p>0.05$.

Fig. 5 RBP inhibited high glucose-induced derangement in the expression of lipogenic genes. HepG2 cells were grown in serum-free DMEM media for 18 h and changed to normal or high glucose DMEM (HG) containing 10% FBS in the absence or presence of the indicated concentrations of RBP for additional 24 h. Compound C (10 μmol L^{-1}) was added to the cultured cells 1 h prior to the incubation with RBP. Total RNA was extracted and relative expression of SREBP-1c (A.) FASN (B.) and CPT-1 (C.) mRNA levels were determined by RT-qPCR. Data are expressed as mean ± SE of three independent experiments. Bars treated with the same letters are not significantly different, $p>0.05$.

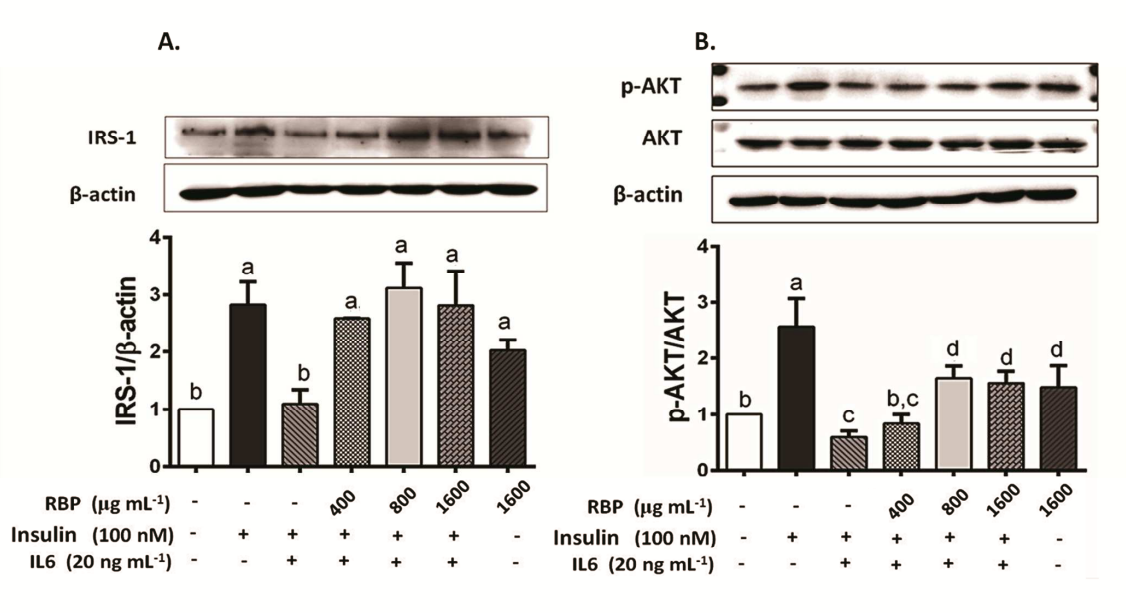

Fig.1

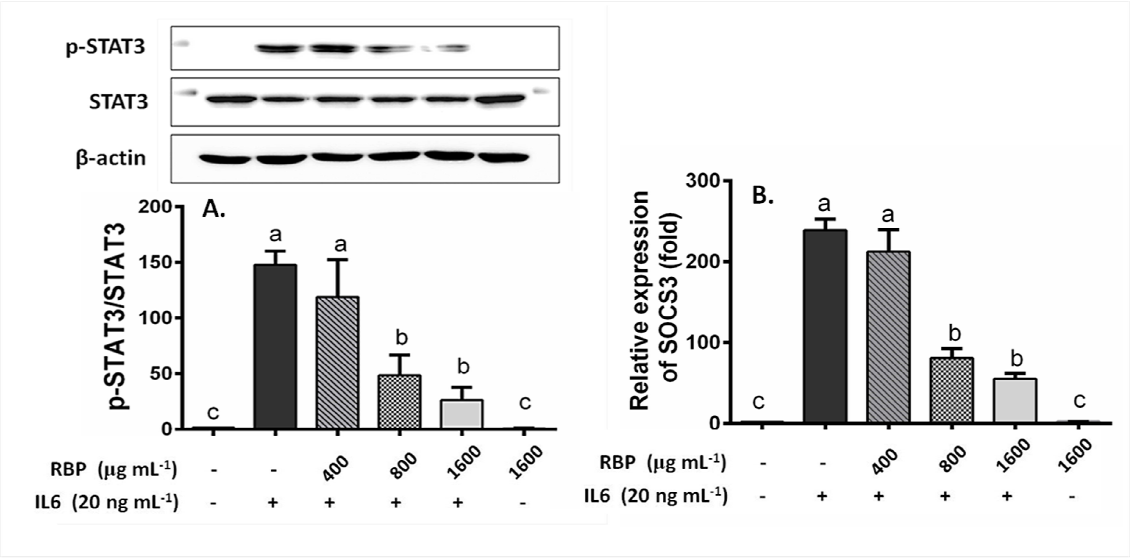

Fig.2

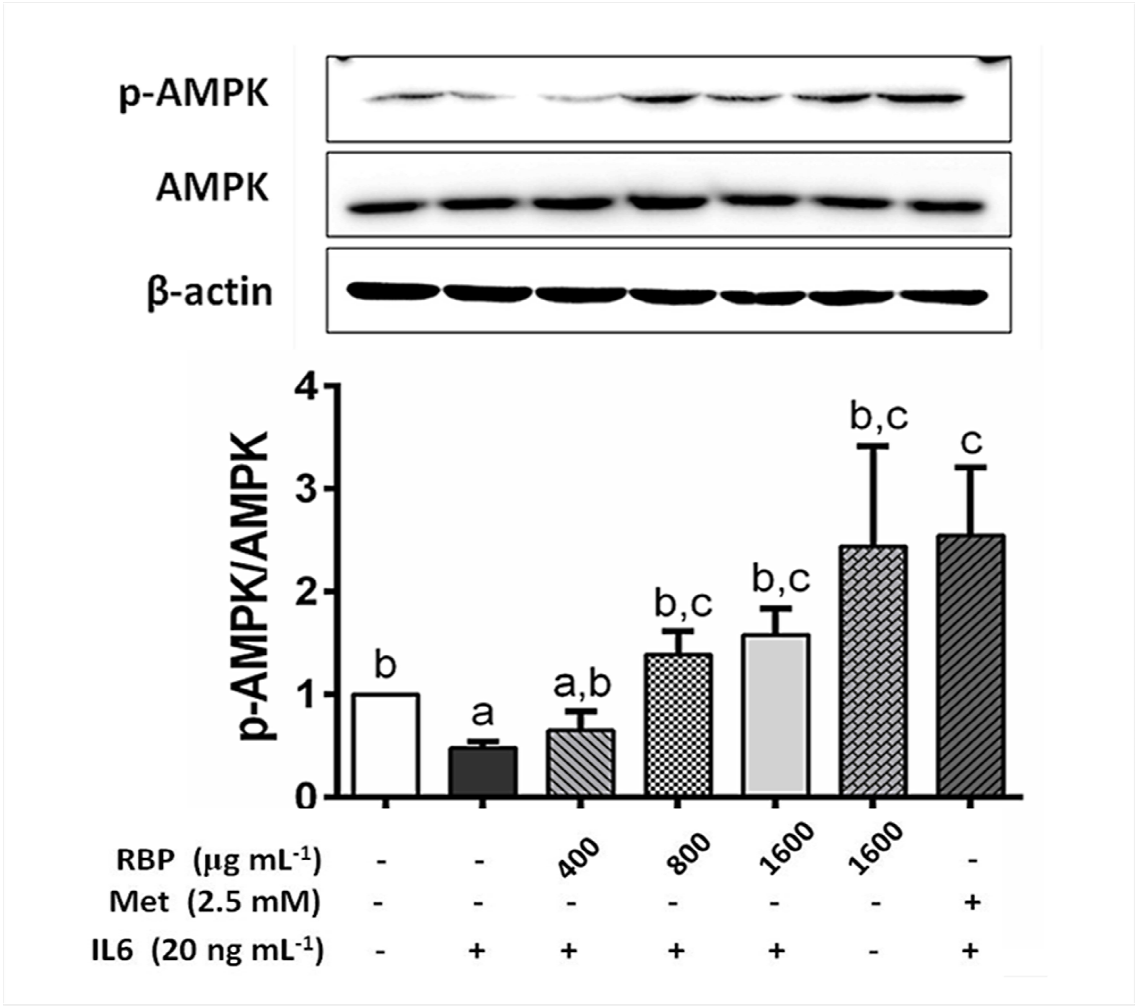

Fig.3

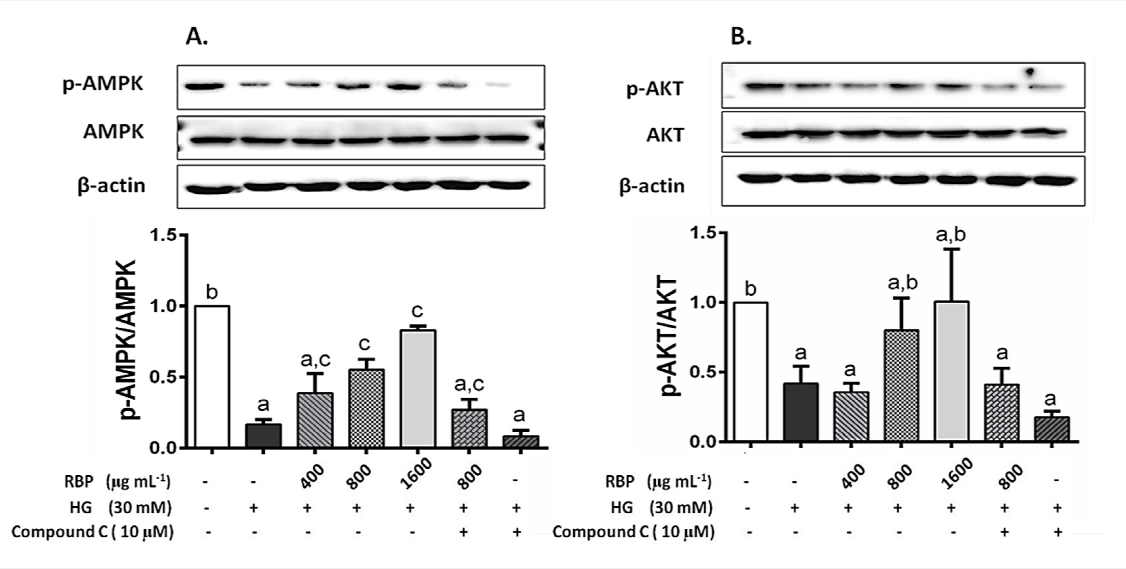

Fig.4

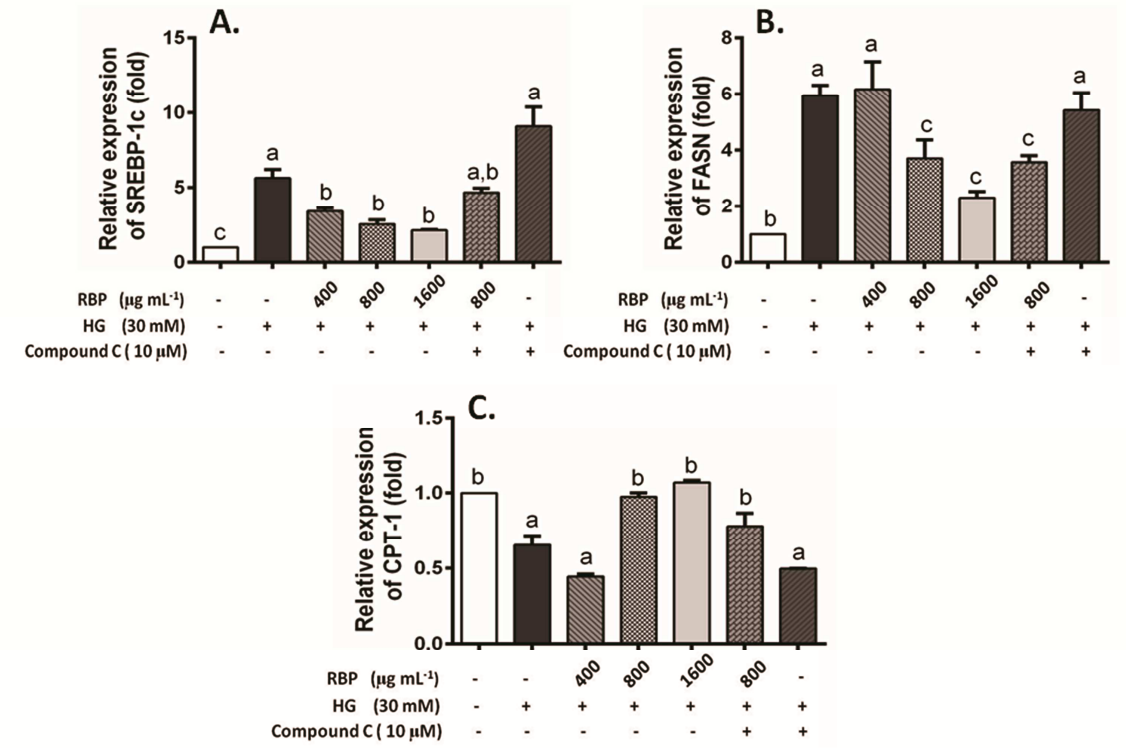

Fig.5