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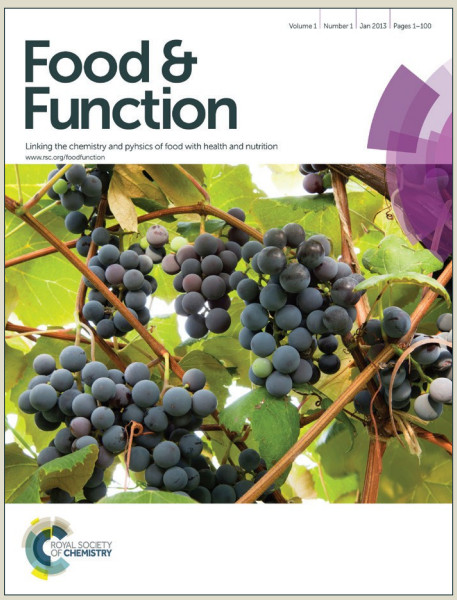

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Tartary Buckwheat Flavonoids protect hepatic cells against high glucose-induced oxidative stress and insulin resistance via MAPKs signaling pathways

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Abstract

Oxidative stress attributes a crucial role in chronic complication of diabetes. In this study, the protective effect of purified tartary buckwheat flavonoids fraction (TBF) against oxidative stress induced by a high-glucose challenge, which causes insulin resistance, was investigated on hepatic HepG2 cells. Oxidative status, phosphorylated mitogen-activated protein kinases (MAPKs), nuclear factor E2 related factor 2 (Nrf2) and p-(Ser307)-IRS-1 expression, and glucose uptake were evaluated. Results suggest that treatment of HepG2 cells with TBF alone improved glucose uptake and antioxidant enzymes, and activated Nrf2, and attenuated the IRS-1 Ser307 phosphorylation, and enhanced total levels of IRS-1. Furthermore, the high glucose-induced changes in antioxidant defences, Nrf2, p-MAPKs, p-IRS1 Ser307, and IRS-1 levels, and glucose uptake were also significantly inhibited by pre-treatment with TBF. Interestingly, the selective MAPK inhibitors significantly enhanced the TBF-mediated protection by inducing changes in redox status, glucose uptake, p-(Ser307) and total IRS-1 levels. This report firstly showed that TBF could recover redox status of insulin-resistant HepG2 cells, suggesting that TBF significantly protected the cells against high glucose-induced oxidative insult, and these beneficial effects of TBF on redox balance and insulin resistance were mediated by targeting MAPKs.

Keywords: Type 2 diabetes mellitus, Oxidative stress, Tartary buckwheat flavonoids, HepG2 cells, Antioxidant defences

Introduction

Type 2 diabetes mellitus (T2DM), a complex metabolic disorder, is the most serious and common cause of morbidity and mortality in modern civilization.[1] Several lines of evidence suggest that oxidative stress plays a main role in the pathogenesis of T2DM,[2,3] where there is a miss-regulation of the glucose homeostasis and the insulin pathway, leading to the decrease in the levels of in vivo antioxidants because of the oxidative stress induced by the hyper-glycaemia.[4-7] However, the modulation of Phase I [glutathione reductase (GR), glutathione peroxidase (GP*x*) and catalase (CAT)] and Phase II [glutathione S-transferase (GST)] enzymes, and glutathione (GSH) levels plays a primary role in the balance of the redox status through the reduction of reactive oxygen species (ROS).[8,9] In this line, the redox-sensitive transcription factor nuclear factor E2 related factor 2 (Nrf2), which is a primary transcription factor responsible for initiating the antioxidative response to reactive oxygen species, could be regulated by dietary flavonoids,[10,11] and its primary control of function lies on its subcellular distribution and/or phosphorylation.[12,13] Moreover, the three major mitogen-activated protein kinases (MAPKs) signaling pathways, including extracellular signal-related protein kinases (ERK1/2), c-Jun NH_2-terminal protein kinases (JNKs) and p38,[14] were regulated by oxidative stress and insulin resistance,[13] and could also be modulated by dietary flavanols,[15,16] and ERK1/2 and p38 have been demonstrated to be main regulators of Nrf2.[13]

Extensive evidence has shown that compounds with strong antioxidant property exert beneficial effects against hyperglycaemia, insulin resistance and oxidative stress,[17,18] which can

be served as a promising approach in the prevention and/or treatment of T2DM.[3,19] Tartary buckwheat (*Fagopyrum tataricum* L., Gaench), a very important edible and medicinal plant in China, have demonstrated to be associated with a lower prevalence of hyperglycemia and improved glucose tolerance in people with diabetes.[20-22] Its beneficial health effects are bound up with its high content of flavonoids, especially rutin and quercetin.[23,24] Additionally, rutin, the major ingredient of buckwheat,[25] has recently been validated as novel strategies for the prevention of type 2 diabetes since it exerts significant anti-diabetic activity by protecting the integrity of pancreatic β cells, restoring the depleted liver antioxidant status, increasing glucose transporter 4 (GLUT4) translocation and reducing plasma glucose in type 2 diabetic rats.[26-29] Quercetin, as the aglycon of rutin, has been demonstrated to exhibit beneficial effects in diabetes mellitus by inhibiting hepatic glucose production, enhancing glucose uptake, potentiating insulin secretion, as well as protecting INS-1 pancreatic β cells against oxidative stress via the ERK1/2 pathway.[19,30-33] All the above indicates that rutin and quercetin are interesting in health protective benefits on T2DM, including insulinomimetic and antioxidant effects. Thereby, it could be hypothesized that tartary buckwheat flavonoids could exert an antidiabetic effect by reducing or even suppressing the oxidative stress of T2DM through the modulation of the cellular antioxidant defences and close-related key proteins for that response. Nevertheless, the enzymatic response of the antioxidant defences and the role of Nrf2 during insulin resistance in T2DM are still remaining unclear.[16] In this regard, the protective effects of tartary buckwheat flavonoids on the response of the antioxidant defence molecular mechanism during insulin

resistance in the liver was firstly studied in terms of antioxidant defences, Nrf2 and regulation by MAPKs.

The aim of the study was therefore to investigate the protective effects of TBF against the oxidative stress in a well-established model of insulin resistant HepG2 cells induced by a high-glucose challenge. Furthermore, we also aimed to determine the mechanisms underlying the process by evaluating the markers of oxidative damage, antioxidant defences and related signals, as well as stress-related signalling pathways and keys features of insulin resistance. This study provided an important clue for substantiating dietary and therapeutic use of tartary buckwheat to prevent the oxidative stress related complication in type 2 diabetes mellitus.

Materials and method

Materials and reagents

Tartary buckwheat powder was obtained from Liangshan Qiongdu Tartary Buckwheat Products Co. Ltd. (Sichuan, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories, Inc. (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliunbromide (MTT), D-glucose, gentamicin, penicillin G, streptomycin, dimethyl sulfoxide (DMSO) and *o*-phthaldehyde (OPT) were purchased from Sigma Co. (St. Louis, MO, USA). 2,7-Dichlorodihydrofluorescein diacetate (DCF-DA) was from Molecular Probes (Carlsbad, CA, USA). The assay kits for GSH, GR, GP*x*, CAT and GST were obtained from Beyotime Co. (Jiangsu, China).

2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose (2-NBDG) was purchased from Molecular Probe (Invitrogen Life Technologies, Carlsbad, CA, USA). Antibodies against ERK1/2, phospho-ERK1/2 (p-ERK) (Thr202/Thy204), JNK1/2, phospho-JNK1/2 (p-JNK) (Thr183/Tyr185), p38 MAPK, phospho-p38 (p-p38) (Thr180/Tyr182), phospho-(Ser307)-IRS-1 and horseradish-peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Cell Signaling Technology, USA). Anti-Nrf2, *β*-actin and anti-lamin B1 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The ERK inhibitor PD98059, p38 MAPK inhibitor SB203580 and JNK inhibitor SP600125 were obtained from Calbiochem (San Diego, CA, USA). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). All other chemicals made in China were of the highest grade available.

Extraction and purification of tartary buckwheat flavonoids

Flavonoids fraction of tartary buckwheat was extracted by methanol-water (75:25, v/v) with reflux for 2 h, and repeated three times.[34] The combined extracts were centrifuged (10 min, 3,000 *g*), and concentrated at 60 °C under vacuum, and then freeze-dried. The dry powder was diluted by deionized water, and then passed through the glass columns (Φ 12 mm × 500 mm) wet-packed with AB-8 resin to purify at the flow rate of 1.5 mL/min. After reaching adsorptive saturation, the column was firstly washed by deionized water, and then eluted by 60% ethanol. The eluate was collected, followed by freeze-drying, and then obtained the purified tartary buckwheat flavonoid fraction (TBF). A detailed description of this TBF has been previously

published.[34] Accordingly, the amount of rutin and quercetin present in the TBF were 536.2 mg/g and 371.6 mg/g, respectively, accounting for up to 90.8% of TBF (determined by HPLC).[34]

Cell culture and treatments

Human hepatic cell line (HepG2), a reliable model, is widely used for biochemical and nutritional studies, where many antioxidants and conditions can be assayed with minor inter-assay variations, and variations of responses to different conditions are more easily detected.[35,36] In the present study, the HepG2 cells were obtained from the Fourth Military Medical University (Xian, China) and cultured in DMEM-F12 medium, supplemented with 2.5% FBS and 50 mg/L antibiotics (gentamicin, penicillin, and streptomycin). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. One day after plating, the medium was changed to DMEM containing 5.5 mM D-glucose, 2 mM glutamine and fetal bovine serum, and the culture was continued. Cells were grown upon reaching 70% confluence and then pre-incubated in serum-free medium for 24 h before treatments.

High dose of glucose was demonstrated to induce insulin resistance, evoked an imbalance of cell redox status, and also induced a response to stress in HepG2 by activating Nrf2 and MAPK pathways.[16] In our study, the dosage of 30 mM glucose was selected to induce oxidative stress and insulin resistance. According to the previously reports,[37,38] the cells were exposed to 100 nM insulin for 10 min after 24 h of incubation with 30 mM D-glucose in serum-free media, and then harvested and tested for ROS production, GSH and carbonyl content, and enzymatic activities (GP*x*, GR, CAT and GST).

To evaluate the protective effect of TBF against high-glucose challenge. TBF was dissolved in DMSO to make a stock solution of 50 mg/mL and further diluted to final concentrations of 25, 50, and 100 μg/mL with serum-free culture medium. The TBF doses were selected based on our previous experiments, since these realistic concentrations were the lowest doses tested that did not induce cellular damage, exhibited a prominent effect on the the ROS and GSH contents.[39] The final concentration of DMSO in culture medium was maintained at 0.05%.[40] Different concentrations of TBF were added to the cells for 24 h, and then, the medium was discarded and fresh medium containing 30 mM glucose was added for additional 24 h. Later, the cells were exposed to 100 nM insulin for 10 min to test the response to insulin and then harvested. In the experiments with the pharmacological inhibitors, cells were pre-incubated with 50 μM PD98059, 10 μM SB203580 or 40 μM SP600125 for 1 h prior to high-glucose challenge. The concentrations of the inhibitors employed have been selected according to the previously used doses in HepG2 cells.[16,18]

Assessment of cell viability and proliferation

Cell viability was determined by using the MTT assay.[41] HepG2 cells were seeded at a density of 1×10^4 cells/mL in 96-well polystyrene culture plates at 37 °C with 5% (v/v) CO_2 for one day. After 24 h of incubation, 100 μL of the medium was removed from each well, and 100 μL of different concentrations of TBF (0, 25, 50, 100 μg/mL) were added and incubated for 24 h. Then 100 μL of 0.5% (w/v) MTT in PBS solution was added to each well and incubated for an additional 4 h at 37 °C in a CO_2 incubator. Then MTT-containing media were removed, and 10%

SDS in DMSO was added to each well and the absorbance at 490 nm of solubilized MTT formazan products was measured using a micro-plate reader (Bio-Rad Laboratories Ltd., China).

For cell proliferation assay, a colorimetric bromodeoxyuridine (BrdU) Cell Proliferation ELISA Kit (Abcam, Cambridge, UK) was used.[15,42] HepG2 cells were seeded in 96-well polystyrene culture plates at a seeding density of 10^4 cells per well counted in a Neubauer chamber. After 20 h of grown, HepG2 cells were either left untreated (control) or exposed to a range of concentrations (0-100 μg/mL) for 24 h. BrdU was added 4 hours before the end of the incubation period. Cells were then fixed, DNA was denatured, and BrdU content was assessed using a monoclonal anti-BrdU antibody following the manufacturer's instructions. Then the immune complexes were quantified by measuring the absorbance at 620 nm in a micro-plate reader.

Detection of ROS production

Cellular ROS were measured by the DCFH assay.[42] This dye is a stable nonpolar compound that diffuses readily into cells and yields DCFH. Intracellular ROS in the presence of peroxidase changes DCFH to the highly fluorescent compound DCF. Thus, the fluorescent intensity is proportional to the amount of ROS produced by the cells. For the assay, the cells were plated in 24-well multi-wells at a rate of 2×10^5 cells per well and changed to FBS-free medium or the different TBF concentrations the day after. Twenty hours later, 5 μM DCFH was added to the wells for 30 min at 37 °C. Then, cells were washed twice with PBS, lysed in cell lysates, and centrifuged at 15,000 *g* for 10 min at 4 °C. The DCF fluorescence intensity of the supernatant

was measured via a fluorescence microplate reader at 485 nm excitation and 535 nm emission (Molecular Devices Co., Sunnyvale, CA). Cellular ROS levels were expressed as relative DCF fluorescence per microgram of protein. This parameter gives a very good evaluation of the degree of cellular oxidative stress.

Measurement of GSH

The content of GSH was quantified by Hissin and Hilf fluorimetric assay.[43] The method is based on the reaction of GSH with *o*-phthaldehyde (OPT) at pH 8.0. After the different treatments, the culture medium was removed and cells (4×10^6) were detached and homogenized by ultrasound with 5% trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells for 30 min at 1,000 *g*, 50 μL of the clear supernatant was transferred to a 96 multi-well plate for the assay. Fluorescence was measured at excitation wavelength of 340 nm and emission wavelength of 460 nm. The results of samples were referred to those of a standard curve of GSH.

Measurement of carbonyl groups

Protein oxidation of cells was measured as carbonyl groups content according to a published method.[44] Treated cells (4×10^6) were collected in PBS and centrifuged at 300 *g* for 5 min to pellet cells. Then, cells were lysed with cell lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China), and centrifuged at 12,000 *g* for 20 min at 4 °C. Absorbance was measured at 360 nm and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22 000 nmol/L/cm. Protein was measured by the Bradford reagent.

Measurement of GP*x*, GR, CAT and GST activities

Treated cells (4×10^6) were collected in PBS and centrifuged at 300 *g* for 5 min to pellet cells to assay the enzymatic activities. Cell pellets were resuspended in 20 mM Tris containing 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at 3,000 *g* for 15 min, then the supernatants were collected and stored at −20 ℃ until biochemical analysis. GP*x* activity was assayed using H_2O_2 and NADPH as substrates. The conversion of NADPH to $NADP^+$ was observed by recording the changes in absorption intensity at 340 nm.[45] GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione.[46] CAT activity was determined by the decomposition of H_2O_2 as a decrease in absorbance at 240 nm.[47] GST activity was analysed by a commercial activity assay kit.[48] The sample was mixed with KH_2PO_4 buffer, EDTA, CDNB and GSH. The reaction was carried out at 37 °C and monitored spectrophotometrically at 340 nm for 5 min.

Preparation of total and nuclear cell lysates

Cells were seeded at a density of 1×10^6 cells/mL in 60 mm polystyrene culture dishes at 37 °C with 5% (v/v) CO_2 for 24 h, and then cells were incubated with TBF for 24 h prior to 24-h glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. After treatments, HepG2 cells were washed twice with PBS (pH 7.4) and lysed in lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing 1% phenylmethylsulfonyl fluoride (PMSF) and 20 mM NaF for 10 min on ice to detect total Nrf2, ERK, p-ERK, JNK, p-JNK, p38, p-p38 and p-(Ser307)-IRS-1. The lysed cells were removed from the culture dish by gentle scraping with a

rubber policeman and transferred to a microcentrifuge tube. To analyse nuclear Nrf2, cells were lysed with nuclear extraction reagent (Xianfeng Biotechnology, Xian, China). Samples were centrifuged for 20 min at 4 °C at 12,000 *g*. The supernatant fractions were collected, and aliquoted and stored at −80 °C until used for Western blot analyses. Protein concentrations were determined with a BCA protein assay kit (Beyotime, China).

Western blot analysis

After boiled with loading buffer for 10 min at 100 °C, proteins (100 μg) were separated by electrophoresis on SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF) (0.45 μm, Millipore) using a semi-dry transfer apparatus (Bio-Rad, Shanghai, China). After blocked with 5% milk for 30 min at room temperature, membranes were incubated at 4 °C overnight with appropriate primary antibodies, followed by incubation with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at 25 °C for 2 h. Blots were detected by enhanced chemiluminescent (ECL) reagent (Pioneer Technology, USA). Normalization of Western blot was ensured by *β*-actin or lamin B1 for nuclear protein extracts, and band quantification was analyzed using Quantity One System (Bio-Rad, Richmond, CA, USA).

Glucose uptake assay

Cellular glucose uptake was assessed using the fluorescent glucose analog, 2-NBDG.[5,28] Briefly, cells were plated in 24-well plates (2×10^5 cells per well counted in a Neubauer chamber) and after the treatments, 2-NBDG was added at 10 μM final concentration for 1 h at

37 °C. Then, cells were washed twice with PBS, serum-free medium was added and fluorescence intensity was immediately measured in a micro-plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. After being taken by the cells, 2-NBDG was converted to a non-fluorescent derivative (2-NBDG metabolite). A fair estimation of the overall glucose uptake was obtained by quantifying the fluorescence.

Statistical analysis

All experiments were performed 3 times, and the data are presented as the mean ± standard error (SE). Statistical comparisons were made using one-way ANOVA, followed by Bonferroni's correction if ANOVAs revealed statistical significances. The p-value < 0.05 was considered to be statistically significant.

Results

Cell viability and proliferation

In order to investigate the potential effects of TBF on cell viability and cell proliferation in HepG2 cells, the cells were cultured with different concentrations (0-100 μg/mL, respectively) for 24 h. As presented in Table 1, treatment of HepG2 cells with TBF up to 100 μg/mL for 24 h did not evoke any changes in cell viability, as determined by the MTT assay, indicating that the concentrations selected for the study did not induce cellular damage during the period of incubation. Similarly, treatment with TBF had no significant impact on cell growth as assessed by a BrdU incorporation assay (p > 0.05), indicating no impairment of cell proliferative

240 machinery and preservation of a regular cell cycle.

241 **Effect of TBF on high glucose-induced oxidative stress**

242 To test the long-term protective effect of TBF on cultured HepG2 cells submitted to oxidative
243 stress, the cells were pre-treated for 24 h with different concentrations of TBF prior to 24 h of 30
244 mM glucose treatment followed by a 10 min chase with 100 nM insulin, and then, parameters
245 related to redox status and antioxidant response were evaluated. As illustrated in Fig. 1, high
246 glucose increased ROS and carbonyl groups levels, and decreased the GSH content when
247 compared to control cells. However, treatment with TBF (100 μg/mL) alone significantly
248 decreased the ROS production, and increased the GSH content ($p < 0.05$), while did not alter the
249 content of tested protein carbonyl. Interestingly, under high-glucose conditions, pre-treatment of
250 HepG2 cells with TBF prevented the GSH depletion and reversed the increases of ROS and
251 carbonyl groups induced by the high glucose in a dose-dependent manner (Fig. 1). Besides, both
252 high glucose and TBF alone increased the GP*x* and GR activities when compared to control cells,
253 while CAT and GST activities did not altered (Fig. 2). Additionally, GP*x* activity was restored to
254 control levels by pre-treatment with all doses of TBF under high glucose conditions, and GR
255 activity was restored to control levels by pre-treatment with TBF (25 and 50 μg/mL) under high
256 glucose conditions, while CAT and GST activities remained unchanged after all incubations (Fig.
257 2). It was also found that when cells were exposed to 100 μg/mL TBF alone, ROS production
258 was reduced to a minimum level, and the GSH content, GP*x* and GR activities reached their
259 maximum levels, showing the best effects compared with treating other doses of TBF (data not

shown). Therefore, 100 μg/mL was selected as the tested treatment concentration for further analysis in this study. These results indicate that high dose of glucose may evoke an imbalance of HepG2 redox status, and TBF protected HepG2 cells against the redox imbalance caused by high glucose.

Effect of TBF on Nrf2 and MAPKs under high glucose condition

To continue the study of the protective effect of TBF against a high-glucose challenge, the levels of Nrf2 and MAPKs were evaluated. As shown in Fig. 3, high glucose significantly increased nuclear and total Nrf2 levels in comparison to control unchallenged HepG2 cells ($p < 0.05$). TBF alone also increased nuclear content and total levels of Nrf2, and showed higher levels of Nrf2 (nuclear) than high glucose-treated cells ($p < 0.05$). Interestingly, the increased nuclear and total Nrf2 levels were unaltered when HepG2 cells were pre-treated with TBF as compared to glucose-challenged cells (Fig. 3). Moreover, phosphorylated levels of ERK, JNK and p38 increased significantly with high-glucose concentrations ($p < 0.05$, Fig. 4). TBF alone did not modify p-ERK and p-JNK levels, whereas decreased the p-p38 levels in comparison to the control cells. It was worth noting that pre-treatment with TBF significantly diminished the enhanced phosphorylated levels of all three MAPKs induced by high glucose ($p < 0.05$, Fig. 4). Total levels of ERK, JNK and p38 were not modified by any treatment. These results suggest that TBF can well modulate the high glucose-induced up-regulations of Nrf2 and MAPKs.

Effect of the MAPKs on TBF-induced change of Nrf2

To further clarify the involvement of MAPKs in the modulation of Nrf2 levels induced by

TBF in high glucose-challenged cells, the effects of MAPKs selective inhibitors on these processes were assayed. First, HepG2 cells were pre-treated for 1 h with ERK and p38 inhibitors (PD98059 and SB203580), respectively, where both kinases have been demonstrated to be main regulators of Nrf2,[13] and then were treated with 100 μg/mL TBF for 24 h. In this regard, it has been reported that the inhibition of the two kinases may reduce nuclear and total levels of Nrf2 caused by both cocoa flavonoids and high glucose in a non-pathological situation.[16] Next, it was examined whether ERK and p38 inhibition would also abolish the increase in the expression of Nrf2 provoked by TBF on high-glucose-challenged cells. As shown in Fig. 5, the blockage of ERK and p38 significantly reduced the nuclear and total levels of Nrf2 in comparison to TBF- and high glucose-treated cells ($p < 0.05$), showing lower values than those of untreated and challenged controls. These results suggested that TBF-induced Nrf2 stimulation was mediated by ERK and p38, and the inhibition of both MAPKs similarly improved the protection induced by TBF.

Effect of the MAPKs on TBF-induced changes of ROS production, and GSH, GP*x* and GR activities

Following, the roles of MAPKs on the generation of ROS, and GSH, GP*x*, and GR activities were analyzed. As shown in Fig. 6, overproduction of ROS induced by high glucose was markedly avoided by TBF treatment, and the presence of MAPKs inhibitors further reduced the ROS production in the TBF pre-treated cells ($p < 0.05$), but did not alter the levels of GSH. Additionally, ERK inhibitor increased GP*x* activity in cells pre-treated with TBF under high

300 glucose condition, whereas blockage of JNK and p38 did not alter this enzymatic activity (Fig.
301 6). In addition, the blockage of p38 significantly increased GR activity in TBF pre-incubated
302 cells under high glucose condition ($p < 0.05$), but ERK and JNK inhibitors did not affect this
303 enzymatic activity (Fig. 6). As a result, blockage of ERK and p38 improved the effects of TBF
304 on GP*x* and GR activities in the high-glucose challenged cells, separately (Fig. 6). These results
305 partly suggested that the protective effect of TBF could be enhanced by partly blocking MAPKs.

306 **Effect of the MAPKs on TBF-induced changes of p-(Ser307)-IRS-1 and IRS-1 levels**

307 Since chronic high glucose treatment may induce the involvement of IRS-1 serine
308 phosphorylation in the desensitization of insulin, and Ser307 is a key regulatory site among
309 several potential serine phosphorylation sites of IRS-1, which is critical to the development of
310 insulin resistance.[37] It has been reported that high-glucose provokes oxidative stress and insulin
311 resistance, and MAPKs are involved in both processes,[16] so the effects of TBF and specific
312 MAPKs inhibitor in insulin-induced Ser307 phosphorylation of IRS-1 and total IRS-1 levels
313 under the high glucose concentration were analyzed. As shown in Fig. 7A and B, the level of
314 insulin-stimulated Ser307 phosphorylation of IRS-1 was significantly increased with high
315 glucose for 24 h, while total IRS-1 level was significantly decreased ($p < 0.05$). However, TBF
316 pre-treatment prevented the enhancement in p-(Ser307)-IRS-1 level and the decrease in total
317 IRS-1 value induced by high glucose, achieving levels similar to the cells of treated with TBF
318 alone ($p < 0.05$). Additionally, the blockage of JNK in high glucose-challenged cells increased
319 the reduction of total IRS-1 expression induced by the high-glucose concentration ($p < 0.05$),

while did not significantly affect the p-(Ser307)-IRS-1 level. Furthermore, the inhibition of JNK in TBF-pretreated cells diminished the p-(Ser307)-IRS-1 to a value lower than the cells incubated with TBF alone. These data indicate that TBF reverses the activation of serine phosphorylation of IRS-1 induced by high glucose, and further JNK is involved in the stimulation of IRS-1 induced by TBF during insulin-resistance in HepG2 cells.

Effect of the MAPKs on TBF-induced changes of glucose uptake

As presented in Fig. 8, high glucose reduced the glucose uptake, whereas TBF alone increased the basal cell glucose uptake, and further pre-treatment of TBF was able to avoid the inhibited glucose uptake caused by the high concentration of glucose, showing high levels to those of TBF-treated cells. In addition, inhibition of all three MAPKs blocked the high-glucose induced reduction of glucose uptake in insulin-resistant cells, showing higher values than the untreated cells ($p < 0.05$). Similarly, the blockage of MAPKs in TBF-pretreated cells completely prevented the diminished glucose uptake of the insulin resistant cells and evoked a higher beneficial effect than that of TBF alone ($p < 0.05$, Fig. 8). These results indicate that TBF protects the hepatic cell functions by improving the glucose uptake, and MAPKs inhibiters may improve the glucose uptake in insulin-resistant cells.

Discussion

Diabetes is strongly combined with increased oxidative stress, which is a consequence of either an increase of free radicals or reduction of antioxidant defenses.[37] Oxidative stress has

been proved as a result of long-term high glucose, and mediates diabetic complications in T2DM.[49] Additionally, the liver, the major modulator in maintaining blood glucose concentration, is particularly susceptible to toxic and oxidative insults.[50] However, numerous studies have demonstrated that tartary buckwheat flavonoids possess beneficial effects on antidiabetic effects in animals and humans,[22] and its main components, rutin and quercetin, which have a protective role against oxidative stress and insulin resistance,[19,26,27,29] may be proposed as potential therapeutic agents in the prevention or treatment of this disease. However, a clear molecular mechanism underlying these effects has not been established. In the present study, we firstly demonstrated that purified tartary buckwheat flavonoid fraction (TBF) could efficiently prevent the unbalance of cellular redox status which was provoked by high glucose via reducing ROS production and modulating the activities of antioxidant enzymes in human liver cells. Furthermore, TBF was also identified as an effective reagent in protecting the insulin resistance induced by high glucose to restore the impairment in glucose uptake which was first steps of the insulin transduction route (p-(Ser307)-IRS-1 and total IRS-1). The observed beneficial effects of TBF on redox balance and insulin resistance were accomplished by restraining signaling pathways related to stress namely MAPKs signal. Here, our data provide a new and detail molecular mechanism how tartary buckwheat can alleviate oxidative stress and insulin resistance under high glucose condition.

Direct evaluation of ROS yields is a good indicator of the oxidative damage to living cells.[50] Glutathione depletion reflects intracellular oxidation, whereas a balanced GSH concentration

could be expected to prepare the cell against a potential oxidative insult.[36,51] In the present study, we revealed that high glucose led to an overproduction of ROS, and as a direct consequence, oxidative damage to proteins was increased and GSH was depleted, which was consistent with the previously reports.[16,52] Changes in the activity of antioxidant enzymes can be considered as biomarkers of the antioxidant response.[35,50] CAT, GST and GP*x* are involved in eliminating peroxides providing an important cellular defence mechanism against oxidative damage, whereas GR recycles oxidized glutathione back to reduced glutathione.[16,53] Therefore, their activities are essential to the intracellular quenching of cell damaging peroxide species, and the effective recovery of the steady-state concentration of reduced glutathione can prevent the cytotoxicity of ROS overproduction.[16,53] Consistent with this, the significant increase in the activity of GP*x* and GR observed in our study clearly indicates a positive response of the cell defense system to face an oxidative insult. However, the GP*x* and GR activities in HepG2 cells treated with high concentrations of glucose were also increased, which evoked an imbalance of HepG2 redox status. These facts might reflect an adaptive mechanism in response to elevated oxidative stress during hyper-glycaemia.[52] Notably, GST activity remained unchanged in HepG2 cells treated with high glucose, while the previous study reported that 30 mM glucose could decrease GST activity.[16] These facts may be related to a diminished hepatic enzymatic expression because of lower insulin stimulation during insulin resistance in T2DM,[54] and further investigations should be carried out. Furthermore, it has been shown that a glucose-induced increase in ROS generation and carbonyl content, and the decrease in GSH concentration were

prevented in cultured cells pre-treated with TBF in the present insulin-resistant model of HepG2 cells. In this regard, it has been reported that both quercetin and rutin induce favorable changes in the antioxidant defense system of cultured HepG2 cells that may prevent or delay cellular oxidative stress.[35,42] In line with these, plant phenolics were reported to provide a parallel protection by increasing the activity of antioxidant enzymes.[50,52] Quercetin was reported to exhibit protective effect on human hepatoma cell line against oxidative stress induced by *tert*-butylhydroperoxide.[55] Numerous evidence suggests that rutin exhibits significant scavenging properties on oxygen radicals and plays a role in modulating oxidative stress by preventing ROS generation.[56-58] Consistent with these, our results revealed that pre-treatment of human HepG2 cells with TBF induced a significant increase in GP*x* and GR activities. Moreover, TBF effectively inhibited the alterations on antioxidant enzymatic activities caused by a high dose of glucose, suggesting that TBF exerted protective effects on the antioxidant defense system in a human HepG2 cells.

Nrf2 regulates the response to cellular stress and cell survival/proliferation, and plays a central role in the induction of phase II enzymes through its binding to the antioxidant response element after its nuclear translocation and phosphorylation.[13] In this regard, it has been described that both nuclear and total Nrf2 can be activated in response to high glucose treatment as an adaptive response to guard against oxidative and inflammatory cell damage,[16] where our result was in full agreement with this. Similarly, it has been shown that quercetin may activate Nrf2 by up-regulating the phosphorylation and translocation of Nrf2, and can modulate the

antioxidant defence, and these effects caused by quercetin seem to play an important role in the regulation of important glutathione-related enzymes.[59,60] In addition, rutin has been reported to significantly reduce oxidative stress and increase Nrf2 expression in injured livers of mice.[61,62] In line with this, it has been described that cocoa flavonoids stimulated Nrf2 with enhancing GP*x* and GR activities.[16] Consistent with these findings, we have found that TBF evokes an increase in GP*x* and GR activities in HepG2 cells in the present study, which correlates with Nrf2 activation. Although this finding deserves further investigation, the results above indicate that TBF-treated HepG2 cells are in favorable conditions to face the increasing generation of ROS induced by the high dose of glucose.

Oxidative stress-induced injury not only results from direct chemical interactions by altering cellular macromolecules, including DNA, proteins and lipids, but also implicates in profound alterations in signal transduction pathways.[63] Signaling cascades involving the MAPKs pathways are key mediators of stress signals and seem to be mainly responsible for protective responses and stress-dependent apoptosis reactions.[18,64] MAPKs are proved to be the upstream signals involved in Nrf2 activation, and would be activated in insulin-resistant HepG2 cells induced by hyperglycaemia.[16] Consistent with this, our study showed that phosphorylated levels of all three MAPKs were increased in insulin-resistant HepG2 cells. Additionally, pretreatment with TBF prevented the activation of MAPKs in hepatic cells treated with a high dose of glucose. In this line, it has been shown that the high glucose (15 mmol/L) induced phosphorylation of ERK and p38 could be significantly inhibited by rutin in human monocytic

THP-1 cells.[65] Moreover, quercetin inhibits lipid accumulation and obesity-induced inflammation by regulation of MAPK signaling factor expression in a dose-dependent manner in adipocytes and macrophages.[66]

MAPKs are also implicated in the up-regulation of antioxidant/detoxificant enzymatic activities together with Nrf2 induction, which constitutes an important pathway to protect cells against oxidative damage.[13] As it has been reported that one of the major contributing mechanisms of Nrf2 activation by phenolic compounds is the phosphorylation of Nrf2 at specific serine and/or tyrosine residues via activation of upstream signaling pathways such as MAPKs.[18] In line with these, there have also been reported that the ERK and p38 inhibitors can decrease the nuclear and total levels of Nrf2 enhanced by the challenge with glucose or cocoa flavonoids, which shows lower values than the challenged hepatic cells.[16] In the present study, the selective inhibitor of ERK prevented the total accumulation of Nrf2 in TBF-pretreated HepG2 cells under high-glucose stress compared with TBF alone and high glucose challenged groups, and p38 inhibitor prevented the nuclear and total accumulations of Nrf2 in TBF-pretreated HepG2 cells under high-glucose stress. Additionally, overproduction of ROS activates the signaling cascades involving in MAPKs pathway.[67] In this regard, ROS generation has been described as a critical upstream mediator for the activation of JNKs, and the persistent activation of JNKs has been directly involved in the development of apoptosis in hepatocytes and non-hepatic cells.[68] Conversely, sustained activation of the ERKs has been shown to confer hepatocyte resistance to death.[18] In the present work, we firstly showed that TBF inhibited the

high glucose-induced increase of ROS, and this effect was improved in the presence of all three MAPKs inhibitors. Moreover, the GP*x* and GR activities restored by TBF in high glucose-challenged cells were modulated by the selective inhibitors of ERK and p38, respectively. These results indicate that the regulation of MAPK pathways by TBF may be implicated in Nrf2, and GP*x* and GR stimulation, and the kinase inhibitors may partly improve the protective effect of TBF. In this regard, it is reported that quercetin regulates GSH-related antioxidant/detoxifying enzymes and Nrf2 by targeting p38-MAPK,[59] and can prevent ethanol-induced hepatotoxicity via p38- and ERK1/2-mediated Nrf2 transcriptional activation.[69] However, other studies suggest that ERKs are implicated in the increased activities of GP*x* and GR in cocoa flavonoids-treated HepG2 cells.[16] In this case, it should be highlighted that further efforts are needed to elucidate the mechanisms of MAPKs involving in the protective effect of flavonoids on the high fructose induced oxidative stress. Altogether, these findings suggest that phosphorylation of all three MAPKs via ROS, glutathione-related enzymes and Nrf2 which probably decrease the oxidative stress, is involved in the protective mechanism of TBF against the oxidative stress induced by the hyperglycemia in HepG2 cells.

Studies have found that the lacking functions of insulin receptor substrate (IRS), a family of docking molecules connecting insulin receptor activation to essential downstream kinase cascades, may be the key molecular lesion signature of hepatic insulin resistance.[37] This defect appears to be a result of insulin-stimulated IRS-1 tyrosine phosphorylation resulting in the reduced IRS-1-associated phosphatidyl inositol 3 kinase activities.[70] Nevertheless, it was

460 demonstrated that serine phosphorylation of IRS-1 was involved in the desensitization of insulin
461 by chronic high-glucose treatment,[71] and our study was consistent with this. However, we have
462 found that TBF prevents the increase of p-(Ser307)-IRS-1 and the decrease of total IRS-1 level
463 induced by high glucose exposure. Additionally, MAPKs have been identified as one of the
464 important proteins in the development of insulin resistance by affecting glucose transporter
465 expression and insulin signaling via modulation of total IRS-1 and phosphorylation of serine
466 residues of IRS proteins.[4,72] It has been reported that ERK and JNK blockages can mediate the
467 inhibition of p-(Ser636/639)-IRS-1 and p-(Ser307)-IRS-1, respectively, displaying negative
468 regulators of hepatic insulin sensitivity,[37] whereas p38 seems to moderately affect p-(Ser)-IRS-1
469 levels.[73] Consistent with this finding, the present study showed that the blockage of JNK could
470 significantly decrease the levels of p-(Ser307)-IRS-1 in insulin-resistant TBF-treated cells. All
471 these results suggest that TBF improves the cellular redox status and insulin resistance in high
472 glucose-exposed HepG2 cells, and these protective effects of TBF are partly enhanced by the
473 JNK inhibitor.

474 In the liver, GLUT2 maintains intracellular glucose in equilibrium with extracellular glucose,
475 although this balance could be altered during insulin resistance.[4,73] It has demonstrated that
476 GLUT2 levels and glucose uptake are decreased in HepG2 cells exposed to a high dose of
477 glucose, and this may be reverted when pre-treated with natural antioxidant compounds.[16]
478 Consistent with the above, the present study showed that TBF prevented the decrease of glucose
479 uptake induced by high glucose in HepG2 cells, displaying a normalization of post-receptor

insulin signalling and a restoration of the hepatic insulin sensitivity. Furthermore, antioxidants have been found to protect the insulin-stimulated glucose transport against oxidative stress in cells,[16] and a role for ERK on the glucose uptake has been reported in hepatic cells.[74] It was also reported that rutin protected oxidative stress-induced insulin resistance in FL83B hepatocytes by improving 2-NBDG uptake through Akt phosphorylation and preventing PPARγ degradation,[75] and quercetin could ameliorate the hyperglycemic effect and oxidative stress parameters by inducing expression of GLUT4 via mRNA expression and translocation to the plasma membrane.[3,76] In the present study, inhibition of all three MAPKs improved the protective effect of TBF on glucose uptake. These results suggest that excess circulating glucose may be cleared by TBF and thereby protects individuals from glucotoxicity damages, and these studies deserve further investigations.

In conclusion, we firstly provide evidence that TBF may be bioactive natural substances with anti-diabetic effect as they can protect hepatic cells from oxidative stress induced by high glucose for enhancing the cellular antioxidant defence capacity. Furthermore, these beneficial effects of tartary buckwheat flavonoids on modulating the translocation of Nrf2 and alleviating this insulin resistance state might be mediated by MAPKs pathways. These findings suggest that TBF possesses protective effect against high glucose-induced oxidative stress and insulin resistance, which is considered as potential therapeutic strategies to preventing or delaying insulin-resistance disease like diabetes, and further experiments directed at the intimate mechanism of TBF are required.

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Figure Captions

Fig. 1 Effects of TBF on the increases of ROS generation and protein carbonyl content, and the deceases of GSH levels induced by high glucose in HepG2 cells. HepG2 cells were treated with 100 μg/mL TBF for 24 h and then were incubated with 30 mM glucose (Gluc) for another 24 h and further exposed to 100 nM (Ins) for 10 min. Data are expressed as percentage of controls. Values are means ± SD of 10 different samples per condition. Different letters over bars indicate statistically significant differences ($p < 0.05$). Different styles of letters (normal, bold and italics) have been used for each parameter depicted within the same graph.

Fig. 2 Effects of TBF on the activities of antioxidant/detoxifying enzymes GP*x*, GR, CAT and GST. HepG2 cells treated with 100 μg/mL TBF for 24 h were incubated with 30 mM glucose (Gluc) for additional 24 h and further exposed to 100 nM (Ins) for 10 min. Values are means ± SD of 10 different samples per condition. Different letters (normal, bold, italics and underlined) denote statistically significant differences, $p < 0.05$.

Fig. 3 TBF induced the nuclear and total Nrf2 levels in HepG2 cells under high glucose condition. Cells were incubated with 100 μg/mL TBF for 24 h prior to 24-h glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. (A) Bands of representative experiments. (B) Nrf2 values are evaluated by densitometric quantification. Values are means ± SD of 8 different samples per condition. β-actin or lamin B1 was used as an internal control.

Means without a common letter differ, $p < 0.05$.

Fig. 4 Effects of TBF on the MAPK signaling pathway in HepG2 cells. Cells were treated with 100 μg/mL TBF for 24 h and were later incubated with 30 mM glucose (Gluc) for another 24 h and further exposed to 100 nM (Ins) for 10 min. (**A**) Bands of representative experiments. (**B**) Densitometric quantification of p-ERK/ERK, p-JNK/JNK and p-p38/p38 ratios as a percentage to the control condition (means ± SD, n = 8). *β*-actin was used as an internal control. Different letters (normal, bold and italics) denote statistically significant differences, $p < 0.05$.

Fig. 5 Effects of TBF and selective inhibitors of ERK (PD, PD98059) and p38 (SB, SB203580) on nuclear and total Nrf2 levels in HepG2 cells. The cells were incubated with 100 μg/mL TBF for 24 h and then treated with 50 μM PD or 10 μM SB for 1 h prior to the 24-h glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. (**A**) Bands of representative experiments. (**B**) Nrf2 values are evaluated by densitometric quantification as a percentage to the control condition (means ± SD, n = 8). *β*-actin or lamin B1 was used as an internal control. Means without a common letter differ, $p < 0.05$.

Fig. 6 Effects of TBF and selective inhibitors of ERK (PD, PD98059), JNK (SP, SP600125) and p38 (SB, SB203580) on intracellular ROS production, and GP*x*, GR and GSH activities in HepG2 cells. The cells were incubated with 100 μg/mL TBF for 24 h and were later treated with

50 μM PD, 40 μM SP, or 10 μM SB for 1 h prior to 24-h glucose (Gluc) challenge, and further exposed to 100 nM (Ins) for 10 min. Intracellular ROS production and activities of GP*x*, GR and GSH are expressed as percentage of control as means ± SD (n = 8). Different letters (plain, bold, italics and underlined) denote statistically significant differences, $p < 0.05$.

Fig. 7 Effects of TBF and selective inhibitors of JNK (SP, SP600125), ERK (PD, PD98059) and p38 (SB, SB203580) on p-(Ser307)-IRS-1 and total IRS-1 levels in HepG2 cells. The cells were incubated with 100 μg/mL TBF for 24-h, followed by further treatment with 40 μM SP, 50 μM PD or 10 μM SB for 1 h prior to 24-h glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. (**A**) Bands of representative experiments. (**B**) Densitometric quantification of p-IRS1(Ser307) and total IRS-1 levels as a percentage to the control condition (means ± SD, n = 8). *β*-actin was used as an internal control. Different letters (plain, bold) denote statistically significant differences, $p < 0.05$.

Fig. 8 Effects of TBF and selective inhibitors of JNK (SP, SP600125), ERK (PD, PD98059) and p38 (SB, SB203580) on glucose uptake in HepG2 cells. Glucose uptake expressed as percentage of control as means ± SD (n = 8). Different letters denote statistically significant differences, $p < 0.05$.

Table 1

Effects of TBF on cell viability and cell proliferation in HepG2 cells. Cell viability was expressed as relative percentage of control cells staining. Cell proliferation was calculated as percentage of the relative increase over the control values of BrdU incorporated into genomic DNA.

	Cell viability (percentage of viable cells)	Cell proliferation (percentage of controls)
Control	100.04 ± 4.97[a]	100.65 ± 6.54[a]
TBF (25 μg/mL)	100.80 ± 8.54[a]	98.45 ± 7.72[a]
TBF (50 μg/mL)	102.52 ± 6.89[a]	102.39 ± 7.04[a]
TBF (100 μg/mL)	104.25 ± 9.07[a]	98.31 ± 10.49[a]

Values are expressed as means ± SD of 10 samples per condition.

Means within a column with a common letter are equal, $p < 0.05$.

Figure 1

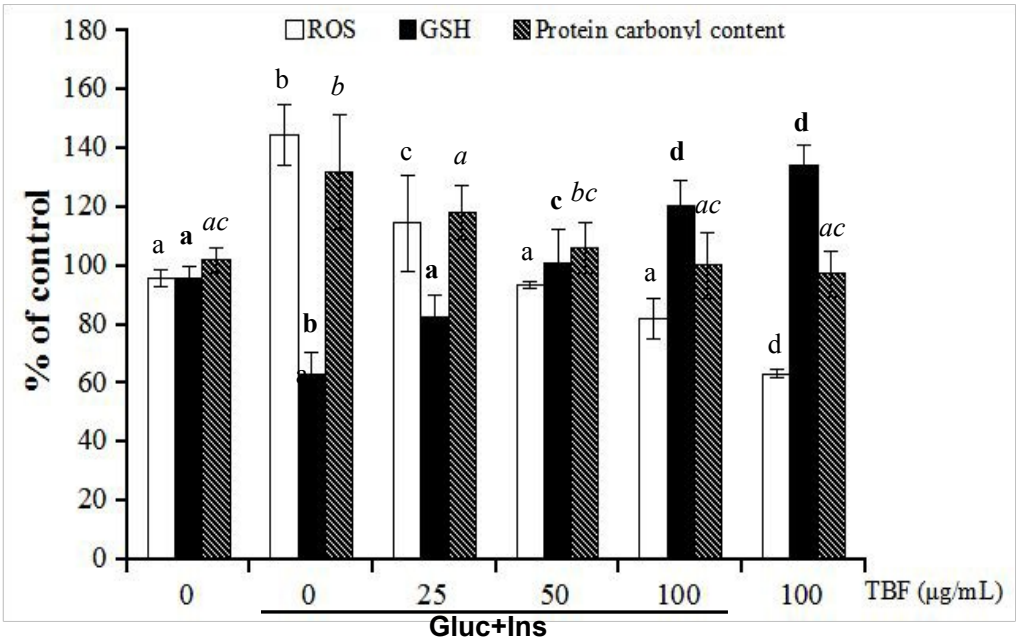
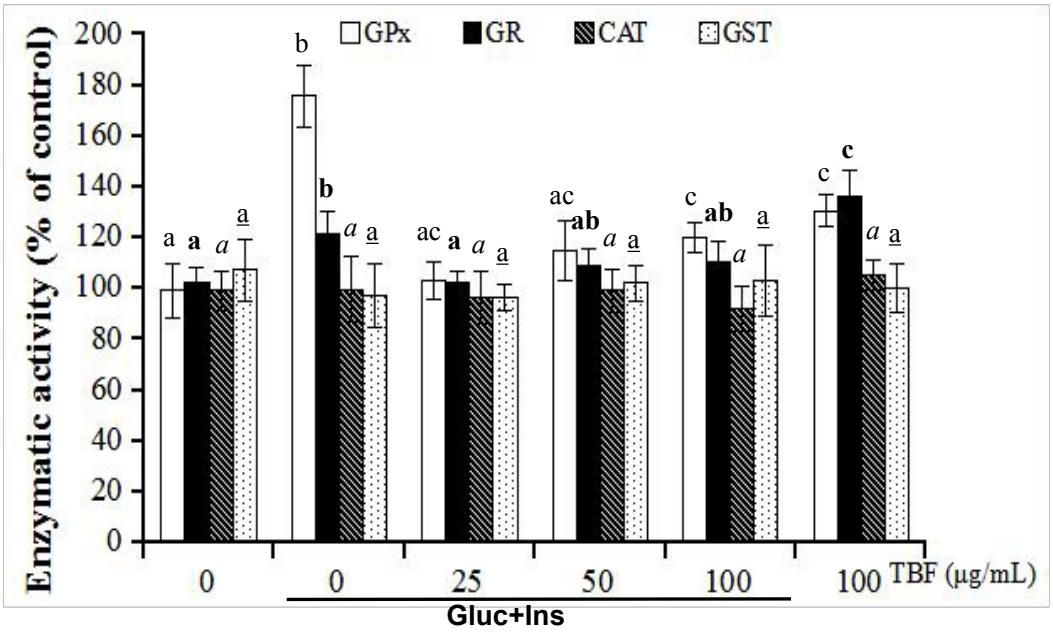


Figure 2



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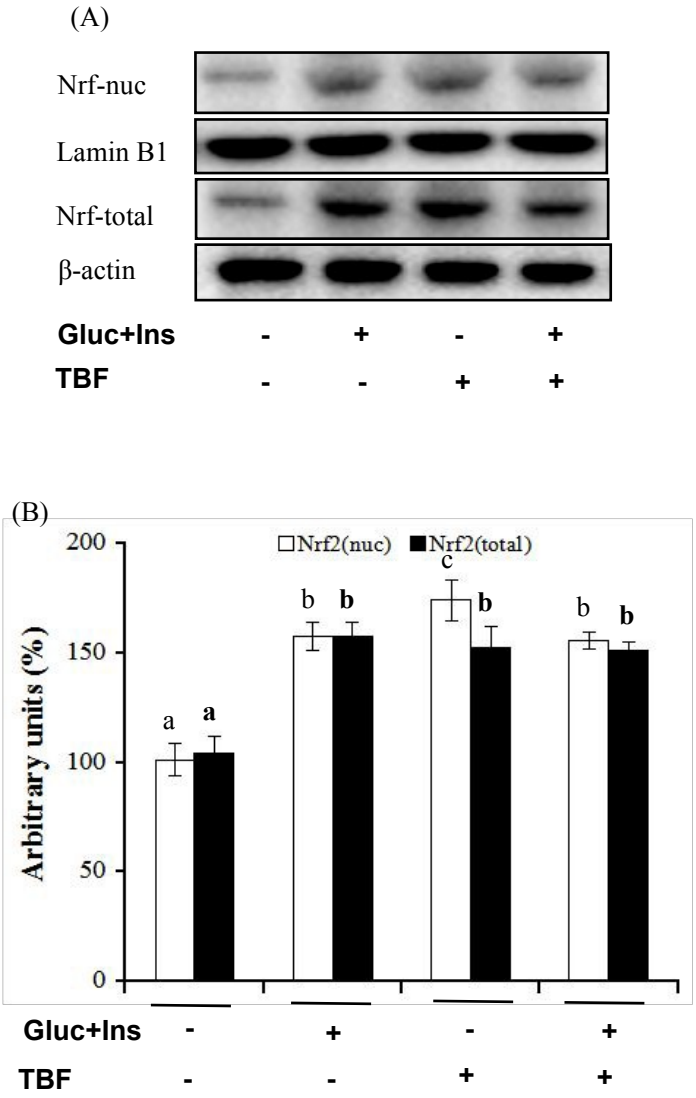

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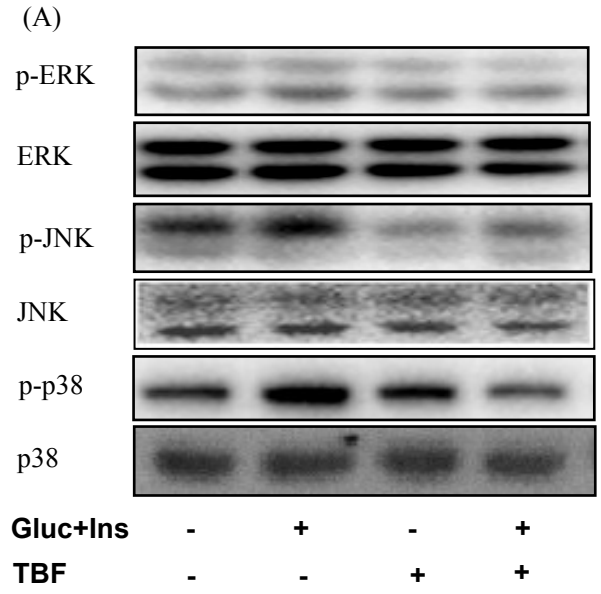


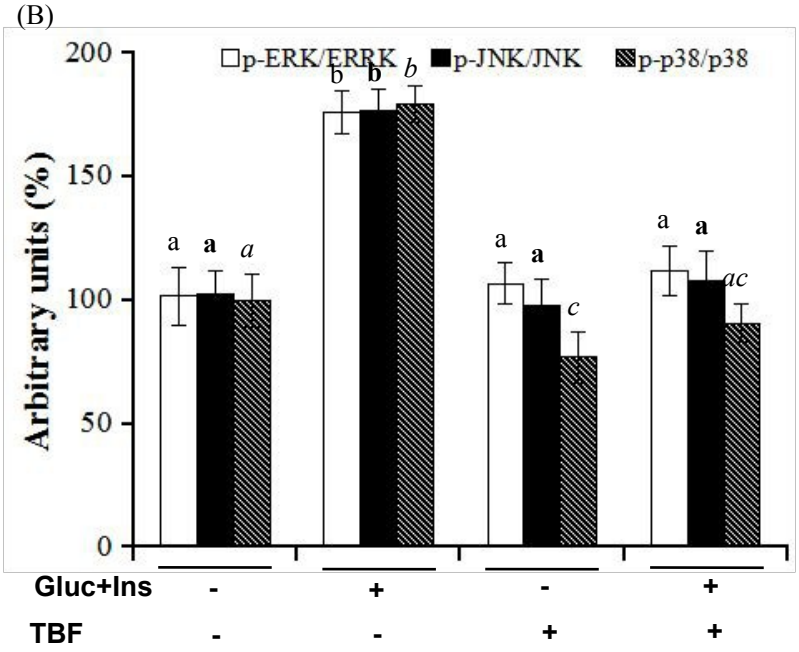


Figure 5

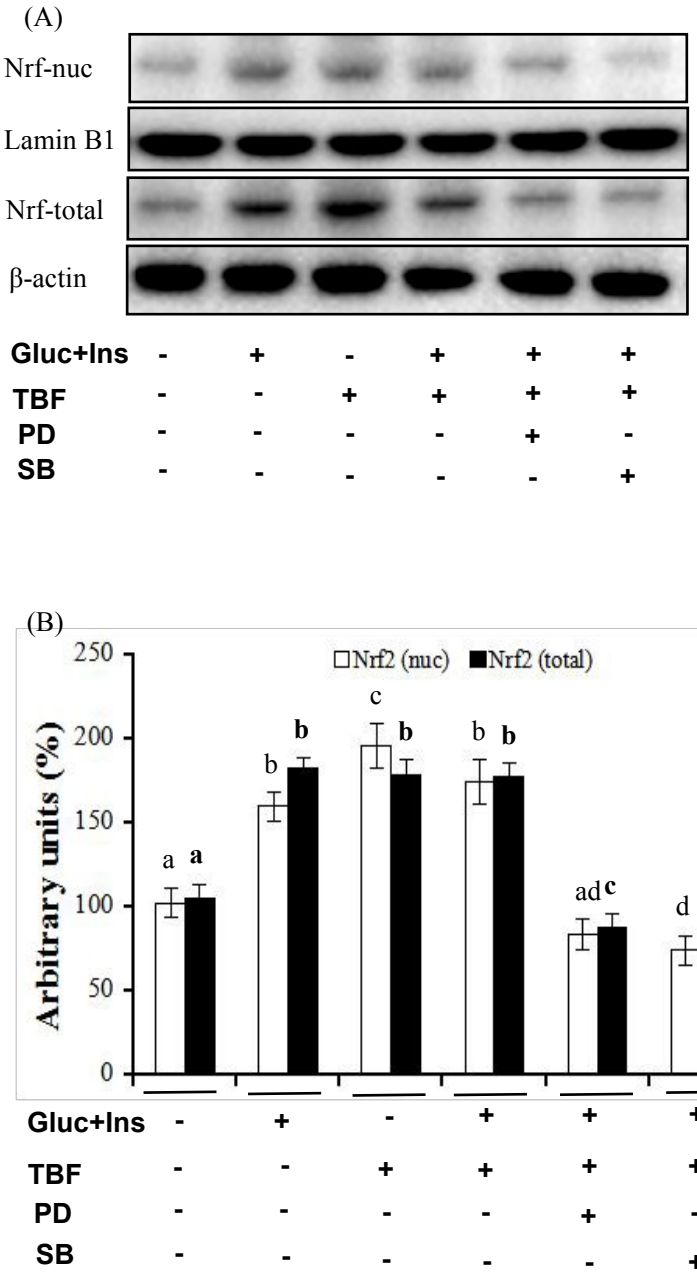


Figure 6

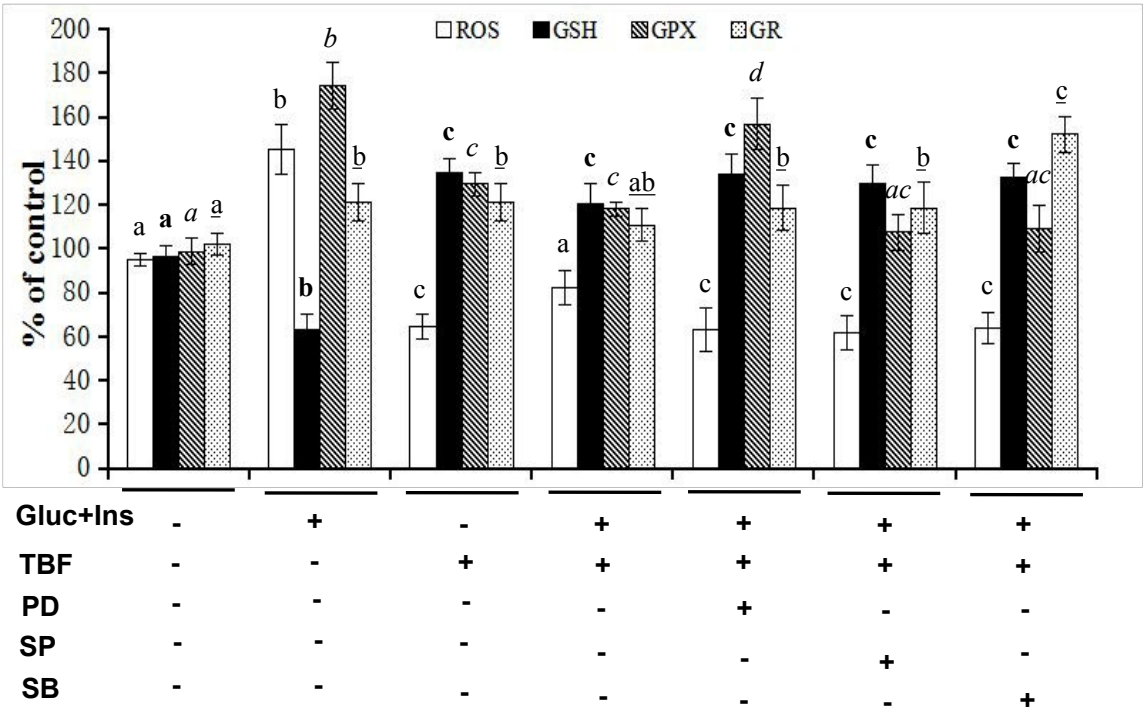
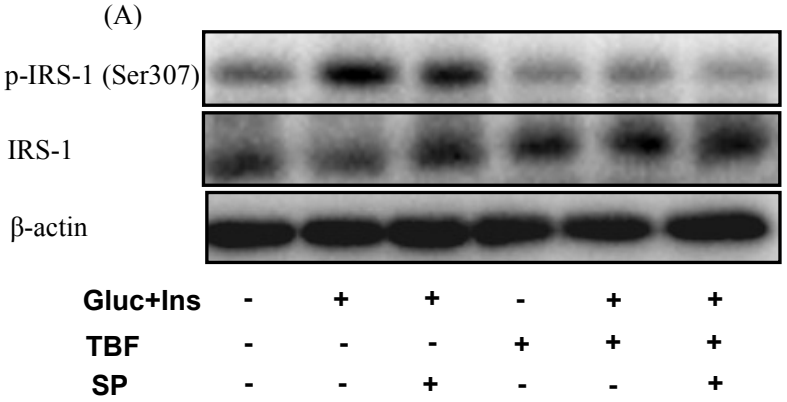


Figure 7



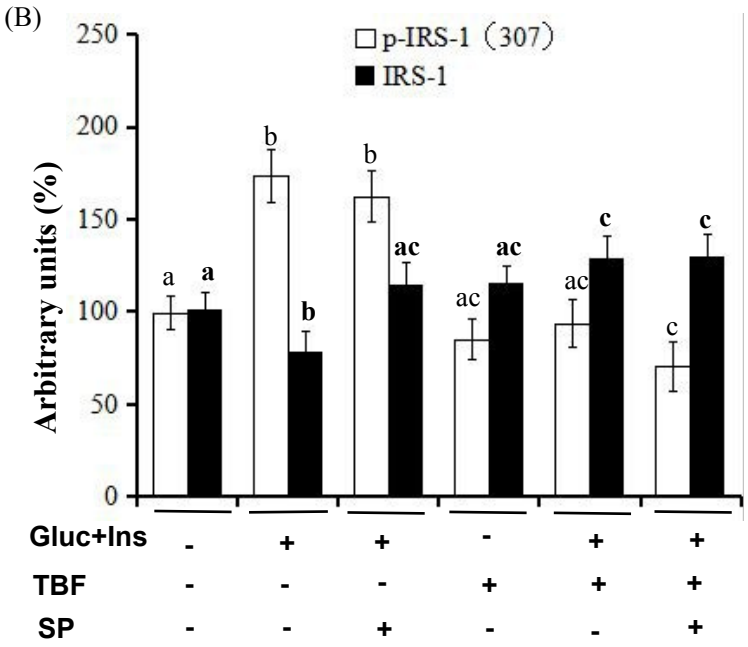


Figure 8