Tartary Buckwheat Extracts Regulate Insulin Sensitivity through IKKβ/IR/IRS-1/Akt Pathway under Inflammation Condition in Mice

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Received April 24, 2014; Revised June 05, 2014; Accepted June 18, 2014

Abstract Tartary buckwheat is rich in flavonoids which have positive effects on preventing chronic disease. But the mechanism of tartary buckwheat ameliorating chronic disease is still poorly understood. This study investigated the regulation of insulin action in skeletal muscle by tartary buckwheat extracts (TBE) under inflammation states in mice. In mice with insulin resistance, glucose intolerance and insulin intolerance was reversed and muscular and hepatic glycogen levels were significantly increased by oral administration of TBE. Furthermore, TBE inhibited inflammation-stimulated IKKβ activation and IRS-1 serine phosphorylation in skeletal muscle tissue, and effectively facilitated IRS-1 tyrosine and downstream molecule Akt phosphorylation, leading to an increase in insulin-mediated glucose uptake in skeletal muscle tissue. The results showed that TBE modulated positively the phosphorylation of IRS-1 function in inflammatory condition to regulation the insulin sensitivity.

Keywords: tartary buckwheat extracts, insulin, inflammation, mice, signal pathway


1. Introduction

Epidemiology has indicated that inflammation is tightly linked with obesity and insulin resistance. Insulin resistance is the fundamental of metabolic syndrome and characterized by specific impairment of insulin phosphatidylinositol 3-kinase (PI3K) signaling, which is attributed to inflammatory molecules [1,2]. Generally, inflammatory cytokines induce insulin resistance via attenuating tyrosine phosphorylation of insulin receptor substrate-1(IRS-1), then inhibiting the downstream PI3K signal transduction [3]. Under inflammation condition, pro-inflammatory cytokines can activate intermediary inflammatory signaling pathways, such as IKKβ/NF-κB pathway, which reduces insulin sensitivity with misleading serine phosphorylation of IRS-1 instead of tyrosine phosphorylation directly or indirectly in the insulin signaling pathway [4]. In IKKβ/NF-κB pathway, IκB kinase β (IKKβ) is a proximal upstream activator of nuclear factor-kappa B (NF-κB), which aggravates insulin resistance through activating NF-κB transcription [5]. Accumulated evidence has established that IKKβ/NF-κB also results in the production proinflammatory cytokines through a feedback regulation mechanism [4]. And this is also confirmed from genetics, that the mice in muscle deficient of IKKβ gene is inhibited the IκB/NF-κB pathway, improved insulin sensitivity [6]. Therefore, the main way to relief the insulin resistance is to control the pro-inflammatory cytokines production or inhibition the inflammatory kinase activity. At present, mounting evidence indicates that flavonoids can available ameliorate inflammation and insulin resistance [7,8].

Tartary buckwheat (Fagopyrum tartaricum Gaertn.) which is a pseudocereal belongs to the Polygonaceae family and is mainly grown in mountainous regions [9,10]. There are affluent flavonoids in tartary buckwheat than common buckwheat and general cereals [11,12]. Meanwhile, studies have showed that flavonoids possess strongly antioxidant ability to scavenge oxygen radicals in vivo and in vitro, based on their structure [13]. Accumulating investigations have revealed that flavonoids have positive effects on the regulation of glucose homeostasis and anti-inflammatory functions alleviating insulin-mediated chronic diseases, such as insulin resistance [14]. Similarly, tartary buckwheat is concerned on the antioxidant activity due to the high level of flavonoids. Therefore, there is now much interested in tatyar buckwheat for its function of improving chronic disease and inflammation. In addition, previous researchers have found that the antioxidant ability of tartary buckwheat is significant linear correlation with the content rutin and total flavonoids [11,15]. Lee et al. [16] also demonstrated that rutin could ameliorate hyperglycemia and hyperinsulinemia in fructose-rich diet-
induced mice. Gong and colleagues [17] and Lee and colleagues [18] found that flavonoids from tartary buckwheat possessed hypoglycemic effect and prevented liver inflammation injury and oxidative stress in mice. But there is little reference on tartary buckwheat extracts regulation the molecular or signal transduction mechanism of inflammatory. In the present study, we investigated the effect of tartary buckwheat extracts (TBE) on the regulation of insulin sensitivity in insulin-resistant conditions in mice and the underlying target molecular of regulation insulin action in IKKβ/IRS-1/Akt-dependent pathway.

2. Materials and Methods

2.1. Materials

2.1.1. Preparation of Tartary Buckwheat Extracts (TBE)  
Tartary buckwheat extracts (TBE) were extracted from Chuanqiao #1. Briefly, TBE were extracted twice with 20 volumes (v/w) of absolute methanol. After vacuum-concentrated, the methanol extracts were removed lipids with petroleum benzene (20 mL) over night, then freeze-dried to obtain powder in which the contents of flavonoids and rutin were respectively 27.51% and 12.90% detected by spectrophotometer (UVmini-1240, shimadzu, Tokyo Japan) and HPLC (Waters, U.S.A). The TBE was stored at 4 °C before use.

2.1.2. Regents

2.1.3. Animals

ICR male mice (6–8 weeks of age), supplied by the Laboratory Animal Center of Nanjing Qinglongshan, were used throughout experiments. The animal care and treatment were maintained in conformity with the Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation.

2.1.4. Preparation of Macrophages-Derived Conditioned Medium

Peritoneal macrophages were prepared as described previously [19]. Mice were killed by cervical dislocation, and injected intraperitoneally of 5 ml of PBS. Two minutes after of gentle abdominal massage, PBS containing peritoneal macrophages was collected, and then incubated in six-well plates at a cell density of 2×10^5/well for 4 h. And then nonadherent cells were removed and adherent cells were washed twice with PBS, and incubated sequentially for 24 h in serum-free DMEM with or without LPS (5 μg/mL). Then, the culture medium with macrophages were centrifuged and the supernatant contained high levels of TNF-α and IL-6 (Interleukin 6) was collected as macrophages-derived conditioned medium (Mac-CM). The Mac-CM was stored at -70 °C before use.

2.2. Methods

2.2.1. Oral Glucose Tolerance Test (OGTT) in Mice

After 12 h of food deprivation, mice (10 mice per group) were treated by oral gavage with either vehicle (normal saline), or TBE (100, 200 and 500 mg/kg), or metformin (200 mg/kg). After 30 min, the mice were intraperitoneally injected with or without Macrophage-CM (0.1 ml/10g, diluted with normal saline, 1:1, v/v, i.p.). Then, mice were orally administered with glucose solution (2 g/kg, p.o.) for another 30 min. Blood was collected from orbital sinus at 0, 30, 60 and 120 min after glucose-load, and levels of blood glucose were determined with a commercial kit based on glucose oxidase peroxidase (GOD-POD) method and blood glucose Area under curve (AUC-G) was calculated as follows: 0.5 × [Bg0 + Bg30]/2 + 0.5 × [Bg30 + Bg60]/2 + 1 × [Bg60 + Bg120]/2 (Bg0, Bg30, and Bg60 referred to the blood glucose concentration at 0,30, 60, and 120 min after glucose load).

2.2.2. Insulin Tolerance Test (ITT) in Mice

The ITT was performed as previously described. Briefly, mice deprived of 12 h were given TBE or metformin and injected Macrophage-CM as same as OGTT. Thirty minutes after the treatment, mice were injected with insulin (0.5 IU/kg, s.c.), and then, blood was collected from orbital sinus before and 30, 60 and 120 min after insulin injection. Glucose concentration was measured by glucose oxidase peroxidase method and the blood AUC was calculated same as a formula above in OGTT.

2.2.3. Determination of Glycogen Content in Mice

Glycogen contents of hepatic and muscle glycogen were measured by commercial kits based on anthrone reagent. Fasting mice for 12 h, then given TBE or metformin (p.o.), injected Macrophage-CM (i.p.), and insulin (0.5 IU/kg, s.c) load as insulin tolerance test. After 30 min of insulin loading, mice was sacrificed by cervical dislocation, and liver and skeletal muscle tissues were treatment as the kit direction.

2.2.4. Western Blot Analysis in Skeletal Muscle Tissue

Mice were performed with TBE or metformin as mentioned earlier in the oral glucose tolerance test. After oral glucose load (30 min), mice were killed by cervical dislocation, and muscular tissue was chopped into small species and then homogenized in ice-cold cell lysis buffer (1:5, w/v, g/mL⁻¹) to extract the protein. The homogenates were incubated at 4 °C for 30 min and then
centrifuged at 12,000 rpm at 4 °C for 15 min and supernatants were collected. The protein content of each sample was determined with Bicinchoninic Acid (BCA) Protein Assay kit (Biosky Biotechnology Corporation, Nanjing, China). Twenty to 40 μg of protein were mixed with sample buffer, boiled for 10 min. Equal amounts of protein samples (20-40 μg) were separated by 10% SDS–PAGE and transferred to PVDF membrane in a MiniTrans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA). The PVDF membranes were blocked with 5% skimmed milk in TBST buffer (Tris–HCl 5 mM, pH 7.6, NaCl 136 mM, 0.05% Tween-20) for 2 h at room temperature or overnight at 4 °C and then incubated with primary antibody (1:800 dilution in TBST buffer) overnight at 4 °C. Following washing three times with TBST buffer, the PVDF membranes were incubated with the secondary antibody to conjugate to HRP at room temperature for 2 h. The intensity of signal was detected by the ECL and levels of protein expression were analyzed quantitatively using Quantity One software (Bio-Rad).

2.3. Statistical Analysis
All data were expressed as mean ± S.D. for three or ten independent experiments. Statistical were analyzed by the SPSS software (version 13). And differences were analyzed by one-way ANOVA and considered to be significant at the $P < 0.05$ level.

3. Results

3.1. TBE Reversed Glucose Intolerance in Mac-CM Treated Mice
The oral glucose intolerance (OGTT) was firstly performed to investigate the effect of TBE at three different doses (100, 200 and 500 mg/kg) on glucose homeostasis under Mac-CM induced insulin-resistant conditions in mice. As shown in Figure 1a, the blood glucose level increased to the maximum at 30 min after glucose loaded, then gradually back to the basal level at 120 min. Administration of TBE p.o. at doses 200 and 500 mg/kg markedly decreased blood glucose at 30 and 60 min by increasing blood glucose disposal, while not significantly change ($P > 0.05$) at dose of 100 mg/kg. As indicated by the increase in AUC (Figure 1b). Metformin, as a positive agent, showed a positive regulation of glucose intolerance by increasing blood glucose disposal ($P < 0.05$).

Figure 1. Oral glucose tolerance in mice under inflammation conditions. Mice were treated by gavage with TBE at concentrations of 100, 200 and 500 mg/kg or metformin (200 mg/kg). After 30 min, mice were intraperitoneally injected with Mac-CM (0.1 mL/10g, diluted with saline, 1:1, v/v), and then mice were orally administered with glucose solution (2 g/kg) 30 min later, and blood glucose was determined at given times. Metformin was taken as a positive control. The results were expressed as the mean ± SD (n = 10)*$P < 0.05$, compare to control.

3.2. TBE Ameliorated Insulin Intolerance in Mac-CM Treated Mice
We further determined insulin intolerance in Mac-CM treatment impaired glucose tolerance in mice. Figure 2(a) showed that the blood glucose levels were sharply reduced after 30 min of insulin loaded. And Figure 2 (a and b) demonstrated that TBE treatment induced the improvement of insulin intolerance in Mac-CM treated mice. In the insulin intolerance test, the blood glucose levels were significantly low in TBE-treatment mice compared to Mac-CM treated group ($P < 0.05$). According to Figure 2(b), the change of blood glucose was not a dose-dependent manner in TBE-treated group. Metformin also demonstrated a similar modulation as the TBE enhancing insulin intolerance ($P < 0.05$).
Figure 2. Insulin tolerance in mice under normal conditions. Mice were treated by gavage with TBE at concentrations of 100, 200 and 500 mg/kg or metformin (200 mg/kg). After 30 min, mice were intraperitoneally injected with Mac-CM (0.1 mL/10g, diluted with saline, 1:1, v/v). After 30 min, mice were injected with insulin (0.5 IU/kg, s.c.), and blood glucose was determined at given times. Metformin was taken as a positive control. The results were expressed as the mean ± SD (n = 10). *p < 0.05 compare to control.

Figure 3. TBE enhanced insulin mediated glycogen synthesis of liver and skeletal muscle tissue in mice. Mice were treated by gavage with TBE at concentrations of 100, 200 and 500 mg/kg or metformin (200 mg/kg). After 30 min, mice were intraperitoneally injected with Mac-CM (0.1 mL/10g, diluted with saline, 1:1, v/v). After 30 min, mice were injected with insulin (0.5 IU/kg, s.c.), and the hepatic and skeletal muscle glycogen were determined after 30 min. Metformin was taken as a positive control. The results were expressed as the mean ± SD (n = 10). *p < 0.05, compare to control.
3.3. TBE Improved the Content of Muscular and Hepatic Glycogen in Mac-CM Treated Mice

As shown in Figure 3(a), the content of hepatic glycogen was reduced in Mac-CM treated group compared to blank group after 30 min of insulin loaded. But this change was reversed by treatment with TBE. And the content of glycogen was significantly increased in medial-dose and high-dose of TBE groups (p<0.05), slightly increased in low-dose group compared to control (p>0.05). Meanwhile, there was a similar change trend on muscle glycogen. And the positive variation was demonstrated that TBE had an effect on modulating of glucose homeostasis in Mac-CM induced mice. Metformin also significantly restored muscular and hepatic glycogen evoked by Mac-CM (p<0.05).

3.4. TBE Inhibited Mac-CM-induced IKKβ Phosphorylation in Muscular Tissue

Research has confirmed that IKKβ is a serine kinase related with inflammation and insulin resistance [20]. IKKβ phosphorylation was notably increased in Mac-CM treatment of mice, but this change was attenuated in TBE and metformin treatment groups (Figure 4). The result indicates that TBE inhibited inflammatory response and enhanced insulin resistance in muscular tissue to some extent.

3.5. TBE Modulated Serine/Tyrosine Phosphorylation at IRS-1 in Mice of Muscular Tissue Exposed to Mac–CM

IRS-1 plays a crucial role in insulin signal pathway, which links inflammation to insulin resistance. Normally, insulin binds to the α-subunit of insulin receptors to induce tyrosine autophosphorylation, then activating the downstream signaling pathway. We inspected the effect of TBE on modulation of IRS-1 serine/tyrosine phosphorylation in Mac-CM treatment of mice. Mac-CM treatment induced IRS-1 serine phosphorylation which disturbed the tyrosine phosphorylation normally. And in our study, TBE treatment group at 100 mg/kg failed to exert influence on IRS-1 serine/tyrosine phosphorylation, while the groups respectively at 200 and 500 mg/kg showed subsequently marked restored tyrosine phosphorylation. Metformin also showed similar positive influence on IRS-1 serine and tyrosine phosphorylation as TBE. On the contrary of serine phosphorylation of IRS-1 by Mac-CM stimulation, tyrosine autophosphorylation was sharply restrained. But the alternation was reversed by treatment with TBE at 200 and 500 mg/kg. It was well demonstrating TBE beneficial modulation of IRS-1 serine/tyrosine phosphorylation (Figure 5). Metformin also showed similar positive influence on IRS-1 serine and tyrosine phosphorylation as TBE.
Figure 5. TBE regulated the IRS-1 serine/tyrosine phosphorylation in mice skeletal muscle tissue under insulin-resistant conditions. Mice were treated by gavage with TBE at concentrations of 100, 200 and 500 mg/kg or metformin (200 mg/kg). After 30 min, mice were intraperitoneally injected with Mac-CM (0.1 mL/10g, diluted with saline, 1:1, v/v). (a) For another 30 min, muscle tissue was separated and homogenized for analyzing Serine (S307) phosphorylation of IRS-1 by Western blot. (b) For another 30 min, mice were injected with insulin (0.5 IU/kg, s.c.), and tyrosine (Py99) phosphorylation of IRS-1 was analyzed by Western blot after 30 min. Metformin was taken as a positive control. The results were expressed as the mean ± SD (n = 3). *p < 0.05, compare to control

Figure 6. TBE enhanced the Akt phosphorylation in mice skeletal muscle tissue under insulin-resistant conditions. Mice were treated by gavage with TBE at concentrations of 100, 200 and 500 mg/kg or metformin (200 mg/kg). After 30 min, mice were intraperitoneally injected with Mac-CM (0.1 mL/10g, diluted with saline, 1:1, v/v) for another 30 min. Mice were injected with insulin (0.5 IU/kg, s.c.). And Akt phosphorylation was detected by Western blot after 30 min. Metformin was taken as a positive control. The results were expressed as the mean ± SD (n = 3). *p < 0.05, compare to control
3.6. TBE Restored Akt Phosphorylation in Mac–CM Treatment Mice

As we all known, insulin modulating the glucose and lipid metabolism is mainly through IRS-1/P13K/Akt signal pathway which is the classical pathway. Normally, Akt is activated by PI3-kinase, and then regulating downstream signaling pathways. So we further observed the effect of TBE on phosphorylations of Akt after insulin-load. Mac-CM treatment attenuated insulin-mediated Akt phosphorylation, but the inhibitory tendency was effectively reversed by TBE at 200 and 500 mg/kg (Figure 6). Metformin also restored Akt phosphorylation.

4. Discussion

Numerous data have demonstrated that flavonoids inhibit insulin-stimulated glucose uptake in muscle cell, liver and adipose tissues [21,22]. Cazarolli et al. [23] reported that flavonoids ameliorated diabetes mellitus by regulation pleiotropic mechanisms of insulin signaling, such as stimulating glucose uptake in peripheral tissue, regulating the activity or expression of the rate-limiting enzymes in carbohyotropic metabolism and modulating insulin secretion or insulin analog. It has recently been reported that flavonoid-rich extract from seeds of Eugenia jambolana (L.) significantly improves glucose tolerance, lipid profile, glycom bioynthesis, glucose uptake and insulin release in vivo and vitro on streptozotocin-induced diabetic mice [24]. It is known that tertiary buckwheat can prevent cardiovascular disease and type 2 diabetes mellitus in favour of abundant flavonoids, especially rutin. In the present study, we observed the effect of TBE on regulation of insulin action in Mac-CM induced mice, and found TBE could exert positive influence on glucose level by acting on IKKβ/IR/IRS-1/P13K/Akt pathway.

To known whether TBE regulates insulin action in vivo through the classical pathway, IKKβ/IR/IRS-1/P13K/Akt signaling cascade, we firstly observed the influence of TBE on glucose tolerance in insulin-resistant mice induced by Mac-CM. Glucose load stimulates insulin secretion from pancreatic islets, and insulin in turn promotes glucose disposal [25]. Administration of TBE significantly restored blood glucose level in OGTT and ITT. These results suggested that TBE had a positive effect on glucose homeostasis in vivo [24,26,27,28,29].

Glycogen exists extensively in bodies and deposits the redundant blood glucose to keep extracellular glucose concentration [30], especially in liver and skeletal muscle tissue. And the levels of hepatic and muscle glycogen are tightly linked with insulin action and directly reflect the activity of insulin. Researches have found that the glycogen levels in skeletal muscle and liver decreased due to the absence of insulin [31], which activates glucose transport, glucose uptake and metabolism and glycogen synthesis to maintain glucose homeostasis. To further verify the effect of TBE on glucose homeostasis, we determined the levels of hepatic and muscle glycogen in insulin resistance mice. In the present study, mice were stimulated with Macrophage-CM to impair insulin signaling, and the concentration of hepatic and muscle glycogen in response to insulin was recuperated by TBE. This result was also consistent with the earlier data, which showed the administration of crude extracts of EJ (Eugenia jambolana) plant improved the hepatic and skeletal muscles glycogen contents levels as compared to control [32]. The increasing of glycogen content might be the flavonoids playing a role in regulating hepatic hexokinase and glucose-6 phosphatase enzyme which tightly linked with insulin signal pathway.

Growing evidence had established that inflammation is closely associated with skeletal muscle insulin resistance [33]. And IKKβ/NF-κB axis is identified as a classical inflammatory pathway that associated inflammation with insulin resistance [34]. As a serine/threonine protein kinase, IKKβ activates NF-κB through transcriptional regulation in involvement of inflammatory pathways. Then NF-κB stimulates the production of numerous markers and potential mediators of inflammation, including TNF-α and IL-6, causing insulin resistance [34, 35]. In return, pro-inflammatory cytokines activate the IKKβ/IKβ/NF-κB inflammatory pathway that results a vicious circle leading to insulin resistance. Macrophage-CM contained inflammatory mediators including TNF-α and IL-6, which was derived from activated macrophages [36]. Studies have proved that TNF-α and IL-6 are the pivotal cytokines tempting serine phosphorylation instead of blunting tyrosine autophosphorylation of IRS-1 in response to insulin induced the inflammation in muscle tissue. Obviously, insulin resistance induced by Macrophage-CM is characteristic of inflammation evidenced by IKKβ activation [36]. As Schenk et al. [37] said, inactivation of IKKβ/NF-κB pathway can control lipid-induced insulin resistance and decrease the proinflammatory stress in skeletal muscle to alleviate insulin resistance. Thus, the pivotal target is to prevent IKKβ phosphorylation to control inflammatory and improve insulin resistance. In this study, the TBE greatly diminished IKKβ phosphorylation under Macrophage-CM induced insulin resistance with (Figure 4), well demonstrating that TBE could blocked the inflammatory cross-talk between macrophages and skeletal muscle tissue.

Many studies have suggested that the serine phosphorylation of IRS-1 is a link between inflammation and insulin resistance, and provide negative feedback to insulin signaling to attenuate insulin-stimulated tyrosine phosphorylation [37]. Meanwhile, serine phosphorylation of IRS-1 can be activated by serine/threonine kinase, inducing insulin resistance. Therefore, keeping the normal phosphorylation of IRS-1 is the key to inhibit insulin resistance and maintain the glucose homeostasis. Thus, we examined the effect of TBE on the phosphorylation of IRS-1 subunits under inflammation condition. Macrophage-CM stimulation induced serine phosphorylation and blunted tyrosine phosphorylation of IRS-1 in respond to glucose load, and these changes were restored by TBE (Figure 5). These results confirmed that inflammation was involved in the impairment of IRS-1 function and TBE played a positive role in IRS-1 function by modification of serine/tyrosine phosphorylation of IRS-
1, leading to improvement of PI3K signaling evidenced by restored Akt phosphorylation (Figure 6). Accordance with the early published reports [38,39,40,41], the tyrosine phosphorylation of IRS-1 activates phosphorylation of downstream signaling events, including Akt, in skeletal muscle. In addition, Shao et al. [36] found that flavonoids ameliorated Akt phosphorylation by modulating IRS-1 phosphorylation rather than PI3-Kinase activity under inflammatory conditions. So, we can deduce that TBE improved the Akt phosphorylation which was the result of regulation of upstream protein molecular phosphorylation of IR/IRS-1/PI3K pathway. What’s more, flavonoids, as multifunctional natural active substance, might also be possible to regulate insulin action in an insulin-independent pathway. Therefore, more studies need to be carried out to clarify the key functional agent and target molecule.

In conclusions, our study indicated that TBE absorption by insulin-resistance mice regulated glucose homeostasis, such as glycemic level and the content of glycogen, via ameliorating insulin sensitivity. Significantly, TBE improved insulin action by blocking IKKβ activation and effectively propagating insulin signaling transduction along IRS-1/Akt pathway (Figure 7). Therefore, TBE can prevent the development of insulin resistance to some extent.

Figure 7. Proposed action pathway of TBE to regulate insulin IRS1/Akt signalling in insulin resistance mice. Under inflammatory conditions, TBE inhibited insulin tyrosine phosphorylation of IRS-1 via suppressed IKKβ phosphorylation, restoring transduction of the insulin IRS-1/Akt pathway.

Acknowledgement

This work was fund by the earmarked fund for China Agriculture Research System (CARS-08-D-2), and National Key Technology R & D Program (2012BAD34B05), People’s Republic of China.

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