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<Original Article>

Anti-diabetic effects of benfotiamine on an animal model of type 2 diabetes mellitus

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Abstract : Although benfotiamine has various beneficial anti-diabetic effects, the detailed mechanisms underlying the impact of this compound on the insulin signaling pathway are still unclear. In the present study, we evaluated the effects of benfotiamine on the hepatic insulin signaling pathway in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which are a type 2 diabetes mellitus model. OLETF rats treated with benfotiamine showed decreased body weight gain and reduced adipose tissue weight. In addition, blood glucose levels were lower in OLETF rats treated with benfotiamine. Following treatment with benfotiamine, the levels of Akt phosphorylation (S473/T308) in the OLETF groups increased significantly compared to the OLETF control group so that they were almost identical to the levels observed in the control group. Moreover, benfotiamine restored the phosphorylation levels of both glycogen synthase kinase (GSK)-3α/β (S21, S9) and glycogen synthase (GS; S641) in OLETF rats to nearly the same levels observed in the control group. Overall, these results suggest that benfotiamine can potentially attenuate type 2 diabetes mellitus in OLETF rats by restoring insulin sensitivity through upregulation of Akt phosphorylation and activation of two downstream signaling molecules, GSK-3α/β and GS, thereby reducing blood glucose levels through glycogen synthesis.

Keywords : Akt phosphorylation, benfotiamine, insulin signaling pathway, OLETF rats, type 2 diabetes mellitus

Introduction

The prevalence of diabetes mellitus and complications associated with this disease are rapidly increasing throughout the world because of changes in lifestyle and increased life spans [13]. Diabetes mellitus is a metabolic disease characterized by hyperglycemia that is linked to various disorders such as neuropathy, nephroopathy, and retinopathy [7]. These complications are important causes of increasing death rates among diabetes mellitus patients [9]. Type 1 diabetes results from a failure of the pancreas to produce insulin whereas type 2 diabetes is caused by insulin resistance. Type 2 diabetic patients develop metabolic syndrome more frequently than individuals with type 1 diabetes, and patients with metabolic syndrome have a high mortality rate [28].

Insulin resistance is a physiological condition characterized by an impaired ability of insulin to regulate blood glucose levels [1] and has been linked to oxidative stress [20, 21]. An excess of glucose products energizing mitochondria results in oxidative stress due to the accumulation of reactive oxygen species (ROS) [8, 11, 18, 19]. One type of ROS, superoxides, switches off the insulin response [8, 18]. Insulin resistance can thus be viewed as a defense mechanism against cellular oxidation damage by blocking increased glucose transport into cells in response to insulin [14, 26]. In the liver, however, insulin resistance causes inactivation of glycogenesis, the process of synthesizing glycogen from glucose [27]. Under these conditions, glucose cannot be converted into glycogen, blood glucose levels gradually increase, and insulin resistance is elevated [27].

Benfotiamine, a lipid soluble thiamine analogue, has greater bioavailability than thiamine and is commonly used to treat diabetic vascular complications [2, 12, 25]. This compound is a thiamine-dependent transketolase activator that directs glucose substrate to the pentose phosphate pathway. Thus, it blocks three major pathways associated with hyperglycemic damage: the hexosamine, advanced glycation end product (AGE) formation, and diacylglycerol (DAG)-protein kinase C (PKC) pathways [12, 25]. All of these mechanisms consequently induce superoxide overproduction by the mitochondrial electron transport chain that causes vascular dysfunction [12, 22, 23]. Although benfotiamine blocks the pathways producing superoxides, no study has demonstrated the effect of benfotiamine on insulin resistance in an animal model of type 2 diabetes mellitus. The Otsuka Long Evas Tokushima Fatty (OLETF) rat is a genetic model of type 2 diabetes mel-

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litus with obesity. OLETF rats show clinically relevant type 2 diabetic phenotypes such as insulin resistance, hyperinsulinemia, hyperglycemia, and mild obesity [15]. In the present study, we evaluated the anti-diabetic effects of benfotiamine on the hepatic insulin signaling pathway in OLETF rats, a well-known genetic model of type 2 diabetes mellitus.

Materials and Methods

Materials

Benfotiamine was purchased from Dalim BioTech (Korea). Anti-phospho-Akt (S473; polyclonal), anti-phospho-Akt (T308; polyclonal), anti-phospho-GSK-3α/β (S21/9; polyclonal), anti-GSK-3α/β (monoclonal), anti-phospho-GS (S641; polyclonal), and anti-GS (polyclonal) antibodies were purchased from Cell Signaling Technology (USA). Anti-β-actin (monoclonal) antibody was from Sigma (USA). Anti-Akt (polyclonal), anti-rabbit immunoglobulin G (IgG), and anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked secondary antibodies were from Santa Cruz Biotechnology (USA).

Animal experiments

Male OLETF and LETO rats were purchased from Otsuka Pharmaceutical (Japan). The animals were housed in rooms kept at 24 ± 1°C with 50% humidity and a 12-h light/dark cycle. The rats were fed a normal diet (5L79; Orient, Korea). The OLETF rats, 27 week old and weighing 540~740 g, were divided into three groups: untreated (n = 7), treatment with 100 mg/kg benfotiamine (n = 7), and treatment with 200 mg/kg benfotiamine (n = 7). LETO rats, 27 week old and weighing 420~520 g, served as controls (n = 7). Benfotiamine was administered with tap water to the OLETF rats for 7 week. Body weight, food consumption and water intake were recorded once a week. All animal experiments were carried out in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals", and were approved by the Institutional Animal Care and Use Committee of Jeju National University (ACUCC; Approval No. 20100016).

Measurement of blood glucose levels

Prior to the experiment (time 0), blood samples were collected from the tail vein after the animals were fasted for 16 h. Blood glucose levels were measured using a OneTouch Horizon system (LifeScan, USA). After treatment with benfotiamine for 7 week, blood glucose levels were again measured using the same methods.

Tissue and organ isolation

At the end of study (7 week), the rats were fasted for 16 h and euthanized in a CO_2 gas chamber. The heart, descending aorta, liver, kidneys, epididymal adipose tissue, and gastrocnemius muscle were removed, weighed, minced, frozen in liquid nitrogen, and stored at −80°C until use.

Sample preparation

Approximately 100 mg of frozen liver were placed in 1.2 mL lysis buffer [20 mM Tris-HCl, (pH 7.4) with 5 mM EDTA (pH 8.0), 10 mM $Na_2P_4O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40, 13.2 μg/mL leupeptin, and 1 mM PMSF] and homogenized for 90 sec at 11,000 rpm with a tissue homogenizer. The homogenates were centrifuged at 800 × g for 15 min. Supernatants were removed and centrifuged at 10,000 × g for 20 min at 4°C. The supernatants from the second centrifugation were stored at −80°C until use. Protein concentrations were measured using the Bradford method [3].

Western blot analysis

Aliquots of liver homogenate protein (60 μg) were separated in a 10% (v/v) SDS-PAGE gel and transferred onto PVDF membranes (Immobilon P; Millipore, USA) for 100 min at 110 V. The membrane was incubated at room temperature for 1 h in blocking buffer [5% (w/v) non-fat dry milk in TTBS; 20 mM Tris-HCl (pH 7.6) with 137 mM NaCl and 0.05% Tween-20], washed four times in TTBS (10 min per wash), and incubated for 1 h with the following primary antibodies diluted 1 : 1,000 in TTBS : anti-p-Akt (S473), anti-p-Akt (T308), anti-Akt, anti-p-GSK-3α/β (S21/S9), anti-GSK-3α/β, anti-p-GS (S641), anti-GS, and anti-β-actin. After washing with TTBS, the membranes were incubated for 40 min with anti-rabbit IgG or anti-mouse IgG HRP-linked secondary antibodies diluted 1 : 10,000 or 1 : 5,000 in TTBS. Reactive bands were visualized using enhanced chemiluminesence (ECL) reagents (Amersham Bioscience, USA). The band intensities were analyzed with Image J 1.42 software (National Institute of Mental Health, USA).

Statistical analysis

All results were represented as the mean ± SEM. Data were analyzed by one way analysis of variance (ANOVA) using SPSS program (SPSS, USA). When the ANOVA test yielded statistical differences ($p < 0.05$ or 0.01), Dunnett's two-tailed *t*-test was used to compare each group against the control group. The value of $p < 0.05$ was used as the criterion for statistical significance.

Results

Body weight gain

The effect of benfotiamine on body weight was evaluated once a week. Weight gain of the OLETF control rats was significantly higher ($p < 0.01$) than that of the LETO animals at the end of the study (7 weeks; Table 1). In contrast, increases in body weight of the OLETF rats treated with both concentrations of benfotiamine (100 mg/kg and 200 mg/kg) were significantly lower ($p < 0.05$ and $p < 0.01$, respectively) than those of the OLETF control group. In particular, administration of benfotiamine (100 mg/kg or 200 mg/kg) significantly reduced adipose tissue weight ($p < 0.05$; Table 1). These results indicate that benfotiamine decreased body weight gain

Table 1. Effect of benfotiamine on the weight of body, adipose tissue, and muscle

Measurement	LETO	OLETF	OLETF B100†	OLETF B200‡
Final body weight (g)§	464 ± 17.2	658 ± 22.3*	608 ± 25.7#	605 ± 19.4#
Body weight gain (g)	49.6 ± 5.2	97.5 ± 12.2*	67.0 ± 7.1#	57.2 ± 10.2##
Adipose tissue (g)§	7.0 ± 0.4	44.8 ± 4.6*	32.9 ± 4.7#	34.8 ± 3.3#
Gastrocnem. muscle (g)§	12.3 ± 0.6	16.4 ± 0.7	15.5 ± 0.5	14.2 ± 1.3
Food intake (g/week)	27.7 ± 0.3	39.8 ± 1.8*	37.4 ± 1.3*	36.2 ± 1.5*
Water intake (mL/week)	35.8 ± 1.8	39.3 ± 2.1	37.3 ± 2.0	38.7 ± 2.3

Data are presented as the mean ± SE. *$p < 0.01$ *vs*. the LETO rats; #$p < 0.05$ and ##$p < 0.01$ *vs*. the OLETF control. †OLETF treated with 100 mg/kg benfotiamine. ‡OLETF treated with 200 mg/kg benfotiamine. §Body weight, adipose tissue and muscle weight were measured at the end of animal experiment (7th week).

Table 2. Effect of benfotiamine on blood glucose levels in OLETF rats

Blood glucose level	LETO	OLETF	OLETF B100†	OLETF B200‡
Initial level (mg/dL)§	112 ± 7.2	135 ± 12.3	137 ± 15.7	142 ± 11.4
Final level (mg/dL)§	124 ± 8.2	182 ± 12.4*	154 ± 9.1#	158 ± 10.2#

Data are presented as the mean ± SE. *$p < 0.01$ *vs.* the LETO rats; #$p < 0.05$ *vs*. the OLETF control. †OLETF treated with 100 mg/kg benfotiamine. ‡OLETF treated with 200 mg/kg benfotiamine. §Initial blood glucose levels were measured at Day 0, and the final levels were measured at the end of animal experiment (7th week).

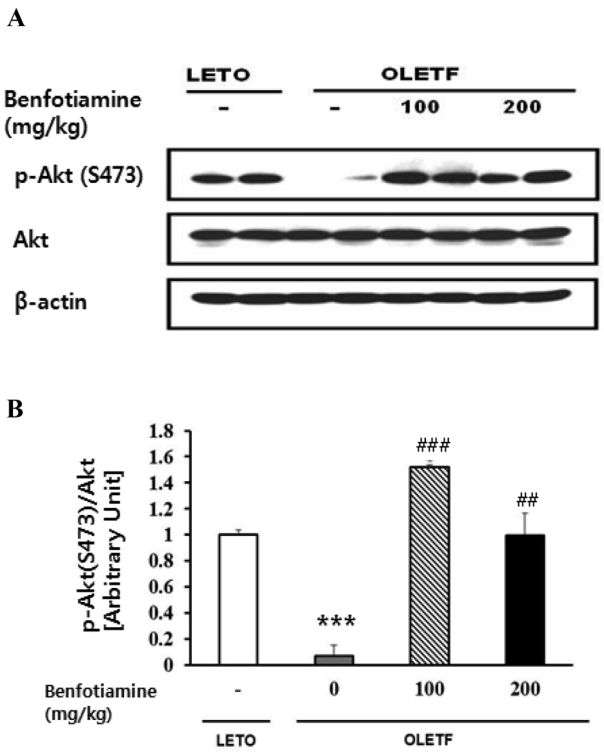

Fig. 1. Effects of benfotiamine on Akt phosphorylation (S473) in rat liver. (A) Akt phosphorylation (S473) was detected by immunoblotting. Representative results from two out of seven rats in each group are shown. (B) The ratio of p-Akt (S473) to Akt band was measured by densitometry. Values are expressed as the mean ± SE. ***$p < 0.001$ *vs.* LETO rats, ##$p < 0.01$ and ###$p < 0.001$ *vs*. the OLETF control.

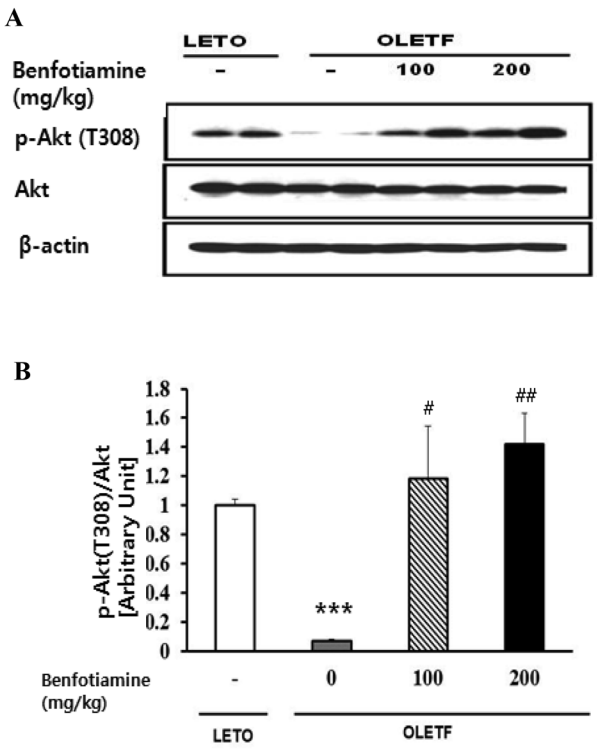

Fig. 2. Effects of benfotiamine on Akt phosphorylation (T308) in rat liver. (A) Akt phosphorylation (T308) was detected by immunoblotting. Representative results from two out of seven rats in each group are shown. (B) The ratio of p-Akt (T308) to Akt band was measured by densitometry. Values are expressed as the mean ± SE. ***$p < 0.001$ *vs*. LETO rats, #$p < 0.05$ and ##$p < 0.01$ *vs*. the OLETF control.

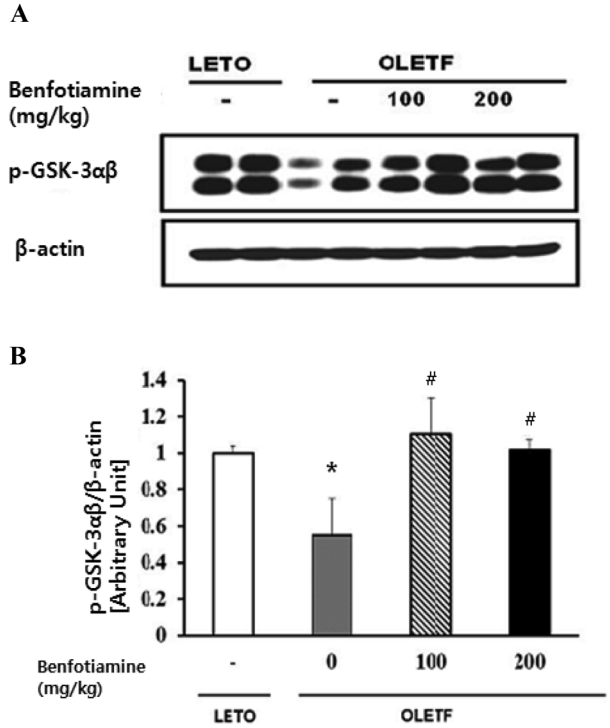

Fig. 3. Effects of benfotiamine on GSK-3α/β phosphorylation (S21/S9) in rat liver. (A) GSK-3α/β phosphorylation (S21/S9) was detected by immunoblotting. Representative results from two out of seven rats in each group are shown. (B) The ratio of p-GSK-3α/β to β-actin band was measured by densitometry. Values represent the mean ± SE. $^{*}p < 0.05$ *vs.* LETO rats, $^{\#}p < 0.05$ *vs*. the OLETF control.

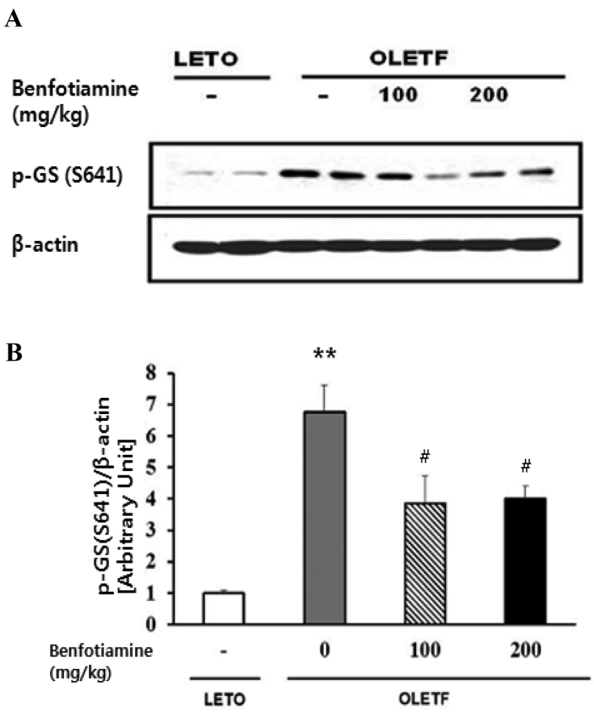

Fig. 4. Effects of benfotiamine on GS phosphorylation (S641) in rat liver. (A) Glycogen synthase (GS) phosphorylation (S641) was detected by immunoblotting. Representative results from two out of seven rats in each group are shown. (B) The ratio of p-GS (S641) to β-actin band was measured by densitometry. Values represent the mean ± SE. $^{**}p < 0.01$ *vs*. LETO rats, $^{\#}p < 0.05$ *vs*. the OLETF control.

by slowing down adipose tissue accumulation.

Blood glucose levels

The effect of benfotiamine on blood glucose levels was assessed before and after treatment with benfotiamine (day 0 and 7 week; Table 2). On day 0, blood glucose levels of the OLETF groups were slightly higher than those observed in the LETO group (Table 2). After 7 week, blood glucose levels of the OLETF control group were significantly greater ($p < 0.01$) than those in LETO group. These changes were significantly reduced ($p < 0.05$) by benfotiamine administration in the OLETF rats (Table 2). Our results indicate that benfotiamine reduces blood glucose levels in OLETF rats.

Akt phosphorylation

To observe the effects of benfotiamine on the insulin signaling pathway in the liver, Akt phosphorylation (S473/T308) was monitored by Western blotting (Fig. 1 and 2). Akt phosphorylation levels of S473 in the OLETF control group were significantly lower ($p < 0.001$) than those observed in the LETO rats. After treatment with both concentrations of benfotiamine (100 mg/kg and 200 mg/kg), Akt phosphorylation of S473 increased significantly ($p < 0.001$ and $p < 0.01$, respectively) compared to OLETF control group so that they were even higher levels than those observed in the LETO group (Fig. 1). Similarly, Akt phosphorylation levels of T308 in the OLETF control group were significantly lower ($p < 0.001$) than those observed in the LETO rats (Fig. 2). After treatment with benfotiamine (100 mg/kg and 200 mg/kg), Akt phosphorylation of T308 increased significantly ($p < 0.05$ and $p < 0.01$, respectively) compared to OLETF control group so that they were almost identical to the levels observed in the LETO group (Fig. 2). The results indicate that benfotiamine restores the phosphorylation of Akt to the normal levels in the liver of OLETF type 2 diabetic rats.

GSK-3α/β and GS phosphorylations

The study also determined the effects of benfotiamine on the phosphorylation of downstream effectors in the insulin signaling pathway: GSK-3α/β (S21/S9; Fig. 3) and GS (S641; Fig. 4). Even though the phosphorylation levels of GSK-3α/β in the OLETF control group were significantly lower ($p < 0.05$) than those observed in the LETO rats, benfotiamine restored GSK-3α/β phosphorylation in the OLETF rats ($p < 0.05$) almost to the same levels observed in the LETO animals (Fig. 3). In contrast, the level of GS phosphorylation

(S641) in the OLETF control group was significantly higher ($p < 0.01$) than that in the LETO group, the levels were significantly decreased ($p < 0.05$) in the OLETF treated with benfotiamine (Fig. 4). The results indicate that benfotiamine restores the phosphorylation of both GSK-3α/β and GS to the normal levels in the liver of OLETF rats.

Discussion

The present study showed that benfotiamine reduced body weight gain, epididymal adipose tissue weight, and blood glucose levels in OLETF rats. The therapeutic effects of benfotiamine on type 2 diabetes mellitus in OLETF rats might result from the restoration of insulin sensitivity in the liver. Benfotiamine increased insulin sensitivity by increasing Akt phosphorylation and modulating the phosphorylation of downstream effector molecules. Phosphorylation of GSK-3α/β was increased by benfotiamine whereas GS phosphorylation was decreased.

Akt phosphorylation is important for glycogen synthesis in the liver [17]. A previous study observed that the phosphorylation level of Akt in adipose tissue from type 2 diabetic mice is decreased by 69% compared to control animals [24]. Growing evidence has suggested that benfotiamine ameliorates diabetic dysfunction *via* mechanisms involving the activation of Akt [10]. Indeed, benfotiamine was found to restore Akt phosphorylation and transketolase activity to normal levels in a mouse model of ischemic diabetic limbs [10]. The results of the present study suggest that benfotiamine can potentially attenuate type 2 diabetes mellitus in OLETF rats by restoring insulin sensitivity through the upregulation of Akt phosphorylation.

Once activated, Akt enters the cytoplasm where phosphorylates and inactivates GSK-3 [4, 5]. A major substrate of GSK-3 is GS, an enzyme that catalyzes the final step of glycogen synthesis [4, 5]. GS phosphorylation by GSK-3 inhibits glycogen synthesis; therefore, the inactivation of GSK-3 by Akt promotes the storage of glucose as glycogen [5, 17]. In contrast, the phosphorylation of Akt is decreased with insulin resistance and GSK-3 is activated [6, 16]. Activated GSK-3 phosphorylates GS that cannot convert glucose to glycogen [5, 6]. The results of the present study suggest that benfotiamine can attenuate type 2 diabetes mellitus in OLETF rats by restoring gluconeogenesis through the upregulation of GSK-3α/β phosphorylation and the downregulation of GS phosphorylation.

Benfotiamine has been used to treat vascular complications associated with diabetes [25]. Although diabetic complications frequently occur with type 2 diabetes mellitus, the mechanism underlying the effect of benfotiamine on insulin resistance has not been fully elucidated. Benfotiamine, a thiamine derivative, can act as a coenzyme for thiamine-dependent transketolase which diverts excess fructose-6-phosphate and glycerhaldeyde-3-phosphate from glycolysis into the pentose-phosphate pathway, thus removing these potentially damaging metabolites from the cytosol [2, 12, 22, 25]. Oxidative stress is therefore reduced by the transfer of upstream metabolites into the non-oxidative pentose-phosphate pathway. Since oxidative stress is an important factor of insulin resistance, benfotiamine might restore insulin sensitivity [12, 25]. Diabetes may be defined as a thiamine-deficient state because reduced thiamine/translocase activity has been described in diabetic patients [22]. Thiamine and/or its derivative, benfotiamine, have been found to counteract the damaging effects of hyperglycemia on vascular cells *in vitro* [12, 25].

Overall, these results suggest that benfotiamine can potentially attenuate type 2 diabetes mellitus in OLETF rats by restoring insulin sensitivity through the upregulation of Akt phosphorylation and the activation of two downstream signaling molecules, GSK-3α/β and GS. Future studies should be conducted to investigate the mechanisms governing the effects of benfotimine on insulin resistance in other organs (*e.g.*, skeletal muscle) and/or other insulin signaling factors (*e.g.*, insulin receptor) in OLETF rats.

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