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Original Article

A herbal extract treats type 2 diabetes mellitus effectively by down-regulating expression of CD14

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Abstract: The incidence of Type 2 diabetes mellitus (T2DM) has increased worldwide. This study investigated the effect of a herbal extract, named as Baihu decoction (BHD), in treating T2DM by using mouse adipose cells and a mouse T2DM model. Mouse 3T3-L1 preadipocytes were cultured in DMEM culture medium and used for experiments when about 90% fat-differentiated cells were mature. The KM male mouse diabetes model was established by injecting streptozotocin. Thirty two KM T2DM mice were randomly divided into 4 groups, with 8 mice in each group. Enzyme-linked immunosorbent assays (ELISA) and Reverse Transcription-quantitative PCR (RT-real time-PCR) were performed to investigate the levels of inflammatory factors and adipokines related to diabetes and their mRNA levels. Western blot experiments were carried out to determine levels of a series of proteins. BHD reduced inflammatory responses induced by lipopolysaccharide in mouse adipose cells and repressed the activity of the CD14/TLR4-NF-κB signal pathway. Our siRNA experimental results demonstrated that decreased *CD14* gene expression reduced the levels of inflammation significantly. The animal experimental results indicated that BHD has an effective effect on reduction of blood sugar level of T2DM mice. Furthermore, this anti-diabetic effect was due to inhibition of the activity of the CD14/TLR4-NF-κB signal pathway, especially CD14. BHD has anti-diabetic effect both *in vitro* and *in vivo* by down-regulating the activity of the CD14/TLR4-NF-κB signaling pathway, especially CD14.

Keywords: Type 2 diabetes mellitus, Baihu decoction, adipose tissue, chronic inflammation, CD14

Introduction

Diabetes is a complex chronic disease associated with high blood sugar levels, induced by deficiencies in insulin secretion, activation, or both. If patients cannot receive high quality care and frequent hospitalization, complications will be resulted [1]. Type 2 diabetes mellitus (T2DM) is a common and increasingly prevalent disease and thus a major public health concern worldwide [2]. Patients with T2DM may develop morbid complications of diabetic ketoacidosis [3]. T2DM is characterized by relative insulin deficiency caused by pancreatic β-cell dysfunction and insulin resistance in target organs. As the sixth leading cause of disability in 2015, diabetes places considerable socio-economic burden, estimated at $825 billion in the world [4, 5]. T2DM patients need intensive managements of sugar and lipid concentrations as well as blood pressure to minimize risk of complications occurring and progression [6]. The incidence of T2DM is affected by genetic and environmental factors. Genetic factors exert their effects following exposure to an obesogenic environment characterized by sedentary behavior and excessive sugar and fat consumption [7, 8]. In patients who are younger than 55 years of age and have a glycated haemoglobin concentration of 6.9% or less, it is twice-fold higher risk of mortality compared with people without diabetes [9]. Previous study have shown that diabetes is associated with increased risk of coronary heart disease, ischemic stroke, and other deaths related to vascular disease [10]. Patients with T2DM can present with established complications such as retinopathy [11].

A recent meta-analysis, which reviewed 4530 studies and analyzed 40 eligible studies, found that obesity is related to the increased prevalence of impaired glucose tolerance and T2DM with polycystic ovary syndrome [12]. It was fou-

nd that one of the mechanisms underlying diabetes and insulin resistance is chronic inflammation [13]. It also reported that dysbiosis could be linked between chronic inflammation, inflammatory bowel disease, colorectal cancer, and T2DM through crosstalk between molecular signal pathways, in particular, NF-κB and TNF-α [14]. The intestinal epithelium has an important function as a protective barrier against luminal antigens. However, the barrier can be breached, therefore disruption of the intestinal homeostasis can promote chronic inflammation [15]. Normally, inflammation is a beneficial activity as it rejuvenates injured tissues and removes the foreign agents disturbing homeostasis. Inflammation is achieved through a complex inflammatory response that may involve a balance between a huge panel of bioactive molecules and autoimmunity. However, chronic inflammation may increase the risk of several pathologic responses such as inflammatory bowel disease, colorectal cancer, and T2DM [14].

The CD14/TLR4-NF-κB pathway are found to be associated with various biological functions implicated in development of inflammation. CD14 is a lipopolysaccharide-binding protein, which functions as an endotoxin receptor. It is anchored to the cell surface by linkage to glycosylphosphatidylinositol. CD14 is strongly positive in monocytes and most tissue macrophages, but is weakly expressed or negative in monoblasts and promonocytes. Myeloblasts and other granulocytic precursors do not express CD14, but neutrophils and a small proportion of B lymphocytes may weakly express. The CD14 receptor is a pattern recognition molecule in the innate immune response against other exogenous and endogenous stress factors [16]. It has been demonstrated that chronic infection by Gram negative microorganisms may contribute to the inflammatory component of atherosclerosis.

Lipopolysaccharide (LPS) or endotoxin generated by Gram negative bacteria exerts proatherogenic effects by contributing to low density lipoprotein oxidation, foam cell formation, and thrombogenesis [17]. LPS binds to the LPS-binding protein, which in turn is coupled to CD14 on the cell surface of monocytes. Then, LPS-CD14 interacts with toll-like receptor 4 (TLR4) and forms a complex with another accessory protein MD-2. The TLR4 signaling cascade is initiated after binding with the adaptor protein MyD88, which leads to a series of events, and subsequently triggers activation and translocation of NF-κB and causes transcription of cytokines, such as TNF-α. The CD14/TLR4-NF-κB pathway can be inhibited by pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor), thus reducing inflammatory reaction.

Many compounds such as insulin secretagogue, α-glucosidase inhibitor, intestinal lipase inhibitor, and insulin sensitizer have been used for treatment of T2DM [18-20]. These drugs have various adverse effects and induce complications such as gastrointestinal discomfort, hypoglycemia, and pulmonary edema, although they can decrease the level of blood glucose [21, 22]. Therefore, it is urgent to study new medicines those have high effectiveness, low adverse effects, and clear action mechanism.

Baihu decoction (BHD), a traditional herb medicine curative for inflammation and high blood sugar, has be used for a long term in China and other Asian countries. BHD with addition of Ginseng has effective hypoglycemic and antioxidant effects [23]. Application of BHD combined with insulin to treat acute hyperglycemia in type 2 diabetes can significantly reduce the levels of TNF-α, IL-6, high-sensitivity C-reactive protein, Leptin, and adiponectin in patients [24, 25]. These findings implies that BHD might have functions to treat T2DM by decreasing blood sugar level.

Adipose cells, a kind of endocrine cells, are an important base for inflammation. Adipose tissue secretes a variety of affinity or anti-inflammatory adipocytokines that can cause, mediate, or antagonize inflammatory responses as well as insulin resistance. In obese human and rodent models, it has been demonstrated that enhanced expression of proinflammatory adipocytokines can induce insulin resistance [26]. In the pathophysiology of inflammation-insulin resistance-type 2 diabetes, dysfunction of adipocyte endocrine regulation plays an important role.

In this study, mouse adipose cells and an animal model were used to study whether BHD can inhibit inflammation and T2DM. It was found that BHD can effectively down-regulate inflammation by inhibit the CD14/TLR4-NF-κB signal pathway, especially expression of *CD14*.

The animal experimental results also demonstrated that BHD efficiently inhibits mouse blood sugar levels by inhibiting the CD14/TLR4-NF-κB signal pathway.

Materials and methods

Cell culture

Mouse 3T3-L1 preadipocytes were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM culture medium containing 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA) at 37°C with 5% CO_2 and 100% humidity. Two days after the cell contact inhibition was observed, cells were cultured in DMEM medium containing 10% fetal bovine serum, 0.5 mM IBMX, 0.25 μM 10 g/ml dexamethasone, and 10 μg/ml insulin for 3 days. When about 90% fat-differentiated cells were mature, cells can be used for the following experiments.

BHD preparation

An extract was prepared by decocting the dry herbs (*Gypsum fibrosum*, *Anemarrhena asphodeloides* Bge, *Oryza sativa* L, and *Glycyrrhiza uralensis* Fisch) with a ratio of 9:3:1.5:1 in boiling water for 60 minutes to a concentration of 1 g/ml. Since *Gypsum fibrosum* is the main ingredient and it is also called "baihu", the extract is named as Baihu decoction (BHD) for convenience. BHD was filtrated with a 2 layer gauze and then membrane with pores of a 0.2 μm diameter, saved in -20°C for use. Before use, BHD was diluted into a series of concentrations, including 0.05 g/ml (5%) and 0.25 g/ml (25%). The 4 kinds of Chinese herbal medicines were purchased from Chinese herbal medicine Pieces Factory (Shenzhen, China).

siRNA experiments

Mouse 3T3-L1 cells were transfected with 80 pmol of siRNA against the mouse CD14 (cat #: sc-29962), TLR4 (cat #: sc-40261), and NF NF-κB p65 (cat #: sc-29411) messages, or a negative control siRNA (cat #: sc-sc-44233 using X-tremeGENE (Roche, Shanghai, China). All siRNAs were purchased from Santa Cruz Biotechnology, Santa Cruz, USA. At 48 hours post-transfection, total protein was harvested, separated on 10% SDS/PAGE gels, and subjected to immunoblot analyses.

Establishment of the mouse diabetes model

All animal experiments were performed in accordance with the guidelines of the Animal Ethics Committee of the University of South China. Forty male KM mice, weighed 20-22 g, were purchased from and maintained in the Animal Biosafety Laboratory of the University of South China. Mice were fed for 1 week before the following experiments. Eight mice were fed continuously as usually, but the left 32 mice were used to establish T2DM model. After 30 days of high fat and high sugar feeding, mice were fasted for 12 hours but water was provided and then mice were intraperitoneally injected with streptozotocin (STZ, 80 mg/kg/mouse). Three days later, the mice received the same treatment as described above one more time. After these treatments, free diets were provided for one week. Then, blood samples were obtained from the mouse tail vein after the mice was fasted for 5 hours. The mouse blood sugar concentration was measured. All mouse grew normally. If mouse blood sugar concentration was greater than 11.1 mM, the mouse diabetes model was considered as successfully established. Blood glucose meter was purchased from Johnson & Johnson (Shanghai) Medical Equipment Co, Ltd, China.

Animal grouping

Eight-week-old male KM T2DM mice were randomly divided into 4 groups, with 8 mice in each group and all mice being given with high fat diet. The 4 T2DM mouse groups were designed as follows: (i) in the phosphate buffered saline (PBS) control group, mice were intragastrically administered with PBS for 8 weeks; (ii) in the 5% BHD group, mice were intragastrically administered with 5% BHD for 8 weeks; (iii) in the 25% BHD group, mice were intragastrically administered with 25% BHD for 8 weeks; and (iv) in the 25% BHD + PDTC group, mice were intragastrically administered with 25% BHD and injected with PDTC (200 mg/kg/d) subcutaneously for 8 weeks.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed using an ELISA kit (Multisciences, Wuhan, China). Briefly, samples and a series of controls were added into wells. Biotin solution was added (100 μl/well), followed by an incubation for 120 minutes in room

Table 1. Primers used in Real-time PCR

Primers	Forward	Backward
Universal primers	F27	R1492
	AGAGTTTGATCMTGGCTCAG	TAGGYTACCTTGTTACGACT
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
TNF-α	GGATCTCAAAGACAACCAAC	ACAGAGCAATGACTCCAAAG
CD14	TTGGCTTGTTGCTGTTGCTTC	GCGGAGGTTCAAGATGTTGAGAT
TLR-4	GAAACTCAGCAAAGTCCCTG	GAAAGGCTTGGTCTTGAATG
NF-κBp65	ATGTGCATCGGCAAGTGG	CAGAAGTTGAGTTTCGGGTAG
Apelin	TGCTCTGGCTCTCCTTGACT	ATGGGTCCCTTATGGGAGAG
Leptin	GGACCAGACATTGGCGATCTAC	CCGGAGGTTCTCCAGGTCA
GAPDH	ATGGGTVAGAAGGACTCCTATG	ATCTCCTGCTCGAAGTCTAGAG

temperature. After plate was washed 4 times, enzyme binding solution (100 μl/well) was added, followed by an incubation for 30 minutes at room temperature. After 4-time washes, coloring solution was added. After 10-20 minutes, stop solution was added, and the OD value of 450 was determined.

Reverse Transcription-quantitative PCR (RT-real time-PCR)

Total RNAs were extracted using TRIzol reagent (Invitrogen, Grand Island, New York, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from the RNA using Reverse Transcription kit (TaKaRa, Tokyo, Japan) with random primers. The levels of mRNAs were quantified by CFX96 RT-real time-PCR detection system using a SYBR Premix Ex Taq kit (Takara, Tokyo, Japan). The relative mRNA levels were normalized to that of the house-keeping gene GAPDH. All primers were given in **Table 1**.

Western blotting

Adipose cells were harvested in RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China), supplemented with 0.5 mM phenylmethylsufonyl fluoride and 0.5% cocktail protease inhibitor (Roche, Basel, Switzerland) using micro-scrapers. After sonication and centrifugation, protein concentrations of samples were determined by the bicinchoninic acid method. Equal amounts of proteins were mixed with 5X loading buffer (250 nM Tris-HCl (PH 6.8), 0.5% BPB, 10% SDS, 50% glycerol, 5% β-mercaptoethanol). Proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene difluride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for 1 hour at room temperature and then incubated overnight at 4°C with anti-mouse primary antibodies as follows: IL-6 (cat. no 12242, 1:1000; Cell Signaling Technology), TNF-α (cat. no 52-B83, 1:1000; Abcam), Apelin (cat. no ab125-213, 1:1000; Abcam), Leptin (cat. no ab3583, 1:500; Abcam), CD14 (cat. no ab133335, 1:500; Abcam), TLR4 (cat. no ab22048, 1:1000; Abcam), NF-κBp65 (cat. no ab16502, 1:1000; Cell Signaling Technology), β-actin (cat. no sc-47778, 1:10,000; Santz Cruz), followed by incubation with horseradish peroxidase-labelled secondary antibodies for 2 hours. Immuno-reactivity was detected using the ECL system (Bio-Rad Laboratories, California, USA). Band gray values were measured by Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

Data are presented as the mean ± standard deviation (mean ± SD) of 3-6 independent experiments. The statistical significance of the difference was determined by the Students' *t* test. $p < 0.05$ was considered to be significantly different. $p < 0.01$ was considered to be very significantly different.

Results

BHD reduces inflammatory responses induced by LPS in mouse adipose cells.

Since T2DM is tightly related to inflammation and BHD has been used to treat inflammation for a long term in China and other Asian countries, we first examined whether BHD could inhibit inflammatory activity. Mouse adipose 3T3-L1 cells were stimulated by LPS to induce the inflammatory responses. The unstimulated cells served as the control group. The LPS-stimulated cells were treated with LPS, LPS + 5% (0.05 g/ml) BHD, LPS + 25% (0.25 g/ml) BHD, LPS + PDTC, or LPS + 25% BHD + PDTC, respectively. After 48 hours, cells were exam-

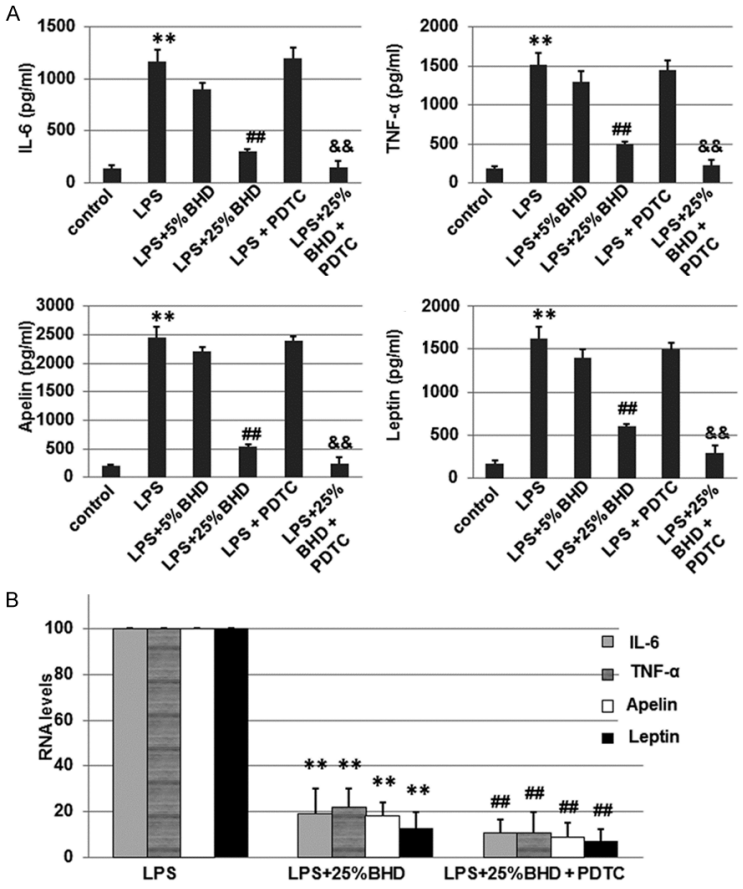

Figure 1. BHD affects secretion of inflammatory factors and adipocytokines of LPS-stimulated 3T3-L1 cells. 3T3-L1 cells were treated with medium (as the control), LPS (1 μg/ml), LPS (1 μg/ml) + 5% BHD, LPS (1 μg/ml) + 25% BHD, LPS (1 μg/ml) + PDTC (10 μM), or LPS (1 μg/ml) + 25% BHD + PDTC (10 μM), respectively. The culture supernatants and cells were harvested for ELISA and RT-real time-PCR experiments, respectively. (A) The levels of IL-6, TNF-α, Apelin, and Leptin were determined by ELISA. Values shown are mean ± SD of 6 independent experiments. $^{**}p < 0.01$, compared to the control group. $^{\#\#}p < 0.01$, compared to the LPS group. $^{\&\&}p < 0.01$, compared to the LPS + 25% BHD group. (B) mRNA levels of IL-6, TNF-α, Apelin, and Leptin were determined by RT-real time-PCR. Values shown are mean ± SD of 3 independent experiments. $^{**}p < 0.01$, compared to the LPS group. $^{\#\#}p < 0.01$, compared to the LPS + 25% BHD group.

ined by microscope. The shape of cells in all 6 groups was observed to be similar, without detectable difference, showing that BHD has no visibly toxic effect on cells. The culture supernatants and cell lysates were harvested separately. The collected culture supernatants were used to examine levels of factors by ELISA. As shown in **Figure 1A**, the levels of IL-6, TNF-α, Apelin, and Leptin in the LPS group were elevated to 8.61, 8.23, 11.69, and 9.25 fold ($p < 0.01$), respectively, in comparison with the unstimulated control group, indicating the inflammatory responses were indeed stimulated successfully. When compared with the LPS group, levels of the 4 kinds of factors in the LPS + 25% BHD group were all decreased significantly ($p < 0.01$), indicating that BHD with a concentration ratio of 25% can effectively inhibit inflammation. The presence of PDTC enhanced the inhibitory effect of BHD by about 50%, when comparing LPS + 25% BHD + PDTC group with LPS + 25% BHD group, suggesting that the function of BHD is related to CD14/TLR4-NF-κB signal pathway since PDTC is an inhibitor of the signal pathway, and PDTC may have an additive effect. But levels of the 4 kinds of factors in the LPS + 5% BHD group were only slightly reduced, indicating 5% BHD is not an effective concentration. When compared with the LPS group, levels of the 4 kinds of factors in the LPS + PDTC group were not changed obviously, showing that the presence of PDTC did not detectably affect the factor levels. Therefore, in the following experiments, the unstimulated control, LPS + 5% BHD, and LPS + PDTC groups were not included.

Cell lysates were used to isolated the total RNAs. As shown in **Figure 1B**, RT-real time-PCR results showed that the mRNA levels of IL-6, TNF-α, Apelin, and Leptin in the LPS + 25% BHD group were reduced to 19.25%, 22.78%, 18.01%, and 13.34%, respectively, relative to those in the LPS groups ($p < 0.01$). However, the addition of PDTC even decreased the levels to 10.51%, 11.02%, 8.98%, and 7.11% ($p < 0.01$), respectively. Since PDTC is a CD14/TLR4-NF-κB signal pathway inhibitor, the function of BHD may be related to the CD14/TLR4-NF-κB signal pathway.

BHD inhibits activity of the CD14/TLR4-NF-κB signal pathway in adipose cells

To further confirm whether the inhibitory effect of BHD on inflammation was related to the

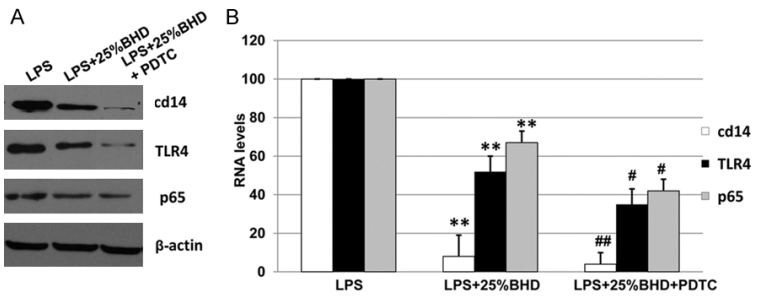

Figure 2. BHD inhibits the activity of CD14/TLR4-NFκB signal pathway in 3T3-L1 cells. Mouse 3T3-L1 cells were treated with LPS, LPS + 25% BHD, or LPS + 25% BHD + PDTC, respectively. Cells were harvested for isolation of the total protein and RNAs. (A) Western blotting was performed to determine the expression of CD14, TLR4, and p65. β-actin served as a loading control. All experiments were performed for 3 times independently and one of them was shown. (B) RT-real time-PCR was conducted to examine the mRNA expression. Values shown are mean ± SD of 3 independent experiments. $^{**}p < 0.01$, compared to the LPS group. $^{\#\#}p < 0.01$, compared to the LPS + 25% BHD group. $^{\#}p < 0.05$, compared to the LPS + 25% BHD group.

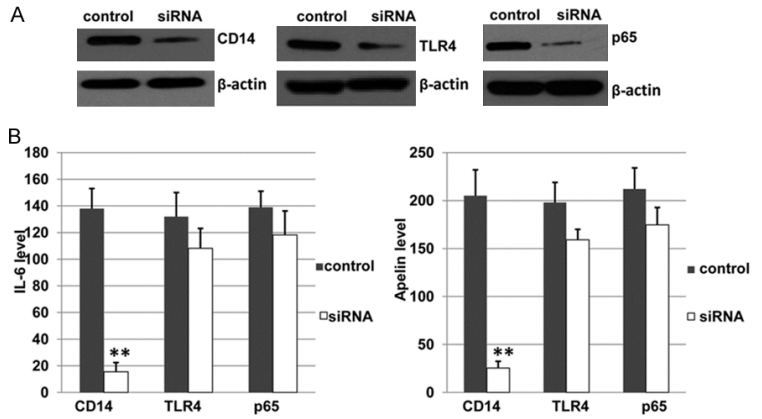

Figure 3. CD14 mRNA expression affects the levels of inflammatory factor IL-6 and Adipokine Apelin. Negative control siRNA and siRNA targeting CD14, TLR4, or p65 were transfected into LPS-stimulated 3T3-L1 cells. After 48 hours, the cells and culture supernatants were harvested for western blotting and ELISA experiments, respectively. (A) The total proteins were extracted for determining protein expression. β-actin served as a loading control. All experiments were performed 3 times independently and one of them was shown. (B) The levels of IL-6 and Apelin were determined by ELISA. Values shown are mean ± SD of 6 independent experiments. $^{**}p < 0.01$, compared to the Negative control siRNA group.

CD14/TLR4-NF-κB signal pathway, 3T3-L1 cells were treated with LPS, LPS + 25% BHD, or LPS + 25% BHD + PDTC, respectively. After 48 hours, microscopic examination showed that cell growth was the same among all groups, confirming both BHD and PDTC have no observable toxic effect on cells. The cell lysates were harvested for Western blot and RT-real time-PCR analyses. As shown in **Figure 2**, protein (**Figure 2A**) and mRNA (**Figure 2B**) levels of CD14, TLR4, and NF-κB p65 were all decreased in the 25% BHD group when compared to the LPS group ($p < 0.01$). Those in the 25% BHD + PDTC were even reduced relative to those in the 25% BHD group ($p < 0.01$ or $p < 0.05$). Importantly, among the 3 proteins, the levels of CD14 protein and mRNA were changed as the highest degree, suggesting that CD14 is possibly the most sensitive one among the 3 proteins in the signal pathway. The results show that the anti-inflammation effect of BHD is due to inhibition of the activity of CD14/TLR4-NF-κB signal pathway, especially CD14.

Reduced CD14 gene expression decreases the levels of inflammatory factor and adipokine

To rule out the possibility that BHD changed levels of CD14 protein and mRNA is due to CD14 is in the upstream of the CD14/TLR4-NF-κB signal pathway and affects levels of TLR4 and p65, siRNAs were used against each protein to investigate their effects on inflammation, respectively. LPS-stimulated 3T3-L1 cells were transfected with siRNA or control siRNA (negative control) against CD14, TLR4, and p65, respectively. After 48 hours, cell lysates were harvested and used to isolate the total proteins. As shown in **Figure 3A**, Western blotting results showed that the protein levels of CD14, TLR4, and p65 were all significantly reduced ($p < 0.05$) as expected.

The culture supernatants were harvested for determining levels of IL-6 and Apelin. In these experiments, only IL-6 and Apelin were determined by ELISA since TNF-α and Leptin were found to be affected at similar degrees with IL-6 and Apelin, respectively in **Figure 1A**. As shown in **Figure 3B**, results showed that IL-6

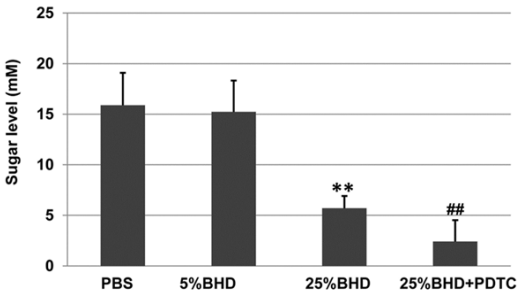

Figure 4. BHD has an anti-diabetic effect on T2DM KM mice. All groups of the mice were treated as described in the METHOD section. Blood samples were obtained from the mouse tail vein after the mice was fasted for 5 hours. The mouse blood sugar concentration was measured using blood glucose meter. Values shown are mean ± SD. $^{**}p < 0.01$, compared to the PBS group. $^{\#\#}p < 0.01$, compared to the 25% BHD group.

level in the siRNA-against CD14 group was decreased to 11.22% ($p < 0.01$) of the negative control siRNA group. However, the levels of TLR4 and p65 in the siRNA groups were decreased to only 81.92% and 85.05% of the negative control siRNA group. A similar situation was found for Apelin. These results suggest that CD14 expression is the most important factor for inhibiting inflammation of LPS-stimulated 3T3-L1 cells. Together with the results in **Figure 2**, it is suggested that BHD decreases inflammation mainly by altering *CD14* gene expression.

BHD has an anti-diabetic effect on T2DM km mice

T2DM, as a complicated metabolic disorder, is associated with inflammation. It has also been found that reduced *CD14* gene expression decreases the levels of inflammatory factor and adipokine in above results, therefore we further investigated whether BHD has an anti-diabetic effect on T2DM. A T2DM animal model was established using KM mice. Blood sugar concentration of every mouse, including 8 mice in the normal non-T2DM group and 32 mice in the T2DM group, was detected. As expected, blood sugar mean level (15.65 ± 0.92 mM) of mice in the T2DM group were increased to 3.39 fold in comparison with that (4.61 ± 0.26) of the normal non-T2DM mice, indicating that the animal model is established successfully.

To determine whether BHD can inhibit the sugar level of mice, T2DM mice were divided into 4 groups, including PBS, 5% BHD, 25% BHD, and 25% BHD + PDTC. Since the presence of PDTC does not affect inflammation as shown above, the animal experiment does include the condition of PDTC only in the following animal experiments. Mice in all groups grew normally, indicating that BHD and PDTC have no detectably toxic effects on mouse. As shown in **Figure 4**, the sugar levels of mice in the 5% BHD group were only slightly reduced, but those of mice in the 25% BHD group were decreased significantly relative to the PBS group ($p < 0.01$). The presence of PDTC enhanced the inhibition of BHD when comparing those of the 25% BHD + PDTC group to the 25% BHD group ($p < 0.01$). These results suggest that BHD has an anti-diabetic effect on T2DM KM mice.

BHD inhibits activity of the CD14/TLR4-NF-κB signal pathway by down-regulating the level of CD14 mRNA level in T2DM km mice, especially CD14

To further determine if the inhibitory effect of BHD on sugar level of T2DM mice was caused by changes in CD14, TLR4, and p65 expression, the mice in the PBS, 25% BHD, and 25% BHD + PDTC groups used in the above experiments excepting the 5% BHD group, were euthanized. Then adipose tissues were collected to isolate total proteins and RNAs. As shown in **Figure 5A**, Western blotting results showed that CD14 protein level in the 25% BHD group were decreased significantly different relative to the PBS group ($p < 0.01$), but TLR4 and p65 levels were decreased not so much ($p < 0.05$). The presence of PDTC enhanced inhibition of BHD when comparing those of the 25% BHD + PDTC group to the 25% BHD group ($p < 0.05$). Although the levels of CD14, TLR4, and p65 were all decreased, the CD14 levels were affected mostly.

To examine the mechanism underlying the altered protein expression, the mRNA levels were investigated by RT-real-time PCR. As shown in **Figure 5B**, the mRNA levels of CD14, TLR4, and p65 in the 25% BHD group were decreased significantly relative to the PBS group ($p < 0.01$). The presence of PDTC enhanced the inhibition of BHD when comparing those of the 25% BHD + PDTC group to the 25% BHD group ($p < 0.01$ or $p < 0.05$). Although the mRNA levels of CD14, TLR4, and p65 were also changed among the 3 groups, the CD14 levels were reduced mostly. These findings suggest that BHD reduces sugar

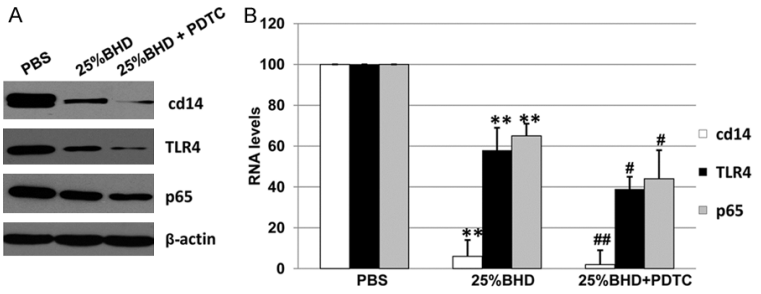

Figure 5. BHD inhibits the activity of CD14/TLR4-NFκB signal pathway in T2DM KM mice. Mice in the PBS, 25% BHD, and 25% BHD + PDTC groups were euthanized, followed by collecting adipose tissues from the mice and isolating of total proteins and RNAs. (A) Western blotting was performed to determine the expression of CD14, TLR4, and p65. β-actin served as a loading control. All experiments were performed for 3 times independently and one of them was shown. (B) RT-real time-PCR was conducted to examine the mRNA expression of CD14, TLR4, and p65. Values shown are mean ± SD of 3 independent experiments. $^{**}p < 0.01$, compared to the PBS group. $^{\#\#}p < 0.01$, compared to the 25% BHD group. $^{\#}p < 0.05$, compared to the 25% BHD group.

levels by decreasing the expression of CD14, possibly by affecting the promoter activity of *CD14*.

Discussion

Diabetes is increasingly occurring worldwide, which causes many complications, such as heart and blood vessel disease, neuropathy, nephropathy, eye and foot damage, hearing impairment, etc. T2DM accounts for more than 90% of diabetes. Although patients can take drugs like insulin secretagogues, diterpenoids, glycosidase inhibitors, and so on, these drugs all have various side effects. Insulin injection might results in hypoglycemia, and even severe hypoglycemic induced coma. Thus new drugs with reduced side effects are urgently needed for patients. Drugs from the natural medicinal herbs are a good resource, since usually those kinds of drugs contains multiple compounds that has many different targets and few toxic effect. In this study, BHD, a traditional herb medicine, was found to have anti-diabetic effects.

The complicated interactions among insulin resistance, adipose tissues, and inflammation were very important for explaining the underlying mechanisms of diseases, including chronic inflammation, T2DM, insulin resistance, atherosclerosis, hyperlipidemia, and nonalcoholic fatty liver. Since inflammation is an important reason to result in T2DM, we first investigated whether BHD could inhibit inflammatory responses induced by LPS in mouse adipose cells. As expected, levels of IL-6, TNF-α, Apelin, and Leptin were all decreased significantly ($p < 0.01$) by BHD. We found that 5% (0.05 g/ml) is not an effective concentration for BHD to decrease the inflammation stimulated by LPS, but BHD with a concentration of 0.25 g/ml (25%) is significantly effective to inhibit levels of inflammatory factor IL-6 and adipokine Apelin. Moreover, BHD at this concentration does not have negative effects on cell growth and shape. The concentration of 0.25 g/ml (25%) is also effective for BHD to reduce blood sugar level of T2DM mice. These results are essential as they implied that BHD may be an effective drug, without obvious side effects, for treating T2DM patients.

The molecular mechanism of BHD inhibiting the sugar level of T2DM mice was also investigated at molecular, cellular, and animal levels. An anti-inflammation effect of BHD was found to be due to inhibition of the activity of CD14/TLR4-NF-κB signal pathway, especially CD14. Furthermore, reduced *CD14* gene expression by siRNA decreases the levels of inflammatory factor and adipokine. It was showed that IL-6 level in the siRNA-against CD14 group was decreased to 11.22% of the negative siRNA group. However, the levels of TLR4 and p65 in the negative control group was decreased to only 81.92% and 85.05%. A similar situation was found for Apelin. These results indicate that CD14 is key factor since CD14 is on the upstream of the CD14/TLR4-NF-κB signal pathway.

A mouse model was established to research the BHD effect on T2DM. As found in the animal experiments, the sugar levels of mice in the 25% BHD group were decreased significantly relative to the PBS group ($p < 0.01$). The presence of PDTC enhanced the inhibition of BHD on sugar levels of mice when comparing those of the 25% BHD + PDTC group to the 25% BHD group ($p < 0.01$). These results suggest that BHD has an anti-diabetic effect on T2DM KM mice. PDTC facilitates the inhibitory effect of

BHD on sugar level of the T2DM mice. PDTC is an NF-κB inhibitor and our results suggest that BHD is a CD14 inhibitor. It is reasonable to imagine that both PDTC and BHD target the CD14/TLR4-NF-κB signal pathway.

BHD reduces the activity of the CD14/TLR4-NF-κB signal pathway by repressing the level of CD14 mRNA level in T2DM KM mice, especially CD14. These results are consistent with those in the adipose cells, suggesting that *CD14* gene expression is essential for BHD to inhibit the T2DM. Although the underlying molecular mechanisms are emerging, it is noted that the interaction among signal pathways are complex. One or multiple components of BHD might inhibit the activity of *CD14* promoter. Further study of the effects of the single component of BHD on T2DM are required in the future.

The extract isolated from 2 kinds of herbs, Huangbai and Zhimu, can reduce the levels of d-glucose, hexadecanoic acid, octadecanoic acid, propanoic acid, 3-hydroxybutyric acid, and 2,3-dihydroxybutanoic acid in urine of T2DM mice [27]. A herbal extract isolated from Hawthorn decreased blood glucose level and increased plasma insulin release from pancreas, representing another agent for prevention or treatment of T2DM found by using a rat model [28]. It was recently reported that Xiexin Tang, an extract prepared by decocting 3 kinds of herbs, can modify of gut microbiota and improve the symptom of type 2 diabetic rats [29]. Although Xiexin Tang can improve the symptom of type 2 diabetic rats, but it is acting by modification of inflammation due to gut microbiota, an indirect mechanism on diabetes. Furthermore, it was not found the clear molecular mechanisms underlying effects of Xiexin Tang on blood glucose levels, like we did in this study.

In this study, BHD would found to repress chronic inflammation as illustrated using mouse cells and hyperglycemia using a mouse model. The effects on inflammation and hyperglycemia were due to the inhibition of activity of the CD14/TLR4-NF-κB signal pathway, especially CD14. Next, human cells will be used to research the influence of BHD on T2DM patients. To furthermore define the mechanism, knock-out mice lacking *CD14*, *TLR4*, and *NF-κB p65* genes will be generated to study activity of their promoters before applying clinical application.

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Disclosure of conflict of interest

None.

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