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Original Paper

Novel Polysaccharide H-1-2 from Pseudostellaria Heterophylla Alleviates Type 2 Diabetes Mellitus

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Key Words
H-1-2 • T2DM • Hypoxia • Sirt1 • Insulin

Abstract
Background/Aims: To investigate the potential therapeutic effect of novel polysaccharide H-1-2 from pseudostellaria heterophylla against type 2 Diabetes Mellitus (T2DM) and elucidate the underling molecular mechanisms. ***Methods:*** Relative expression of HIF1α and Sirt1 in T2DM patients was determined via real-time PCR. The direct binding of HIF1α on Sirt1 promoter was validated by ChIP assay. The inhibitory regulation of Sirt1 by HIF1α was analyzed using luciferase reporter assay. The endogenous protein of HIF1α and Sirt1 in response to H-1-2 treatment was quantified by western blotting. The blood glucose, secreted insulin and serous lipid profiles were measured with ELISA kits. ***Results:*** We consolidated that HIF1α and Sirt1 was dysregulated in T2DM patients and subjected to H-1-2 modulation. H-1-2 significantly inhibited hypoxia and up-regulated Sirt1 expression in EndoC-βH1 cells. Accordingly, H-1-2 enhanced glucose-stimulation insulin secretion and improved blood glucose and lipid profiles in T2DM cells, and elevated the glucose and insulin tolerance simultaneously. Furthermore, we demonstrated that H-1-2 alleviated T2DM via inhibition of hypoxia and up-regulation of Sirt1 in isolated pancreatic β-cells from T2DM rats. ***Conclusion:*** Our data unambiguously demonstrated H-1-2 administration alleviated T2DM by enhancing Sirt1 expression through inhibition of hypoxia.

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Introduction

Type 2 Diabetes mellitus (T2DM) is a chronic metabolic disorder that features in high blood sugar, insulin resistance and relative lack of insulin, and with long-term complications including heart disease, strokes, diabetic retinopathy-associated blindness, kidney failure and poor blood flow in the limbs [1]. Clinically, the T2DM accounts for approximately 90% of cases with diabetes, the incidence of which increased over the past decades and intimately associates with obesity and lack of exercise [2]. As of 2015, 392 million cumulative patients were diagnosed worldwide and imposed heavy burden on individual health and social economy [3]. Diagnosis of diabetes was commonly performed by blood tests such as fasting plasma glucose, oral glucose tolerance test or glycated hemoglobin and clinical managements included exercise, dietary change, medication metformin and augmentation of insulin [4].

Hypoxia refers to the pathological conditions that oxygen supply is deprived or limited, which has been characterized in diversity of human diseases [5]. The hypoxia response has been increasingly recognized involving in the pathogenesis and disease course of T2DM as well [6]. It's indicated that hypoxia represented the early molecular event and the dysregulated Hypoxia-Inducible Factor 1 Alpha Subunit (HIF1α) in the initiation and progression of diabetic nephropathy (DN), the leading cause of T2DM-related mortality [7]. HIF1α is stabilized and accumulated under hypoxic conditions and plays critical physiological and pathological roles as transcriptional activator [8].

Sirt1 encodes a member of the sirtuin family of NAD-dependent protein deacetylases, which senses intracellular energy state and intimately links to transcriptional modulations, and consequently participates in coordination of diverse cellular processes such as cell cycle, DNA damage response, metabolism, apoptosis and autophagy [9]. The recent report has disclosed the beneficial effect of Sirt1 in resveratrol-mediated enhancement of hypoxia-induced autophagy in type 2 diabetic nephropathy rat, which immediately prompted us to investigate its potential involvement in our system [10].

Pseudostellaria heterophylla, also known as Prince Ginseng, is historically used in the traditional Chinese medicine (TCM) to tonify the qi and generate yin fluids [11]. The herb is commonly prescribed to treat lung damage associated with asthma, pleurisy, bronchitis, bacterial pneumonia, wheezing, dry cough and emphysema. The pharmacological actions of pseudostellaria heterophylla have currently been ascribed to the protection on the mucin layer lining the respiratory tract and functioning as an immune defense system [12]. In addition, in the formula of Li Gan Zi Shen Tang (Decoction to Regulate the Liver and Enrich the Kidney), pseudostellaria heterophylla is exploited to treat yin deficiency-associated diabetes mellitus [13]. The composition analysis of pseudostellaria heterophylla identified the abundance of amino acids, fatty acids, polysaccharides, heterophyllin and diversity of trace elements, among which the polysaccharides are regarded as the fundamental constituents underlying its therapeutic values [14]. Until recently, a novel homogenous polysaccharide named as H-1-2 was isolated from the polysaccharide fractions in the crude extracts of pseudostellaria heterophylla, which has been further demonstrated to improve glucose uptake and ameliorate T2DM complications [15]. Here we sought to elucidate the molecular mechanism underlying the beneficial effect of H-1-2 on T2DM both *in vitro* and *in vivo*, and hypothesized H-1-2 influenced Sirt1 expression via modulation the hypoxia response.

Materials and Methods

Participants and blood sample collection

Totally, 20 T2DM patients (male:female=1:1) and 14 healthy individuals (male:female=1:1), of comparable age, were enrolled in this study and the written informed consents were obtained. The prospective single-center study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui University of Traditional Chinese Medicine. The adults with type 2 diabetes at least 1 year and serous HbA1c of 7.5% or higher were included and the patients with pregnancy, history of drug/alcohol

abuse, diagnosis of AIDS or type 1 diabetes, secondary hypertension, cardiovascular (CVD) disorders, and especially previous usage of pseudostellaria heterophylla were excluded from this study. The blood samples (20 mL) from patients subjected to overnight fasting were collected, the serum was immediately isolated via cryo-centrifugation and preserved at -80℃ for future use.

Cell line

The human pancreatic β-cell line EndoC-βH1 was established following the previously described protocol. The culture petri dish was coated with Matrigel (100 μg/mL) and fibronectin (2 μg/mL) prior to use, and cells were cultured in DMEM medium supplemented with 5.6 mM glucose, 2% BSA fraction V, 50 μM 2-mercaptoethanol, 10 mM nicotinamide, 5.5 μg/mL transferrin, 6.7 ng/mL selenite, 100 U/L penicillin and 100 μg/mL streptomycin. The cells were maintained in humidified 5% CO_2 incubator and sub-passaged every week with trypsin digestion.

Luciferase assay

The promoter region of Sirt1 spanning the putative hypoxia response element was sub-cloned into the pGL3 luciferase reporter plasmid and transiently transfected into EndoC-βH1 using Lipofectamine 2000 in accordance with the manufacturer's instruction. The recipient cells were subjected to either hypoxia or normoxia stimulations. The relative luciferase activities were determined 24-hour post-transfection with the commercial available Bright-Glo Luciferase Reporter System (Promega, MA, USA) following the provider's guide.

Chromatin immunoprecipitation (ChIP)

The ChIP was performed with commercial available kits purchased from Abcam (Ab500, UT, USA) in accordance the manufacturer's instruction. Briefly, the exponentially growing cells subjected to hypoxia mimic stimulations were first crosslinked with 1% formaldehyde for 20 min at room temperature and then lysed in lysis buffer on ice for 10 min. After centrifugation, the supernatant was completely removed and the chromatin pellet was resuspended and subjected to ultrasonic shearing with 3 15-second pulses and 30-second interval rest on ice. The target chromatin fragments were immunoprecipitated with HIF1α antibody overnight at 4°C and released by incubation with RNase and proteinase K in DNA Release Buffer for 30 min at 42℃. The enrichment of candidate promoters was determined with subsequent PCR.

Quantitative real-time PCR

Total RNA was extracted from patient samples and cultured cells using Trizol reagent following the manufacturer's instructions. The quantity and integrity of isolated RNA was determined with the BioAnalyzer 2100 (Agilent, CA, USA). The cDNA was prepared from 1 μg RNA with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, MA, USA). The real-time PCR was performed with the SYBR Green Real-Time PCR Master Mix (ThermoFisher, MA, USA) in accordance with the manufacturer's instruction. The relative expression of target genes was calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin. The primers were listed as follows:

Human-HIF1α-forward: 5'-GTGGAAGTGGCAACTGATGA-3'
Human-HIF1α-reverse: 5'-ATTCACCATGGAGGGCG-3'
Human-Sirt1-forward: 5'-TGTGACAGAGAGATGGCTGG-3'
Human-Sirt1-reverse: 5'-GCTCGCCTTGCTGTAGACTT-3'
Human-β-actin-forward: 5'-CCTTGCACATGCCGGAG-3'
Human-β-actin-reverse: 5'-GCACAGAGCCTCGCCTT-3'
Rat-HIF1α-forward: 5'-GATGACGGCGACATGGTTTAC-3'
Rat-HIF1α-reverse: 5'-CTCACTGGGCCATTTCTGTGT-3'
Rat-Sirt1-forward: 5'-GCTGACGACTTCGACGACG-3'
Rat-Sirt1-reverse: 5'-TCGGTCAACAGGAGGTTGTCT-3'
Rat-β-actin-forward: 5'-GGCTGTATTCCCCTCCATCG-3'
Rat-β-actin-reverse: 5'-CCAGTTGGTAACAATGCCATGT-3'

Western blot

The cell lysates were prepared in RIPA lysis buffer and protein content was quantified with the BCA Protein Assay Kit (ThermoFisher, MA, USA). The equal amount of protein samples was resolved by SDS-PAGE electrophoresis and subsequently transferred onto PVDF membrane on ice. The PVDF was briefly blocked with 5% skim milk dissolved in TBST buffer on shaker at room temperature for one hour, and hybridized with primary antibodies (anti-HIF1α, CST#14179, Cell Signaling Technology, 1:1, 000; anti-Sirt1, CST#2310, Cell Signaling Technology, 1:1000; anti-β-actin, CST#4970, Cell Signaling Technology, 1:1, 000) at 4°C overnight. After rigorous wash with TBST for 5 min by 6 times, the target proteins were blotted with secondary antibody (anti-rabbit, CST#7074, Cell Signaling Technology, 1:5, 000) at room temperature for one hour and visualized with the commercial enhanced chemiluminescence kit (ECL, Millipore, MA, USA). The house-keeping gene β-actin was employed as internal loading control.

Glucose-stimulated insulin secretion assay

The glucose-stimulated insulin secretion was determined with 1-2-3 UltraSensitive Human Insulin ELISA Kit (ALPCO Diagnostics, NH, USA) in accordance with the manufacturer's instruction. Briefly, EndoC-βH1 cells were first washed with Krebs buffer and the residual insulin was completely removed by incubation with 2 mM glucose for 2 h. Cells were then washed twice with PBS and subjected to the second round of incubation with 2 mM glucose for 30 min, and conditioned medium was collected. The adherent cells were washed again and followed by the third round of incubation with 20 mM glucose for 30 min, and conditioned medium was sampled for further analysis. The cell number at endpoint was counted for normalization purpose.

Rat model of T2DM

The Wistar rats were obtained from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). All animals were housed in the pathogen-free environment with temperature of 22 ± 1°C and relative humidity of 65-70%. The protocol conformed to the Guideline for Experimental Animal from NIH and was approved by the Animal Use and Care Committee of The First Affiliated Hospital of Anhui University of Traditional Chinese Medicine. To establish type II diabetic rat model, the adult male rats were administrated with high-fat and high-carbohydrate diet (10% lard, 2.5% cholesterol, 1% sodium cholate, 20% sucrose, and 66.5% of the basic feed) and received intraperitoneal STZ injection (≤50 mg/kg body weight in citrate buffer, pH 4.5) after 4 weeks culture. The rats with sugar level > 11.1 mmole/L were selected for following study. Three groups of rats, with 12 rats in each group, including normal control, diabetic with vehicle and diabetic with H-1-2 were subjected to either vehicle or H-1-2 administrations. The H-1-2 was dosed via oral gavage at 1.5 g/kg body weight for consecutive 30 days and controlled by drinking water, according to previously established method [13].

Blood glucose, insulin level and lipid profile assays

The blood glucose, total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were determined using the commercial available kits (Jiancheng Institute of Biotechnology, Nanjing, China) in accordance with the directions from the manufacturer.

Oral glucose tolerance and insulin tolerance test

The experimental animals were first subjected to overnight of fasting and the baseline glucose levels were determined in blood sampled from tail vein. After administration of either glucose (2 g/kg body weight) or insulin (1 U/kg body weight), the blood samples were collected via distal venesection of the tail vein at interval of 30 min up to 2 h for glucose quantitation. The glucose tolerance test (OGTT) and insulin tolerance test (ITT) was evaluated respectively.

Statistical analysis

All results represented at least three independent repeats and expressed as mean ± standard deviation (SD). Sample size of treatment groups was determined using established statistical power analysis [16]. Difference between mean of each compared treatment group was divided by their SD to determine the standardized effect size. Next, setting significance level at 5% and student t-test power at 90%, respectively, the minimum sample size was found to be 8. Data analysis was performed with the SPSS 23.0 (IBMSPSS,

IL, USA) and the one-way ANOVA was employed for statistical comparison. The p value was calculated and $p<0.05$ was considered as significantly different.

Results

HIF1α and Sirt1 expression levels were dysregulated in T2DM patients

Here we first sought to determine the relative expression of HIF1α and Sirt1 in clinical T2DM patients who fulfilled the inclusion criteria described in the methods section. As shown in Fig. 1A, the median level of HIF1α in T2DM group was significantly higher than control group (0.65 ± 0.12 vs. 0.39 ± 0.12, $p<0.0001$). On the contrary, the average expression of Sirt1 was remarkably suppressed in T2DM patients in comparison with healthy counterparts (0.09 ± 0.05 vs. 0.16 ± 0.06, $p<0.001$, Fig. 1B). Our results exactly consolidated the previous observations that hypoxia response was provoked in T2DM disease setting and accompanied with downregulation of Sirt1.

H-1-2 upregulates Sirt1 expression through the hypoxia response element (HRE) in its promoter region

Our previous results demonstrated that both HIF1α and Sirt1 were dysregulated in T2DM milieu, whereas the causal relationship was still to be defined. With close inspection of the promoter region of Sirt1, we have identified two putative hypoxia response elements as shown in Fig. 2A. Next, we sought to experimentally validate the direct association between HIF1α and the candidate cis-elements via chromatin immunoprecipitation (ChIP). The PCR amplification of HIF1α antibody-immunoprecipitated chromatin fragments

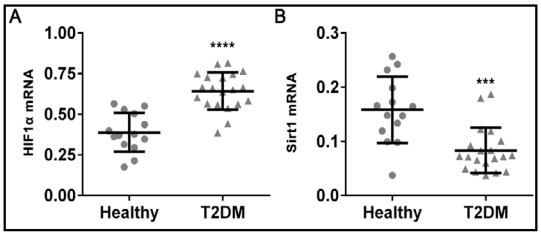

Fig. 1. HIF1α and Sirt1 expression levels were dysregulated in T2DM patients. Serum mRNA expressions of HIF1α (A) and Sirt1 (B) were analyzed in both healthy control (n=14) and T2DM patients (n=20). Data were shown as mean±SD, as normalized by β-actin as internal control. **** $p<0.0001$, *** $p<0.001$, between healthy and T2DM.

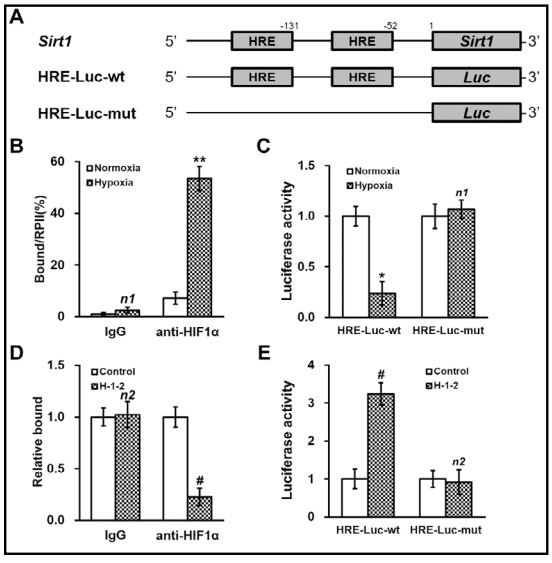

Fig. 2. H-1-2 upregulates Sirt1 expression through the hypoxia response element (HRE) in its promoter region. (A) Promoter region of Sirt1 contains putative hypoxia response element (HRE). Wild type (HRE-Luc-wt) or mutated (HRE-Luc-mut) HRE sites from miR-519d promoter were cloned at the upstream of a luciferase reporter gene open reading frame (Luc). (B) EndoC-βH1 cells were incubated with media containing 0 (normoxia) or 100 μM (hypoxia) of $CoCl_2$, respectively, followed by ChIP assay using control IgG or HIF1α antibody (anti-HIF1α). (C) Luciferase activities of HRE-Luc-wt or HRE-Luc-mut constructs were measured in EndoC-βH1 cells treated as in (B). (D) EndoC-βH1 cells were treated with control or H-1-2, respectively, followed by ChIP assay using control IgG or HIF1α antibody (anti-HIF1α). (E) Luciferase activities of HRE-Luc-wt or HRE-Luc-mut constructs were measured in EndoC-βH1 cells treated as in (D). Data were shown as mean±SD. ** $p<0.01$, * $p<0.05$, n1 not significant, between normoxia and hypoxia. # $p<0.05$, n2 not significant, between control and H-1-2.

identified significant enrichment of the putative HRE sequences under hypoxic mimic conditions (Fig. 2B). We further confirmed the inhibitory regulation of HIF1α on Sirt1 expression in response to hypoxia stimulation via luciferase activity reporter assay. To this purpose, we constructed either wild-type or scramble promoter sequences of Sirt1 into luciferase reporter plasmids and transfected EndoC-βH1 under normoxia or hypoxia conditions. The luciferase activity driven by the wild-type Sirt1 promoter was significantly suppressed in comparison with scrambled counterpart in response to hypoxia mimic (0.23 ± 0.1 vs. 1 ± 0.11, $p<0.05$, Fig. 2C). Consistent with its known effect in suppression of hypoxia response, treatment with H-1-2 significantly impaired the direct association between HIF1α and Sirt1 promoter (0.23 ± 0.07 vs. 1 ± 0.08, $p<0.05$, Fig. 2D). Consequently, H-1-2 significantly stimulated the wild-type Sirt1 promoter-driven luciferase activity (3.2 ± 0.3 vs. 1 ± 0.3, $p<0.05$), while this effect was completely abrogated by the mutation introduced in Sirt1 promoter (Fig. 2E). Our data unambiguously demonstrated the direct inhibitory regulation of HIF1α on Sirt1 expression, which could be readily relieved with H-1-2 treatment.

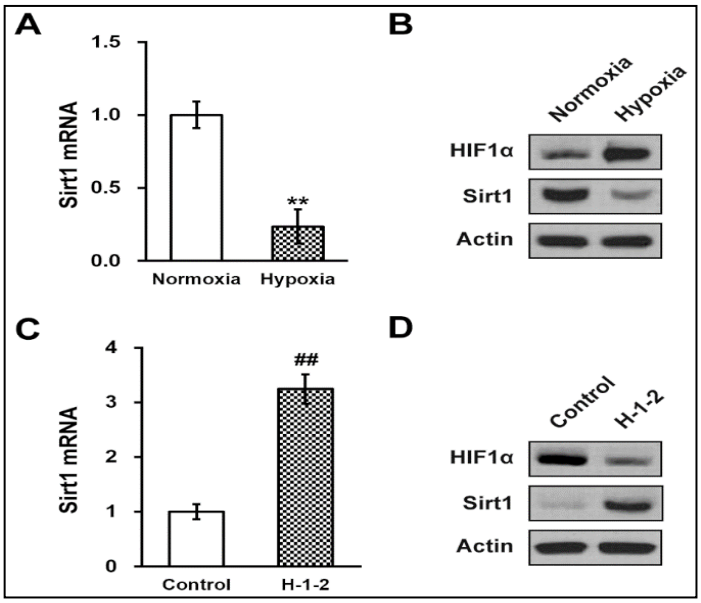

Fig. 3. H-1-2 inhibits hypoxia and upregulates Sirt1 expression in EndoC-βH1 cells. (A, B) EndoC-βH1 cells were incubated with media containing 0 (normoxia) or 100 μM (hypoxia) of $CoCl_2$, respectively, and expressions of Sirt1 mRNA (A), as well as HIF1α and Sirt1 proteins (B), were examined. (C, D) EndoC-βH1 cells were treated with control or H-1-2, respectively, and expressions of Sirt1 mRNA (C), as well as HIF1α and Sirt1 proteins (D), were examined. Data were shown as mean±SD. ** $p<0.01$, between normoxia and hypoxia. ## $p<0.01$, between control and H-1-2.

H-1-2 inhibits hypoxia and upregulates Sirt1 expression in EndoC-βH1 cells

Our previous results characterized the direct association between HIF1α and Sirt1 promoter, which was subjective to H-1-2-mediated destruction. Next, we sought to determine the endogenous expression of Sirt1 in response to hypoxia and the potential effect of H-1-2 on this. As shown in Fig. 3A, the transcription of Sirt1 was remarkably inhibited in response to hypoxia mimic in EndoC-βH1 cells, which was consistently in agreement with our previous results from the luciferase reporter assay. Likewise, the Sirt1 protein was tremendously decreased with increase of HIF1α in $CoCl_2$-treated cell (Fig. 3B). Upon treatment with H-1-2 in the hypoxic cells, both the transcriptional and translational level of Sirt1 was greatly stimulated (Fig. 3C, D). Notably, in line with previous observation that H-1-2 possessed the property to suppress hypoxia, our data consolidated this notion and demonstrated that HIF1α was markedly decreased in response to H-1-2 treatment. Our data suggested that the endogenous Sirt1 was subjected to the hypoxia regulation and significantly induced by H-1-2.

H-1-2 enhances glucose-stimulated insulin secretion in EndoC-βH1 cells

Along with the direction that H-1-2 significantly stimulated the activation of Sirt1, next we sought to determine the potential influence of H-1-2 treatment on glucose-stimulated insulin secretion in the *in vitro* cultured EndoC-βH1 cells. As shown in Fig. 4, high concentration of glucose treatment significantly stimulated insulin secretion in EndoC-βH1 cells (2.65 ± 0.37-fold increase, $p<0.05$). Notably, this effect was tremendously enhanced while the cells were co-treated with H-1-2 (4.1 ± 0.4, $p<0.05$).

H-1-2 improves blood glucose levels of T2DM rats

Next, we sought to investigate the potential effect of H-1-2 treatment on blood sugar, secreted insulin and the lipid profiles in T2DM model rats. The blood samples were collected from either vehicle or H-1-2 groups and the related paramenters were determined with commercial available kits. The comprehensive influences were summarized in Table 1 and 2. The blood glucose concentration was dramatically higher in T2DM rat in comparison with control, which indicated our success in establishment of this disease model, and was decreased over time to the comparible level with healthy rats at day 30 post-treatment of H-1-2 (5.9 ± 0.8 vs. 6.2 ± 1.5, $p<0.05$). The insulin resistance indicated by the serous insulin concentration was greatly ameliorated by H-1-2 treatment (10.34 ± 2.47 in H-1-2 group vs. 17.83 ± 2.71 in T2DM group, $p<0.05$). Consistently, the lipid profiles of T2DM rats were significantly improved upon H-1-2 administration. The TC content was decreased from 3.24 ± 0.61 mM in diabetic rats to 2.07 ± 0.52 mM in H-1-2 group. Likewise, the TG concentration was decreased from 2.67 ± 0.46 mM in diabetic rats to 1.56 ± 0.29 mM in H-1-2 group, and LDL concentration was decreased from 0.52 ± 0.13 mM to 0.39 ± 0.08 mM as well. In contrast, the HDL content was restored from 1.37 ± 0.24 mM to 2.18 ± 0.19 mM with H-1-2 treatment. With aid of the disease model animals, here we demonstrated that administration with H-1-2 significantly improved the insulin resistance, glucose responsiveness and lipid profiles.

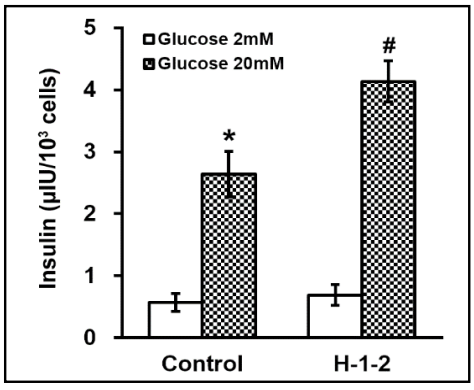

Fig. 4. H-1-2 enhances glucose-stimulated insulin secretion in EndoC-βH1 cells. EndoC-βH1 cells treated with control or H-1-2 were challenged with 2 and 20 mm glucose, respectively, and insulin secretion into the media was measured.

Table 1. H-1-2 improves blood glucose levels of T2DM rats. Data were shown as mean±SD, in mmol/L, n=12 each group. * $p<0.05$, compared to normal group on the same day. # $p<0.05$, compared to T2DM group on day 15 and T2DM+H-1-2 group on day 0. $ $p<0.05$, compared to T2DM group on day 30 and T2DM+H-1-2 group on day 0 and 15

	Normal	T2DM	T2DM+H-1-2
Day 0	5.2±1.7	24.9±4.2 *	23.4±3.8 *
Day 15	5.4±0.9	23.1±4.6 *	12.4±2.3 *#
Day 30	6.2±1.5	25.3±3.5 *	5.9±0.8 #$

Table 2. H-1-2 improves serum insulin and lipid profiles of T2DM rats. TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Data were shown as mean±SD, n=12 each group. * $p<0.05$, compared to normal group. # $p<0.05$, compared to T2DM group

	Normal	T2DM	T2DM+H-1-2
Insulin (mU/L)	8.71±1.84	17.83±2.71 *	10.34±2.47 #
TC (mmol/L)	1.87±0.50	3.24±0.61 *	2.07±0.52 #
TG (mmol/L)	1.35±0.28	2.67±0.46 *	1.56±0.29 #
HDL (mmol/L)	2.31±0.27	1.37±0.24 *	2.18±0.19 #
LDL (mmol/L)	0.32±0.07	0.52±0.13 *	0.39±0.08 #

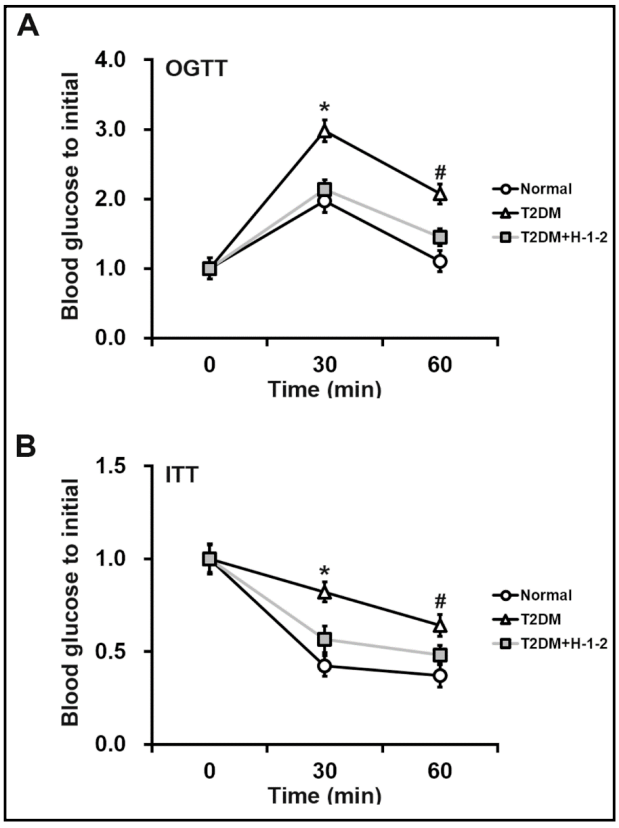

Fig. 5. H-1-2 improves glucose and insulin tolerance of T2DM rats. (A) Oral glucose tolerance test (OGTT) was performed on three experimental groups of rats, followed by blood glucose measurements at indicated time points. (F) Insulin tolerance test (ITT) was performed on three experimental groups of rats, followed by blood glucose measurements at indicated time points. Data were shown as mean±SD, in mmol/L, n=12 each group. * $p<0.05$, compared to both normal and T2DM+H-1-2 groups at 30 min. # $p<0.05$, compared to both normal and T2DM+H-1-2 groups at 60 min.

H-1-2 improves glucose and insulin tolerance of T2DM rats

We further evaluated the glucose and insulin tolerance of T2DM rats and potential influence imposed by H-1-2 treatment. All rats were fed with glucose and serum were collected at interval of 30 min up to 1 h for blood glucose measurement. As shown in Fig. 5A, the blood sugar level was dramatically increased at 30 min post-administration of glucose and dropped back to normal at 1 h in control rats. However, in the T2DM rats, the blood glucose was significantly higher during our observation windows (2.95 ± 0.15 vs. 1.98 ± 0.15, $p<0.05$ at 30 min; 2.05 ± 0.11 vs. 1.12 ± 0.10, $p<0.05$ at 1 h), and this discrepancy was completely reversed by H-1-2 co-administration (2.15 ± 0.17 vs. 1.98 ± 0.15, $p<0.05$ at 30 min; 1.35 ± 0.16 vs. 1.12 ± 0.10, $p<0.05$ at 1 h). Similarly, the insulin tolerance was determined via measurements of blood glucose in insulin-injected T2DM rats. As shown in Fig. 5B, the blood sugar was tremendously decreased until 1 h post-injection in healthy rats (0.45 ± 0.05 at 30 min; 0.44 ± 0.08 at 1 h). However, this effect was greatly compromised in T2DM rats wherein the insulin injection led to much moderate decrease of blood glucose (0.82 ± 0.13 at 30 min; 0.65 ± 0.11 at 1 h), which was significantly improved by co-administration of H-1-2 (0.61 ± 0.18 vs. 0.82 ± 0.13, $p<0.05$ at 30 min; 0.57 ± 0.09 vs. 0.65 ± 0.11, $p<0.05$ at 1 h). Our data indicated that treatment with H-1-2 potently improved the oral glucose tolerance and insulin tolerance.

H-1-2 alleviates T2DM by inhibiting hypoxia and upregulating Sirt1 expression in pancreatic β-cells

Although our previous results demonstrated the remarkably beneficial effects of H-1-2 against T2DM and elucidated the potential molecular mechanisms via derepression of the inhibitory regulation of Sirt1 by HIF1α, the *in vivo* scenario was still to be defined in this setting. To this purpose, the pancreatic β-cells were isolated from experimental rats and relative expression of HIF1α and Sirt1 was determined at both transcription and translation level in response to H-1-2 treatment. Consistent with our *in vitro* results, we consolidated the

observations the down-regulation of Sirt1 and up-regulation of HIF1α in the T2DM rats (Fig. 6A and B), which was remarkably reversed by H-1-2 treatment.

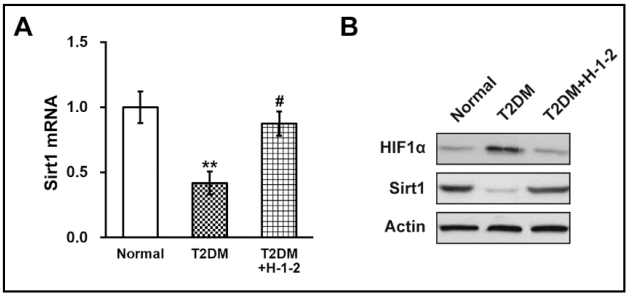

Fig. 6. H-1-2 alleviates T2DM by inhibiting hypoxia and upregulating Sirt1 expression in pancreatic β-cells. Pancreatic β-cells were isolated from three experimental groups of rats, and expressions of Sirt1 mRNA (A), as well as HIF1α and Sirt1 proteins (B), were examined. Data were shown as mean±SD. ** p<0.01, between normal and T2DM. # p<0.05, between T2DM and T2DM+H-1-2.

Discussion

Here we exploited the potential therapeutic value of the novel identified polysaccharide H-1-2, which was isolated from the traditional Chinese medicine pseudostellaria heterophylla and implicated in improvement of glucose uptake [15], against T2DM with related disease animal model. We first consolidated the previous observations that hypoxia response was activated in T2DM milieu [17] and the corresponding down-regulation of Sirt1. With the aid of bioinformatics tools, we further identified two putative hypoxia response elements in the Sirt1 promoter, and experimentally validated the direct binding between HIF1α and Sirt1. In contrast to its well-known role as transcription factor [18], here we demonstrated that recruitment of HIF1α significantly suppressed the downstream Sirt1 expression. To our best knowledge, our report for the first time disclosed the potential that HIF1α functioned as transcriptional repressor in this pathological condition, which definitely warranted further clarifications. Noteworthily, the currently preliminary observations could not conclude whether HIF1α was solely engaged in the inhibitory regulation or functioned in combination with other factors. In line with results interrogated from luciferase activity reporter assay, the endogenous alterations in respect with HIF1α and Sirt1 were consolidated in the EndoC-βH1 cells as well.

The previous study has demonstrated the protective effects of polysaccharides fraction against cobalt chloride-stimulated hypoxic injury in myocardial cell line H9c2 [19], which was suggestive of the existence of hypoxia-antagonizing agents in the crude extract. In agreement with this effect, here we treated the cultured human pancreatic β-cells with H-1-2, which significantly alleviated the intracellular hypoxia milieu as indicated by the remarkable decrease of HIF1α and the Sirt1 was reversely activated. The precise role of Sirt1 in mediating the anti-hypoxia effects of H-1-2 was still to be defined in this setting. However, our data resembled the previous investigation showed that Sirt1 was essential for resveratrol enhancement of hypoxia-induced autophagy in the T2DM nephropathy rats, wherein Sirt1 was up-regulated by resveratrol and restored physiological autophagy which was impaired by hypoxia stimulations [10]. At least, our data suggested the increase of Sirt1 in response to the inhibition of hypoxia imposed by H-1-2 treatment and might further underlaid its beneficial effect against T2DM. Undoubtedly, the radical involvement of Sirt1 with predominant role in this setting necessitated further elucidations.

Pseudostellaria heterophylla has been historically used for treatment of lung damage-associated complications. Until recently, the potential therapeutic values of pseudostellaria heterophylla against diabetes have increasingly exploited. For example, Hu *et al.* demonstrated the hypoglycemic effect of polysaccharides with different molecular weight and characterized the PF40 fraction effectively prevented the cascade of inflammatory events and blocked overweight progressing to obesity [13]. Chen *et al.* isolated and identified a pectic polysaccharide in the aqueous phase of pseudostellaria heterophylla with potency to stimulate insulin secretion of INS-1 cell [20]. Likewise, here we showed administration of the single-component polysaccharide, H-1-2, via oral gavage significantly enhanced the glucose responsiveness and therefore glucose-stimulated insulin secretion, which was

accompanied with decrease of blood glucose, insulin and obvious improvement in respect to the lipid profiles. Furthermore, H-1-2 treatment remarkably improves the oral glucose tolerance and insulin tolerance. Our data for the first time systematically characterized the therapeutic benefit of H-1-2 in T2DM milieu and observed none of apparent reverse effects associated with this compound. Our preliminary cell-based and disease model-based study unambiguously indicated the clinical potential of H-1-2, which urgently warranted for further investigations and clinical trials.

Although we experimentally confirmed the anti-hypoxia effect of H-1-2 both *in vitro* and *in vivo*, the fundamental mechanism underlying down-regulation of HIF1α was still elusive currently. HIF1α was persistently subjected to hydroxylation by prolyl hydroxylase [21] and subsequent ubiquitination by VHL under normoxia condition [22], and consequently led to degradation through ubiquitin-proteasome system. In response to the limitation of oxygen supply, HIF1α was stabilized, accumulated and heterodimerized with HIF1β to transcriptionally activated a variety of downstream genes [23]. In the light of decline of HIF1α protein upon H-1-2 treatment, here we hypothesized that H-1-2 inhibited HIF1α activity via either activation of prolyl hydroxylase or ubiquitin ligase, which convergently conveyed HIF1α to proteasome-mediated degradation. Another potential mechanism rooted in the impairment of heterodimer of HIF1α and HIF1β by direct blockade the interface. Anyway, more experimental verifications were required to fully uncover the molecular events.

Conclusion

Here we demonstrated that polysaccharide H-1-2 alleviated T2DM in rat model, which might function via enhancement of Sirt1 expression by inhibiting hypoxia.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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