Regulation on SIRT1-PGC-1α/Nrf2 pathway together with selective inhibition of aldose reductase makes compound hr5F a potential agent for the treatment of diabetic complications

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ABSTRACT

(R,E)-N-(3-(2-acetamido-3-(benzyloxy) propanamido)propyl)-2-cyano-3-(4-hydroxy phenyl)acrylamide (**hr5F**) was design-synthesized based on bioactivity focus strategy as a potential agent to treat diabetic complicates. With *in vitro* enzyme assay, it is confirmed that **hr5F** is an effective ALR2 inhibitor with IC_{50} value of 2.60 ± 0.15 nM, and selectivity index of 86.0 over ALR1, which is a little bit better than the reference Epalrestat (Epa). **hr5F** inhibits the increase of ALR2 enzyme activity and expression in human lens epithelial cells (HLECs) induced by high glucose. By applying western blot, it was found that **hr5F** alleviates the high glucose-induced superoxide overproduction insults by regulating SIRT1-PGC-1α/Nrf2 pathway, together with regulating NRF-1, mtTFA, Bax/Bcl-2 to ameliorate cell apoptosis. The *in vivo* effects of **hr5F** on short term streptozocin (STZ)-induced diabetic mice confirm the same functions disclosed *in vitro*. All the evidences support that **hr5F** may serve as a promising agent in the treatment of diabetic complications with close efficacy and broader indication than the reference Epa.

1. Introduction

Diabetes mellitus (DM) is a chronic disease characterized by glucose metabolic dysfunction and hyperglycemia. And long-term diabetes will trigger serious related diabetic complications (DC) including macrovascular and microvascular disease, which have brought heavy economic burden to many developed and developing countries [1]. DM together with DC is actually a worldwide health threat.

Many mechanisms, including polyol pathway, advanced glycation end product pathway, protein kinase C (PKC) pathway and hexosamine pathway, have been demonstrated with promotional effect on DC. Initially, there has been no apparent common pathway linking the four pathways of hyperglycaemia-induced complications [2]. However, recent discoveries have suggested that hyperglycaemia-induced mitochondrial superoxide plays a leading role in initiating the four pathways [3,4]. Hyperglycaemia-induced increase of proton gradient results in overproduction of electron donors by tricarboxylic acid cycle, which, in turn, induces a significant increase of superoxide [5]. Other studies also found that hyperglycaemia-induced overproduction of

mitochondrial superoxide induces a 66% decrease of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), bringing about activation of the four pathways [3]. Meanwhile, the reactive oxygen species (ROS) overproduction causes the DNA damage and subsequently the poly ADP ribose polymerase (PARP) activation, which inhibit GAPDH activity and activate various pro-inflammation pathways [6,7]. All these changes will promote the process of tissue damage, and finally develop into diabetic complications such as diabetic cataract.

As we know, the activated aldose reductase (AR, E.C. 1.1.1.21, AKR1B1) (ALR2) plays a key role in polyol pathway. Under hyperglycemia, ALR2 catalyzes glucose to sorbitol, which is subsequently converted to fructose by sorbitol dehydrogenase [8]. Studies have shown that the activation of ALR2 in the target tissues may involve in oxidative stress, pseudohypoxia, osmotic stress, protein glycation and PKC activation, which will finally lead to DC [9,10]. Moreover, hyperglycaemia-induced ALR2 activation also brings about the imbalance of $NAD^+/NADH$. As the cofactor of silence information regulator 1 (SIRT1), NAD^+ can regulate its deacetylation activity. It has been displayed that SIRT1 takes part in many physiological and cellular

processes. It exerts positive effect in cancer, diabetes, cardiovascular disease and aging by regulating various transcription factors such as forkhead box O-type protein (FOXO), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and *ether-à-go-go*-related gene 1 (Erg-1), p65 and p53 [10,11]. As one of the downstream targets of SIRT1, the activation of PGC-1α alleviates the damage of high glucose by promoting the biosynthesis of mitochondrial [12]. As mentioned above, no doubt, inhibition of ALR2 is an attractive strategy to counteract the development of long-term DC.

Nowadays, it is recognized that nuclear factor-erythroid 2-related factor 2 (Nrf2) is the most important pathway to respond to ROS inside life body. Nrf2 is a transcription factor modulating the cellular oxidative system. When encountered with ROS, the conformation change of Kelch-like ECH-associated protein 1 (Keap1) results in Nrf2 dissociation from Keap1 and translocation from cytoplasm to the nucleus. The activated Nrf2 interacts with the antioxidant response element to promote the transcription of the antioxidant gene, such as hemeoxygenase 1 (HO-1), superoxide dismutase (SOD), glutathione S-transferase and nicotinamide adenine dinucleotide phosphate (NADPH)-quinone oxidoreductase [13]. Many investigations disclosed that Nrf2 possesses impressively protective effect on diabetes [14], multiple sclerosis [15] and cancer [16]. Interestingly, more and more evidences support that Nrf2 may provide a new therapeutic target for treatment of diabetes and diabetic complications [17–19].

Therefore, it is reasonable to postulate that if one agent plays both the roles as aldose reductase inhibitor (ARI) and Nrf2 activator, it may probably possess better and wider spectrum activity against DC. Fortunately, in our previous work, we did design-synthesize a compound, chemically named as (*R,E*)-*N*-(3-(2-acetamido-3- (benzyloxy) propanamido)propyl)-2-cyano-3-(4-hydroxy phenyl)acrylamide (**hr5F**). It was confirmed not only as an excellent ARI, but also a significant anti-oxidant in a chick embryo model of hyperglycemia [20]. However, the exact mechanism of how **hr5F** mediates the cellular oxidative system and what its effects *in vivo* remain still unknown. And herein, in the current paper, we tried to elucidate the potential mechanism of **hr5F** on SIRT1-PGC-1α/Nrf2 pathway in high glucose- impaired human lens epithelial cells (HLECs) model. Furthermore, the protective effect of **hr5F** in streptozocin (STZ)-induced diabetic rats will also be disclosed.

2. Materials and methods

2.1. Research governance

All animal care and experimental procedures were approved by the Laboratory Animal Ethics Committee of Jinan University (20,141,112,017), and were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (7th edition, USA).

2.2. Materials

RNAiso Plus, PrimeScript™ RT reagent Kit (Perfect Real Time) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) were obtained from Takara (Shenyang, China); MDA kit, SOD kit and GSP kit were purchased from Jiancheng bioengineering institute (Nanjing, China); Dichlorodihydrofluorescein diacetate (DCFH-DA), sodium D-glucuronate, streptozocin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma (Santa Clara, CA, USA); SIRT1 rabbit polyclonal antibody, PGC-1α rabbit polyclonal antibody, AR rabbit polyclonal antibody, Nrf2 rabbit polyclonal antibody, SOD-1 rabbit polyclonal antibody and β-actin rabbit polyclonal antibody HRP labeled goat anti-rabbit IgG (H + L) were got from Proteintech Group (Chicago, USA); Bax rabbit polyclonal antibody and Bcl-2 rabbit polyclonal antibody were supplied by Cell signaling technology (Boston, USA); Glucose and mannitol were purchased from Aladdin (Shanghai, China); NADPH and $NADPNa_2$ were obtained from Roche (Basel, Switzerland); DL-glyceradehyde was got from Solarbio (Beijing, China); Edavarone (Eda) and Epalrestat (Epa) were provided by TCI (Shanghai, China); Epalrestat tablet was got from Nanjing Hailing Pharmaceutical Industry Co., Ltd. (Nanjing, China). Compound **hr5F** was synthesized by our group according to the procedure described in reference at greater than 95% purity [20].

2.3. Cell culture

Human lens epithelial cells (HLECs) were purchased from Cellbio (Shanghai, China), cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Beijing, China) containing 10% fetal bovine serum (Gibco, California, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were grown at 37 °C in a humidified incubator with 5% CO_2.

2.4. MTT assay to determine cell viability

The cell viability of HLECs was assessed using the MTT assay [21]. The process was described below: HLECs were divided into eight groups: control group (5.5 mM glucose), high glucose group (80 mM glucose), mannitol group (5.5 mM glucose + 74.5 mM mannitol), Epa group (80 mM glucose + 100 μM Epalrestat), Eda group (80 mM glucose + 100 μM Edaravone), **hr5F**-1 μM group (80 mM glucose + 1 μM **hr5F**), **hr5F**-10 μM group (80 mM glucose + 10 μM **hr5F**), **hr5F**-100 μM group (80 mM glucose + 100 μM **hr5F**). Epalrestat and Edaravone were treated as ARI and antioxidant positive control, respectively. The cells were seeded onto 96-well plates at a density of 5×10^3 cells/well. After 48 h of culture, 20 μL 0.5 mg/ml MTT was added and then incubated for 4 h in incubator. After the incubation, the medium was removed carefully, and the insoluble formazan was dissolved by adding 200 μL DMSO. Cell viability was quantified by measuring the absorbances at 490 nm using a multi-detection microplate reader (Synergy™ HT, BioTek, USA). The cell viability was estimated by the formula: $OD_{treated}/OD_{control} \times 100\%$, where the cell viability of control group was set as 100%.

2.5. Measurement of ALR2 activity

ALR2 activity was determined by the method described previously with slight modifications [22]. Crude ALR2 was prepared from HLECs and rats lens of each group. The cells were collected and re-suspended with 100 mM potassium phosphate buffer (PBS) (pH7.4), following ultrasonic disrupted (300 Hz, sonication 3 s with interruption 2 s, total 5 min) on ice. The lenses were homogenized in 10 volumes of 100 mM PBS on ice. The homogenates were centrifuged at 15,000 r/min for 30 min and the supernatants were kept in -20 °C refrigerator. The protein concentration of the supernatant was determined using BCA kit. ALR2 activity was assayed according to the method described by reference [22]. The assay mixture containing 50 mM phosphate buffer, 2 mM DL-glyceradehyde, 5 mM β-mercaptoethanol, 0.1 mM NADPH and enzyme solution, with a total volume of 200 μL. The mixture was put on ice, and the reaction was initiated by the addition of NADPH and incubated at 37 °C for 15 min immediately. And then 100 μL 0.1 M HCl was added to the mixture on ice for 10 min to stop the reaction. Finally, 300 μL 6 M NaOH containing 10 mM imidazole was added to the mixture at 60 °C for 10 min to generate fluorescent derivative of $NADP^+$. Fluorescence was excited at a wavelength of 360 nm and emitted light was observed at 420 nm by multi-detection microplate reader (Synergy™ HT, BioTek, USA). The content of $NADP^+$ derivative was calculated by standard curve made by $NADPNa_2$. The activity unit of ALR2 (U) was defined by the generation of 1 μM $NADP^+$ per min of 1 g protein.