Study on Isoquercitrin Relieving Insulin Resistance of RNF6 Overexpressing db/db Mice

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Abstract: Insulin resistance is the main pathogenesis of type 2 diabetes. Previous studies have proved that isoquercitrin, a natural flavonoid extracted from Bidens bipinnata, can alleviate insulin resistance, but its mechanism is still unknown. Ring finger protein 6 (RNF6) is related to the degradation and ubiquitination of target protein, and it is a potential target for the treatment of insulin resistance. db/db mice were constructed with RNF6 overexpression, and their body weight and fasting blood glucose levels were recorded. After isoquercitrin administration, the body weight, fasting blood glucose and fasting insulin content of mice in each group were measured, and the pancreatic tissue morphology of mice in each group was observed by HE staining. The protein expressions of RNF6, IRS1 and PIK3R1 in cells were detected by Western blot. The results showed that RNF6 overexpression mice were successfully constructed. Overexpression of RNF6 would aggravate the insulin resistance of mice and inhibit the expression of IRS1 and PIK3R1, while isoquercitrin administration could reduce the expression of RNF6, up-regulate the expression of IRS1 and PIK3R1 and relieve the insulin resistance of mice. In a word, the results indicate that isoquercitrin can target RNF6, up-regulate the expression of IRS1 and PIK3R1 decreased by RNF6, and relieve insulin resistance in db/db mice.

Keywords: Insulin resistance, Isoquercitrin, Type 2 diabetic, RNF6, db/db mice

1. Introduction

As the most important blood glucose regulating hormone in human body, insulin can promote the uptake and utilization of glucose in tissues and cells and the synthesis of fat, protein and glycogen [1]. Insulin resistance is a defect state of insulin effect caused by many reasons, and it is the main pathogenesis of type 2 diabetes mellitus (T2DM), accompanied by the whole process of diabetes occurrence and development [2]. T2DM has become a heavy burden for patients all over the world, and its incidence rate is rising, accounting for more than 90% of the global diabetes cases [3]. According to the prediction of the World Health Organization, diabetes will become the seventh leading cause of death by 2030 [4]. At present, the clinical intervention for T2DM mainly depends on insulin supplementation or hypoglycemic drugs, but it cannot be eradicated [5]. Therefore, it is very important to find a new method to treat insulin resistance.

In recent years, people are paying more and more attention to the medicinal value of natural compounds and their extracts. These compounds have high research value and potential because of their multi-targets, multi-pathways and lower toxic and side effects [6]. Isoquercitrin is widely distributed in Bidens bipinnata, a composite plant, and as an effective component of medicinal plants, it has hypoglycemic, antihypertensive, anti-inflammatory and sedative effects [7]. RING finger protein 6 (RNF6)
is a ring-type E3 ubiquitin ligase of RNF family, which is related to target protein degradation and ubiquitination. Many studies have shown that RNF6 is helpful to regulate cell proliferation, metabolism and apoptosis [8]. Activation of IRS1/PI3K signaling pathway can enhance the utilization rate of insulin, while inhibition of the pathway will show insulin resistance [9]. PIK3R1 can promote PI3K to play a role, and can be used as a linker for the interaction between insulin receptor substrate (IRS) protein and growth factor receptor [10]. Therefore, PIK3R1 plays an important role in this pathway. At present, as a potential target of diabetes, RNF6 has been paid more and more attention. In this experiment, the mechanism of isoquercitrin acting on RNF6 to alleviate insulin resistance was studied.

2. Materials

2.1 Experimental animal

24 SPF db/db mice and 6 dbm mice, all 5 weeks old, were purchased from Jiangsu Huachuang Cigna Pharmaceutical Technology Co., Ltd., male. The license number is SYXK Gui 2020-0005. It is kept in the Experimental Animal Center of Guilin Medical College (Lingui Campus), and the basic feed is provided by the Experimental Animal Center of Guilin Medical College.

2.2 Experimental drug

(1) Isoquercitrin: purchased from Nanjing Yuanzhi Biotechnology Co., Ltd. (No.:B21529).

(2) RNF6 overexpressed adeno-associated virus and empty adeno-associated virus were constructed in cooperation with Shanghai Jikai Gene Chemical Technology Co., Ltd.(No.:GOSV0396238)

2.3 Main reagents and consumables

RNF6 antibody, PIK3R1 antibody, IRS1 antibody (Abcam Company, USA); MonScript RTIlI All-in-One Mix for reverse transcription kit and MonAmp SYBR Green for fluorescence quantitative kit (Mona Biotechnology Co., Ltd.); Heparin Anticoagulant Blood Collection and ELISH Kit (Jiangsu Kangcheng biological Biotechnology Co., Ltd.) 4% paraformaldehyde tissue fixative, xylene, hematoxylin, eosin, neutral resin, goat serum sealing solution (Beijing SolarBio Biotechnology Co., Ltd.)

2.4 Main instruments

Rotary slicer, spreading and baking machine, paraffin embedding machine, frozen workbench produced by Hubei Kangqiang Medical Equipment Co., Ltd., gel electrophoresis instrument, protein transfer membrane instrument, gradient PCR amplification instrument produced by BIO-RAD company of America, and fluorescence quantitative PCR detector produced by ABI company of America.

3. Experimental method

3.1 Preparation of db/db mouse model

Six db/db mice were raised in a cage, and they were free to eat and drink. The room temperature was 22 2 degrees Celsius, and the relative humidity was 40-70. 12 hours in light/12 hours in darkness. After feeding for 7 days, the mice were fasted for 6 hours, and the fasting blood glucose value of each db/db mouse was more than 11.1mmol/L, and then the follow-up experiments were carried out.

3.2 Grouping of mice and drug intervention

The mice were divided into five groups, with 6 mice in each group, namely Normal group (dbm mice), Model group (db/db mice), Model+EPV group (db/db mice+empty adeno-associated virus), Model+AAV-RNF6 group (db/db mice +RNF6 adeno-associated virus), Model+AAV-RNF6 group (db/db mice +RNF6 adeno-associated virus +40IS group (db/db mice +RNF6 adeno-associated virus +40mg/kg isoquercitrin), 200uL (titer/concentration: 1.0e+12 v/g/ml) of corresponding adeno-associated virus was injected into the tail vein of mice on the 0 th day, and the other groups were injected with the same volume of physiological saline. On the 21st day, the Model+AAV-RNF6+40IS group was given isoquercitrin by gavage for 14 days, with 200uL each time, while the other groups were given the same volume of normal saline. On the 35th day, the feeding
was stopped and the materials were taken.

3.3 Determination of fasting blood glucose and body weight in mice

Tail-pinching adaptation training should be given to mice in advance to avoid stress hyperglycemia. After fasting for 6 hours on the 0th, 21st, and 35th day, the fasting blood glucose and weight of each mouse were measured, and the actual data were read and recorded.

3.4 Mouse sampling

Mice were fed for 35 days, and animal samples were taken. After taking blood from eyeball, the mice were killed by dislocation of cervical vertebra. The pancreas and part of the liver of the mice were washed with dust-free filter paper and put in 4% tissue fixative for later use. The liver was sectioned with paraffin and the pancreas was stained with HE. The remaining liver was frozen at -80°C.

3.5 Detection of fasting insulin by ELISA

The blood of mice was centrifuged at 12000 rpm/min at 4°C for 10 min, and the serum was collected. The sample was added by ELISA insulin detection kit according to the instructions, and the absorbance at 450 nm was measured, and the standard curve was drawn to calculate the fasting insulin content of mice.

3.6 Histopathological observation of pancreas

The pancreatic tissue was taken, fixed, dehydrated, transparent, soaked in wax and embedded, and then sliced to obtain tissue paraffin sections. After adding hematoxylin dye and eosin dye, the sections were sealed with neutral resin, and the images were taken under a microscope for image analysis.

3.7 Western blot was used to detect the protein expression of related factors

Take out 50mg of mouse liver, grind the tissue with a mortar after autoclaving, add 500ul of cell lysate to extract total protein, determine the protein concentration, add 5× protein loading buffer according to the sampling amount, mix well, and take a water bath at 100°C for 15 min to fully denature it. Electrophoresis, film transfer and luminescence were carried out to observe the expression of related factors.

3.8 Statistical analysis

The data are expressed as mean standard deviation. ANOVA was used to analyze multiple data sets, and T-test was used for pairwise comparison. P < 0.05 is considered statistically significant. Use GraphPad Prism v8.0 software for data analysis.

4. Results

4.1 RNF6 overexpressed adeno-associated virus was successfully transfected and expressed in mouse liver

Immunofluorescence photographs were taken of mouse liver pairs. As shown in Figure 1, the normal group and model group showed no fluorescence, while the empty adeno-associated virus group and RNF6 adeno-associated virus group showed immunofluorescence, indicating that adeno-associated virus was successfully transfected into mouse liver. The expression of RNF6 was detected. The results showed that the expression of RNF6 in the model RNF6 adeno-associated virus group was significantly higher than that in the model group, but there was no significant difference between the empty adeno-associated virus group and the model group. These results indicated that RNF6 adeno-associated virus was successfully transfected and expressed in the mouse liver.
4.2 Effect of isoquercitrin intervention on state and biochemical indexes of mice with RNF6 overexpression

The body weight and blood sugar of mice were measured on the 0, 21 and 35 days after injecting adeno-associated virus into the tail vein. From the results in Figure 2, we can observe that on the 0th day, the blood sugar and body weight of mice in the model group were significantly higher than those in the normal group. After injecting RNF6 adeno-associated virus for 21 days, the blood sugar and body weight of the Model+AAV-RNF6 group were significantly higher than those in the model group. Isoquercitrin was administered for 14 days from the 21st day, and the blood sugar and weight of mice were measured on the 35th day. The results showed that isoquercitrin administration could significantly improve the blood sugar level of mice and reduce the weight of mice. The results of fasting insulin in mice in Figure 2.C and Figure 2.D show that the high expression of RNF6 can increase the fasting insulin content and insulin resistance index of mice, and isoquercitrin can decrease the fasting insulin level and insulin resistance index of mice. These results indicate that over-expression of RNF6 will aggravate insulin resistance in mice, and isoquercitrin administration can alleviate insulin resistance.
4.3 Effect of isoquercitrin on pancreatic pathology in mice

The pancreas of mice was analyzed by HE staining. As shown in Figure 3, the islet tissue structure of normal mice was normal, the boundary was obvious, and the cells were densely arranged and evenly distributed. The islets of mice in model group and no-load adeno-associated virus group showed loose tissue, disordered structure, irregular shape and inconspicuous editing. After injection of adeno-associated virus, it can be seen that the morphological changes of islets in mice are more obvious, the structure is looser, and the size of cells tends to be more discrete and unevenly distributed. Isoquercitrin administration can improve the islet morphology in mice, with relatively clear and regular islet morphological contour, uniform pancreatic nuclei size and relatively regular arrangement. These results indicate that over-expression of RNF6 will aggravate the islet pathology in mice, and isoquercitrin administration can inhibit the pathological results of RNF6 on mouse islets and significantly improve the islet tissue morphology in mice.

![Figure 3: Staining results of pancreatic tissue of mice in each group (X100)](image)

4.4 Isoquercitrin inhibited the expression of RNF6 protein and up-regulated the levels of IRS1 and PIK3R1 protein

In the result of Western blot in Figure 4, we observed that the expression of RNF6 protein in the injection group of RNF6 adeno-associated virus increased, which also indicated that the adeno-associated virus was successfully constructed and expressed, and the overexpression of RNF6 inhibited the expression of PIK3R1 and IRS1 proteins. Isoquercitrin administration can effectively reduce the expression of RNF6 and up-regulate the expression of IRS1 and PIK3R1. This may be a way of isoquercitrin to relieve insulin resistance. By inhibiting the expression of RNF6, the IRS1/PIK3R1 pathway inhibited by RNF6 overexpression is activated, which increases the utilization rate of insulin, thus relieving insulin resistance.

![Figure 4: Western blot detection results of RNF6, PIK3R1 and IRS1 in mice of each group.](image)

Note: A: Protein bands. B: RNF6 protein expression. C: IRS1 protein expression. D: PIK3R1 protein expression. Compared with Model group, **p<0.01, * * p < 0.001; Compared with Model+AAV-RNF6 group, ###p<0.001.
5. Discussion

Recent studies have shown that different epigenetic signals may get together and affect the occurrence of IR. Genetic, epigenetic and environmental factors interact and contribute to the development of diabetes [11]. Ubiquitin, as a kind of histone modification, can select specific target protein molecules under the action of a series of special enzymes, and modify the target protein specifically [12], thus affecting the phenotype and disease development by regulating protein expression. It is worth noting that IR is the result of complex interaction between genes and environment, and ubiquitination is reversible. Uncovering the relationship between IR and ubiquitination will provide a new method for improving the management and prevention of IR [13].

In this experiment, we established a mouse model by injecting RNF6 adeno-associated virus into the tail vein of db/db mice. After the model was established successfully, the mice were treated with isoquercitrin by gavage, and the changes of various physiological indexes and biochemical indexes of mice were observed, as well as the morphology of mouse islets. It was found that the over-expression of RNF6 could increase the weight and blood sugar of mice, and aggravate the insulin resistance of mice. After the administration of isoquercitrin, the effects of RNF6 on mice could be inhibited, so that the mice could be improved. As a member of E3 family, RNF6 has certain substrate specificity, which is particularly important for mediating the ubiquitination of specific protein [14]. IRS1, as an insulin receptor substrate, is easy to be superficially modified [15] and ubiquitinated by E3 ubiquitin ligase [16] [17], which is one of the reasons why we consider that RNF6 can exert an inhibitory effect on IRS1. In the results of Western blot, we found that RNF6 inhibited the signal pathway of IRS1/PIK3R1, and the expression of RNF6 was inhibited after administration, and the IRS1/PIK3R1 pathway was activated, which may be a way for isoquercitrin to alleviate insulin resistance.

To sum up, in this experiment, we found that over-expression of RNF6 can aggravate the insulin resistance of mice and inhibit the expression of IRS1 and PIK3R1, while isoquercitrin can reduce the expression of RNF6, activate the IRS1/PIK3R1 pathway and alleviate the insulin resistance of mice.

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