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Mechanisms of Metformin Improving vasodilation of coronary artery in Type 2 Diabetic Rats

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Abstract In recent years, the incidence of type 2 diabetes has gradually increased. Coronary artery-related disease is one of the most common and serious complications of this. Endothelial dysfunction has become a risk factor for cardiovascular disease in patients with coronary heart disease. In this study, the protective effect of metformin on coronary artery in type 2 diabetic rats was investigated.

Keywords: metformin; type 2 diabetes; coronary diastolic function

1. Introduction

In recent years, due to the significant improvement in living standards and changes in dietary structure, the incidence of metabolic diseases represented by diabetes has increased significantly and has shown a trend of younger age.The study found that the incidence of type 2 diabetes (T2DM) is the highest in diabetic patients, and the pathological harm is also the greatest. In people with type 2 diabetes, obesity and other factors are the main causes of insulin resistance1,2. This is different from the pathological resistance of insufficient insulin secretion in type 1 diabetes,T2DM is mainly due to decreased sensitivity of tissues or cells to insulin, while insulin levels in blood tend to be normal or slightly high1,3. As the increase or fluctuation of blood sugar level seriously endangers the function of normal tissues and organs, in clinical treatment, besides stabilizing blood sugar level, prevention and treatment of various complications, especially cardiovascular diseases, which caused by diabetes mellitus, is an important issue of clinical concern4-6. Diabetes is a factor in many complications, such as myocardial infarction, vascular disease and coronary artery disease. In our study, we found that the incidence of cardiovascular disease is 2-4 times that of normal people, which is a very high rate, which is also the main cause of death in diabetic patients4. Endothelial dysfunction is the main cause of vascular-related complications in diabetes and plays a major role in the development of diabetes.7,8.

Metformin (MET) is commonly used in the clinical treatment of diabetes mellitus, usually in patients with uncontrolled type 2 diabetes mellitus and obese patients9-12. In clinical treatment, it has the pharmacological effects of lowering blood sugar and improving insulin sensitivity, and in application, it can effectively reduce the mortality rate of patients. Its hypoglycemic mechanism is mainly related to the reduction of hepatic glycogen production. In addition to lowering blood sugar, many studies have shown that metformin also plays an important role in the treatment of tumors, nonalcoholic fatty liver disease, polycystic ovary syndrome, and metabolic syndrome. In addition, more and more studies have confirmed that metformin has significant advantages in reducing endoplasmic reticulum stress, anti-oxidative stress and anti-inflammatory 12.

Although the cardiovascular protective effect of metformin has been widely recognized, the specific mechanism by which it reduces cardiovascular events such as myocardial infarction, especially the protection of coronary function, remains unclear. This paper studies the protective mechanism of dimethicone on arterial relaxation and its role in diabetic complications.

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2. Methods and Material

2.1 Animals

In the study, 4-week rats with a body weight of 100g-200 were used as the normal group; the type 2 diabetic rats were used as the control group. All rats were housed in a standard ultra-clean environment to establish normal circadian rhythms. The rats in the T2DM group and the MET group were intraperitoneally injected with streptozotocin 30 mg/kg for 3 consecutive days, and the NC group was injected with the same dose of citrate buffer as the control group. Rats in NC group were given T normal diet. The T2DM and MET groups were given a high-fat diet (20% fat, 62% carbohydrate, 17% protein, 1% other). After 6 weeks, the rats with blood glucose levels higher than 11.1 mmol/Lof OGTT test in the MET group in the T2DM group were included in the follow-up experiment, with 6 rats in each group. The rats in the MET group were given metformin 300 mg/kg/d orally, and the T2DM group and the NC group were given the same amount of normal saline as the control group. Rats body weight and blood glucose were measured after 8 weeks and sacrificed for subsequent experiments.

2.2 Vasodilation measurement of coronary artery

The animals were anesthetized with sodium pentobarbital. After taking blood from the ventricular, the heart tissue was saved in PSS buffer with the composition of (nM): ,KCl 4.7, CaCl2·H2O 2.5， KH2PO4 1.18, MgSO4·7H2O 1.17 , NaCl 119， NaHCO3 25, EDTA 0.02, D-glucose 5.5, pH 7.4, preheated to 37 ° C with a mixture of 5% CO2 and 95% O2. After careful separation of the coronary artery septum under the stereoscope, the coronary artery with a length of 2 mm was prepared in the vascular perfusion vessel of the microvascular tension instrument DMT, and the vascular was balanced at the tension of 1.8-2.1 mN for 60 minutes, and then the activity of artery was determined by 60 mM KCl buffer. Vascular activity and endothelial integrity were measured with 1 mM acetylcholine (ACh). After pre-constricted the artery with 10-5M Endothelin 1 (ET-1), gradient concentration of 10-9~10-5M ACh, 10-9~10-6M sodium nitroprusside and 10-10~10-7M insulin (INS) was added in the perfusion tank respectively, the tension of coronary artery under different concentrations of diastolic agent was recorded, and the percentage of vasodilation at this concentration was calculated, percentage of vasodilation= (tension under ET-1 －tension under vasodilator) / (tension under pre-constriction－tension under balance) × 100%.

2.3 Nitric oxide (NO) concentration determination

After 5 minutes of addition of vasodilator (ACh 10-5M, INS 10-7M), the buffer in the vascular perfusion tank was collected to determine the NO concentration in the buffer by nitrate reductase method. The specific operation was strictly in accordance with instruction of the kit (purchased from Nanjing). After the operating instructions were completed, the absorbance (OD) of the sample at 550 nm excitation light was measured. NO concentration (μmol/g) = (OD value in sample tube－ blank control) / (standard control - blank control) × 100 × dilution ratio / mass of blood vessel.

2.4 Cell culture

Human umbilical vein endothelial cells (HUVECs) cultured at 37 ° C under 5% CO2, and passaged when the cells were grown to 70%. The NC group was cultured in normal HUVECs cell culture medium. The T2DM group and the MET group were cultured in HUVECs medium containing 50 μM glucose and 500 μM sodium soft acid for 48 hours. The MET group was added with 30 μM metformin, NC group and T2DM group. The same dose of the medium was added as control, and after 48 hours of culture, the cell culture supernatant was harvested and the NO concentration in the supernatant was determined, and the total cell protein was extracted.

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2.5 Western Blot

The Western-Blot experiment was performed under standard procedures. HUVECs cells was digested and total protein was extracted for quantification. The concentration of protein in each sample was adjusted and denaturation. The sample was electrophoretic separation under 5% upper gel and 10% lower gel, and then the protein was transformed to membrane under 300 mA for 90-150 minutes. Prepare a 3% bovine serum albumin solution, put the membrane in it and seal it, and keep it at 4° C for 12h. The membrane was then washed 3 times with PBST solution and finally incubated at room temperature. After 60 minutes incubation in the secondary antibody for 60 minutes, the membrane was washed in PBST buffer for three times and then Detection of these signals in the Bio-Rad ChemiDoc XRS Imaging System.

2.6 Statistical analysis

The results of this study were analyzed by software Prism 7.0. Results are expressed as mean ± standard deviation. Multivariate ANOVA was used for multiple comparisons between multiple samples. A t-test was used to compare the differences between two independent groups. $p<0.05$ was considered statistically significant.

3. Results

3.1 Metformin can significantly reduce body weight and blood glucose levels in type 2 diabetic rats

Compared with the normal control group, the T2DM group had a significant increase in body weight (483.68 ± 14.77 vs. 421.21 ± 17.23, $p<0.01$). The rats showed obesity, decreased activity, and typical diabetes symptoms of polydipsia and polydipsia. The blood glucose at 2 hours in OGTT was significantly increased (15.25 ± 2.36 vs. 7.11 ± 0.61, $p < 0.01$). Compared with the T2DM group, the MET group had a relatively reduced body weight (452.59 vs. 483.68±14.77, $p<0.05$), and the OGTT results at 2 hours was significantly lower (11.26±1.93 vs. 15.25±2.36, $p<0.01$, Table 1), but still higher than the NC group. The results indicated that the experimental dose of metformin could significantly control the blood glucose level of the type 2 diabetic rat model, and had a certain therapeutic effect.

3.2 Metformin could significantly improve endothelium-dependent vasodilation of coronary artery in type 2 diabetic rats

Acetylcholine mediates endothelium-dependent vasodilation. In the NC group, the coronary arteries were pre-constricted by ET-1, and vasodilated after addition of acetylcholine. After the final concentration of acetylcholine was added, the relaxation reached 71.69±4.49% (Fig. 1A). Compared with the NC group, the endothelium-dependent vasodilation of coronary artery in the T2DM group was significantly impaired. After the final concentration of ACh, the maximum vasodilation was only 14.96±3.49% (Fig. 1A, $p<0.05$). Significantly, compared with the T2DM group, coronary endothelium-dependent vasodilation was improved in the MET group, with a maximum vasodilation of 60.77 ± 7.14% ($p < 0.01$). The coronary artery vasodilation results of the three groups were statistically different.

Unlike acetylcholine-mediated endothelium-dependent vasodilation, there were no significant differences in vasodilation mediated by sodium nitroprusside, which is the endothelium-independent vasodilator, in the three groups. (Figure 1B)

3.3 Metformin increased coronary acetylcholine-mediated NO release in type 2 diabetic rats

NO was an important vasodilator. Compared with NC rats, after adding 10-5M acetylcholine, the concentration of NO in the perfusion of coronary artery in T2DM group was significantly decreased ($p<0.01$), while the diabetic rats treated with metformin was improved. After treatment with

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acetylcholine, the level of NO in the perfusate was higher than that in the T2DM group, but lower than that in the NC group ($p<0.01$, Figure 2A).

After the HUVECs cells cultured in vitro, 10-5M ACh was added, and the supernatant was collected to determine the NO concentration in the cell culture medium. The results showed that the NO content in the high glucose and high fat cultured group was significantly reduced. After the addition of metformin, the level of NO release increased, similar to the control group. ($p<0.05$, Figure 2B)

3.4 Metformin could increase insulin sensitivity of coronary endothelial cells in type 2 diabetic rats

Insulin could mediate vasodilation through endothelial cells, and type 2 diabetes was characterized as insulin resistance. Compared with NC group, vasodilation is greatly reduced in coronary T2DM rats (3.58±6.3% vs. 45.63±8.22%, $p <0.01$), and after metformin treatment, the coronary insulin sensitivity of the MET group was improved, with significant improvement of vasodilation contrasted with T2DM group ($p < 0.05$, Figure 3A).

The results of in vitro experiments also showed that after metformin treatment, compared with the high-sugar and high-fat cultured group, the NO release level in the cell culture supernatant was significantly increased after the addition of insulin 10-7M. ($p<0.05$, Figure 3B)

3.5 Metformin could enhance the phosphorylation of AMPK and eNOS in HUVECs cultured with high glucose and high fat

The protein expression and phosphorylation levels of HUVECs were detected by western-blot. As shown in the results, HUVECs were cultured with 50 μM glucose and 500 μM sodium soft acid for 48 hours, and there was no significant difference in the expression of AMPK among each group, but the phosphorylation level was significantly decreased, which could be elevated through treated with metformin ($p < 0.05$, Figure 4A). Consistent with the results of AMPK, metformin significantly increased the phosphorylation level of eNOS in HUVECs cultured with high glucose and high fat acid. ($p<0.05$, Figure 4B).

4. Discussion

In our study, it was found by in vitro vascular function experiments that metformin can significantly improve the endothelium-dependent vasodilation function of coronary artery in type 2 diabetic rats. It was known that acetylcholine can act on the M receptor of endothelial cells, causing an increase in intracellular Ca2+ concentration, activation of nitric oxide synthase (NOS) to produce NO, and NO acting on vascular smooth muscle to convert GMP into cGMP to cause vasodilation. 21,22 Sodium nitroprusside is a potent vasodilator, which can directly produce NO in vascular smooth muscle cells when dissolved and mediates vasodilation. This study found that coronary artery responsiveness to acetylcholine was decreased in type 2 diabetic rats and vasodilation response to sodium nitroprusside was normal, which suggesting that the decrease of coronary diastolic function in type 2 diabetic rats may be related to the decrease of NO produced by endothelial cells. The coronary artery function was improved after metformin treatment, and the possible mechanism was that metformin increased the release of NO by vascular endothelial cells. The results of NO quantitative detection in the supernatant confirmed our speculation.

In order to study the possible mechanism of metformin in improving coronary artery relaxation in diabetic rats, the response of coronary artery to insulin was further studied. Insulin can activate the intracellular PI3K/Akt signaling pathway and activate nitric oxide synthase (NOS) to generate NO-mediated vasodilation by acting on the insulin receptor of endothelial cells. The responsiveness of vascular endothelial cells to insulin in type 2 diabetic rats decreases, leading to insulin resistance. After metformin treatment, the vascular reactivity increased, showing increased vasodilation and increased NO release, which may be related to the activation of PI3K signaling pathway and the

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increase of nitric oxide synthase activity. The cellular composition of vascular tissue is very complicated. Therefore, we used HUVECs as the research object in further mechanism research. Under the condition of high glucose and high fat culture, the phosphorylation level of AMPK was significantly decreased as the cell damage performance. The results showed that the change of NO release was consistent with the vascular function test, which further clarified the protective effect of metformin on endothelial cells. More importantly, protein immunoblotting experiments have found that the protective effect of metformin is related to the increase of eNOS phosphorylation level, but its specific molecular mechanism still needs to be further explored. For example, how metformin regulates eNOS phosphorylation level by upstream molecules remains unclear.

In this study, we demonstrate for the first time that metformin can protect vascular diastolic function by protecting coronary endothelial cells in type 2 diabetic rats, which may by increasing of endothelium-dependent NO release. This study demonstrates for the first time the role of metformin in protecting cardiovascular function in patients with type 2 diabetes from the perspective of metformin protecting coronary diastolic function and improving hemodynamic stability of cardiac perfusion, providing experimental basis for clinical rational drug use.

5. Methods

Rats model of type 2 diabetes were established by high glucose-high fat diets combined with STZ injection. The coronary artery vascular was isolated in vitro and the relaxation response to acetylcholine, sodium nitroprusside and insulin was measured. The vasodilation factor NO was determined. The protein levels of NO release were detected by immunoblotting.

6. Results

In our study, we found that compared with the normal group, the relaxation response mediated by coronary acetylcholine and insulin was inhibited, and the release of NO was also inhibited. While the release of NO was increased with improved diastolic function after metformin treatment. After high glucose and high fat induction, the release level of NO in HUVECs supernatant was decreased, and the phosphorylation levels of AMPK and eNOS were decreased too. However, an increase in NO release and the phosphorylation of AMPK and eNOS were increased after metformin treatment.

7. Conclusion

Metformin can improve coronary diastolic function and insulin sensitivity in type 2 diabetic rats. The possible mechanism may be related to the increase of AMPK, eNOS phosphorylation and increase of NO release in endothelial cells.

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