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Silybin reduces obliterated retinal capillaries in experimental diabetic retinopathy in rats

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ABSTRACT

Silybin has been previously reported to possess anti-inflammatory properties, raising the possibility that it may reduce vascular damage in diabetic retinopathy. Present study was designed to investigate this potential effect of silybin and its underlying mechanisms in experimental diabetic retinopathy. Diabetes was induced with streptozotocin (STZ) plus high-fat diet in Sprague-Dawley rats, and silybin was administrated for 22 weeks after the induction of diabetes. Histochemical and immunofluorescence techniques were used to assess the obliterated retinal capillaries, leukostasis, and level of retinal intercellular adhesion molecule-1 (ICAM-1). Western blot was performed to quantitate the expression of retinal ICAM-1. Results showed that silybin treatment significantly prevented the development of obliterated retinal capillaries in diabetes, compared with vehicle treatment. In addition, leukostasis and level of the retinal ICAM-1 were found to decrease considerably in silybin-treated diabetic groups. In conclusion, these results indicate that silybin reduces obliterated retinal capillaries in experimental diabetes, and the recovered retinal vascular leukostasis and level of ICAM-1 at least partly contributes to the preventive effect of silybin.

1. Introduction

One of the characteristic features of the early diabetic retinopathy is obliterated retinal capillaries, which initially occur in single, isolated capillaries, however, as more and more capillaries become occluded, retinal perfusion decreases, at least locally, leading to retinal ischemia, subsequently increasing production of growth factors, and eventually leading to development of proliferative diabetic retinopathy.

Although the exact pathogenesis of obliterated retinal capillaries remains unclear, excess adherent leukocytes in retinal vasculature or leukostasis (Joussen et al., 2004) is considered to play a causal role in the development of obliterated retinal capillaries in diabetes (Miyamoto et al., 1999).

The process of leukocyte adhesion to retinal vascular endothelial cells is mediated by intercellular adhesion molecules, such as ICAM-1, a crucial participant in retinal vascular leukocyte adhesion or leukostasis in diabetes (Joussen et al., 2001; Miyamoto et al., 1999).

Silybin, a bioactive polyphenolic flavonoid in the seeds of milk thistle (Silybum marianum), has been used as a traditional drug for over 2000 years to treat a range of liver disorders (Flora et al., 1998; Saller et al., 2001). Recent studies also demonstrated that silybin possessed anti-inflammatory properties (Al-Anati et al., 2009; Chittezhath et al., 2008; Giorgi et al., 2012; Gu et al., 2007; Kim et al., 2012; Prabu and Muthumani, 2012; Salamone et al., 2012; Youn et al., 2013). Therefore, silybin might be a suitable protective agent for early diabetic retinopathy.

The aim of our study was to investigate the potential protective effect of silybin on retinal vasculature and its underlying mechanisms in experimental diabetes.

2. Materials and methods

2.1. Experimental diabetes

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Sichuan University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA. Male Sprague-Dawley rats ($n=40$, 180 ± 10 g) obtained from the Laboratory Animal Centre of Sichuan University were housed under controlled room temperature (22 ± 2 °C), and humidity ($55\% \pm 5\%$) with a 12/12 h light/dark cycle. In order to simulate the pathogenesis of type 2 diabetes, the most common clinical type of diabetes,

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our diabetic rat model was induced with STZ plus high-fat diet. After a 1 week of habituation, animals were randomly divided into high-fat diet ($n=30$) and normal chow ($n=10$) groups. Rats in high-fat diet group were fed on a modified high-fat diet (10% grease, 20% sucrose, 1% bile salt, and 2.5% cholesterol) (Wang et al., 2010), while rats in normal chow group were fed on basic normal rat chow. At the end of week 9, all rats in high-fat diet group were injected with a single dose of 30 mg/kg STZ via abdominal cavity to induce diabetes, while all rats in normal chow group were injected with the drug vehicle citric acid buffer to serve as non-diabetic control. 1 week after STZ injection, fasting blood glucose was measured and only animals with >16.7 mmol/l of blood glucose were included in the diabetic group. Rats in diabetic group were randomly subdivided into vehicle-treated ($n=10$) and silybin-treated ($n=20$) groups. At the end of week 10, silybin, suspended in 1% carboxymethylcellulose sodium salt, began to be administrated orally at 15 mg/kg/day ($n=10$) and 30 mg/kg/day ($n=10$), respectively. The total course of treatment was 22 weeks. 4 weeks after STZ injection, high-fat diet was withdrawn, and rats in all groups were fed on the normal rat chow until they were killed.

2.2. Reagents

Streptozotocin, silybin and trypsin were from Sigma-Aldrich. Anti-ICAM-1 and anti-CD45 rabbit polyclonal antibodies were from Santa Cruz Biotechnology. Fluorescent isothiocyanate (FITC)-labeled Concanavalin A was from Nanocs (New York, NY, USA). Horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG antibody, anti-β-actin mouse monoclonal antibody, and Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody were from ZSGB-BiO (Beijing, China). Modified RIPA buffer and SDS sample buffer were from Beyotime (Shanghai, China). All solutions were prepared just before use and protected from light if necessary.

2.3. Metabolic characteristics, body weight and white blood cell count measuring

Blood samples were collected, after 12 h fast, from tail vein at the end of week 10 and from aortaventralis at the end of week 32, respectively. Fasting serum glucose and lipid profiles were detected by a micro-plate reader (Bio-Rad Laboratories, Inc., USA) with kits (Changchun Huili Biotech Co. Ltd, China). Glycosylated hemoglobin was measured by a DCA Vantage GlycoHemoglobin Analyzer (Siemens AG, Germany). White blood cell count was measured by an automatic blood cell analyzer (Mindray Medical International Ltd, China).

2.4. Immunofluorescence and histochemical staining

We labeled leukocytes with FITC-conjugated concanavalin A and anti-CD 45 antibody and isolated retinal vasculature with digesting technique as described previously (Ishida et al., 2003; Kern and Engerman, 1995; Yamashiro et al., 2003), with a slight modification. At the end of week 32, rats were killed and immediately perfused with sterile normal saline (500 ml/kg) with heparin (0.1 mg/ml) in it from a needle inserted into the left ventricle (the descending aorta was clamped) to wash out non-adherent blood cells. One eye was ligatured from retrobulbar vessels and removed for analysis of ICAM-1. The other eye was perfused with 30 ml FITC-conjugated concanavalin A (30 μg/ml in phosphate-buffered saline (PBS), pH 7.4) to label the adherent leukocytes and retinal vascular endothelial cells. Residual concanavalin A was removed by washing with PBS. All perfusions were performed under physiological pressure. After removal of rat eyes, they were fixed with 4% paraformaldehyde for 24 h, and then washed with PBS for 12 h. Isolated retinas were digested with 3% trypsin at 37 °C for 1.5 h, making an easy isolation of retinal vasculature from retinal tissue. Anti-CD45 antibody was used to confirm the identity of the adherent leukocytes in vessel walls, as CD45 is a marker of leukocytes. After permeated with 0.5% Triton X-100 in PBS (4 °C) for 12 h and blocked with 3% albumin in PBS, the retinal vasculature was incubated in anti-CD45 rabbit polyclonal antibody (1:300) overnight at 4 °C, and then incubated in Alexa Fluor 594-coupled anti-rabbit antibody (1:300) for 2 h at 20 °C after 5 washes with PBS. The adherent leukocytes, labeled with FITC-conjugated concanavalin A and anti-CD45 antibody, in retinal vasculature were observed and imaged under a fluorescence microscope (Olympus X71, Japan). ICAM-1 in retinal vasculature was analyzed with the same procedure. Only adherent leukocytes labeled by both FITC-conjugated concanavalin A and anti-CD45 antibody were counted for quantification. In order to quantify obliterated retinal capillaries, the retinal vasculature was stained with Periodic acid-Schiff (PAS) and hematoxylin. Adherent leukocytes and obliterated retinal capillaries were counted in 4 quadrants of the mid-retina.

2.5. Western blot analysis

The retinas were homogenized in ice-cold modified RIPA buffer. Lysates were then centrifuged at 12,000 g for 5 min at 4 °C. After transferring the supernatant to a fresh ice-cold tube, protein concentration was determined with a Bio-Rad protein assay kit. Equal concentrations of proteins were mixed with SDS sample buffer and denatured at 95 °C for 5 min. The Equal amounts of protein were loaded in a 6% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) followed by a 10% SDS-PAGE gel. The gels were run for 30 min at 80 V and 50 min at 120 V, respectively. Then the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 300 mA for 1.5 h in a transfer buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. After blockage, the membranes were incubated overnight with primary antibody for ICAM-1 (1:300) at 4 °C, followed by incubating with horseradish peroxidase-conjugated secondary antibody (1:3000) for 1.5 h at room temperature. Signals were visualized by the molecular imager ChemiDocTMXRS+ detection system (BIO-RAD Laboratories Inc., USA). Protein Bands on the membranes were analyzed by Quantity One software (BIO-RAD Laboratories Inc., USA). To control sample errors, the ratio of band intensity to β-actin was obtained to quantify the relative protein expression level.

2.6. Statistical analysis

All data were expressed as means ± S.E.M. Analysis of data was accomplished with SPSS 19.0. Statistical comparisons were made using analysis of variance (ANOVA), followed by Dunnett's test wherever appropriate. P value <0.05 was considered significant.

3. Results

3.1. Effect of silybin on metabolic characteristics, body weight, and white blood cell count

Diabetic rats were characterized with increases in levels of fasting serum glucose ($n=8$, $P<0.05$) (Fig. 1A), total cholesterol ($n=8$, $P<0.05$) (Fig. 1B), and triglyceride ($n=8$, $P<0.05$) (Fig. 1C), and body weight ($n=8$, $P<0.05$) (Fig. 1E) at the end of week 10. However, there were no statistical differences in levels of fasting serum glucose ($n=8$, $P>0.05$) (Fig. 1A), total cholesterol ($n=8$,