·Basic Research·

Expression and effect of proline hydroxylase domain 2 in retina of diabetic rats

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Abstract

• AIM: To observe the expression of proline hydroxylase domain 2 (PHD2) in the retina of diabetic rats and investigate the relationship between PHD2 and relevant intraocular vascular proliferation factors.

• METHODS: Sixty male specific pathogen free (SPF) Sprague-Dawley (SD) rats were randomly divided into two groups: the diabetic group and the control group. The rats in the diabetic group were intraperitoneally injected with 60 mg/kg (0.60 mL/100 g) of streptozotocin to induce a diabetic rat model. The rats in the control group were injected with an equal volume of sodium citrate buffer solution by the same method. Hematoxylin-eosin (HE) staining and immumofluorescence (IF) method were adopted to observe the pathological changes of retinal tissues and the expression of PHD2, glial fibrillary acidic protein (GFAP), vascular endothelial growth factor (VEGF) by 8wk. RT-PCR method was applied to detect the expressions of mRNA of PHD2, VEGF and GFAP. The relationship between PHD2 and other vascular proliferation factors was analyzed.

• RESULTS: HE staining showed that there was the retinal tissue edema in the diabetic group, and the arrangement was in disorder, and proliferation could be seen. IF staining: in the retina of normal rats, PHD2 was not expressed, GFAP and VEGF were mainly expressed in astrocytes; while in the diabetic rats, PHD2, GFAP and VEGF staining showed strong positivity in all retinal layers, mainly in neurogliocytes. PHD2 was co-expressed with VEGF and GFAP. The mRNA expression levels of PHD2, GFAP and VEGF in the diabetic group were obviously higher than that in the control group, respectively 1.83 times, 1.75 times and 2.08 times. The difference had statistical significance (P<0.01).

• CONCLUSION: The high expression of PHD2 in the retina of early-stage diabetic rats might result from secretion of neurogliocytes induced by local high-concentration blood glucose, thus promoting the expression of VEGF and GFAP. PHD2 plays an important role during the occurrence of diabetic retinopathy.

• KEYWORDS: proline hydroxylase domain 2; diabetic retinopathy; retinal neovascularization
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INTRODUCTION

Diabetic retinopathy (DR) is one of the most important microvascular complications of diabetes. It has become a primary cause for blindness of diabetics [1]. However, the mechanism for DR is still unknown. Many researches indicate retinal anoxia results in the high expression of angiogenesis factors, such as vascular endothelial growth factor (VEGF), which promoting division and proliferation of vascular endothelial cells and formation new vessels. At present, the drug therapy against DR mainly inhibits VEGF, which inhibits the growth of new vessels. But the therapeutic effect only against VEGF is limited. Therefore, it is necessary to find a more effective method to treat retinal neovascular diseases. Proline hydroxylase domain 2 (PHD2) is regarding as enzyme of hypoxia-inducible factor-1α (HIF-1α), and HIF-1α regulate the expressions of many factors, such as VEGF and erythropoietin (EPO)[2-3]. In recent years, some research indicates PHD2 plays a role in promoting normalization of tumor neovascularization[4-6], but the expression of PHD2 in eyes and its relationship with VEGF and other proliferation factors is not definite. This research will observe the expression of PHD2 in the retina of diabetic rats[7].

MATERIALS AND METHODS

Animals Sixty healthy male specific pathogen free (SPF) Sprague-Dawley (SD) adult rats were purchased from the Animal Center of Inner Mongolia University with a weight of 190-200 g and an age of 6-8wk. This research has been approved by the Animal Experiment & Ethics Committee of Inner Mongolia Medical University.

Reagents and Antibodies Rabbit anti-mouse PHD2 polyclonal antibody (Abcom, USA); anti-mouse VEGF polyclonal antibody, goat anti-mouse IgG-FITC antibody, goat anti-rabbit IgG-CY3 antibody, goat anti-rabbit IgG-TRITC antibody, anti-mouse glial fibrillary acidic protein (GFAP) antibody (Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd.); 5% calf blocking serum (Beijing Boaosen Biotechnology Ltd.); DAPI labeling kit (Beijing Solarbio Science & Technology Co., Ltd.); RNAiso Plus, RT kit, RT-PCR kit (TaKaRa Japanese); primers [TaKaRa Biotechnology (Dalian) Co., Ltd.]

Establishment Rat Diabetes Model Streptozotocin (STZ) was dissolved in a 0.1 mol/L citrate buffer solution (pH 4.5). SPF SD rats were fasted for 12h. Then they were weighed and blood was taken from caudal vein. Fasting blood glucose (FBG) was examined in the morning. The rats were randomly divided into two groups: the diabetic group (45 rats) and the control group (15 rats). Before modeling, the FBGs of the two groups were both less than 6.2 mmol/L. The rats in the diabetic group were intraperitoneally injected STZ with 60 mg/kg (0.60 mL/100 g). Twenty-four hours later, blood was taken from caudal vein. The rats with blood glucose were 16.7 mmol/L or above were included. The rats in the control group were injected with an equal volume of sodium citrate buffer solution. Blood glucose was examined once a week. In the 8th week of modeling, the rats were killed by intraperitoneal injection of 10% chloral hydrate. The eyeballs were removed and the retinal issues were separated and stored at -80℃ for future use. Meanwhile, 5 eyeballs were randomly selected from each group. After fixation, dehydration and embedment, frozen sections were prepared.

HE Staining The frozen retinal sections were washed with distilled water 5min×3 times; stain it with hematoxylin for 5min, and wash it with tap water for 1min; differentiate it by 1% hydrochloric-alcohol solution for 20s and wash it with tap water for 1min; turn it back to blue by using 1% weak aqua ammonia for 1min, and wash it with distilled water for 1min; stain it with eosin for 20s and wash it with tap water for 30s; dehydrate it with ethanol by gradient, hyalinize it with xylene for 5min and mount it with neutral gum; observe HE staining result under an optical microscope and take photos.

Immunofluorescence Staining The frozen retinal sections were washed with PBS 5min×3 times; block the section with 5% normal calf serum BSA and 1%Triton X-100 0.01mol/L PBS at 37℃ for 1h, then spin away surplus liquid; dropwise add primary antibody and incubate it in a 4℃ wet box for 12h; wash it with PBS 5min ×3 times; dropwise add secondary antibody at a ratio of 1:200, incubate it at room temperature for 2h; wash it with PBS 5min ×3 times; incubate it in 6-diamidino-2-phenylindole (dihydrochloride, DAPI) at normal temperature, keep it in a dark place and stain nuclei for 10min; add anti-quenching mounting medium 50% glycerol, rinse it with PBS 5min×3 times and mount it; the frozen sections were observed under Nikon fluorescence microscope and statistics were analyzed by using the supporting image processing software.

The mRNA Expression of Proline Hydroxylase Domain 2, Glial Fibrillary Acidic Protein and Vascular Endothelial Growth Factor Design and synthesis of primers: Look for the gene sequences of PHD2, GFAP and VEGF of rats from PubMed/Nucleotide GenBank, use GAPDH as a reference gene and apply Primer Express 5.0 software to design primers as follows. The primers were synthesized by Invitrogen Trading (Shanghai) Co., Ltd. under entrustment and are PAGE purified products. GFAP sequence: forward: 5'-CCCCATTCCCTTTCTTAT-3'; reverse: 5'-TCCTCACCTGCCCACCAA-3' (169 bp). VEGF sequence: Forward: 5'-CGAAACCATGAACTTTCTGC-3'; reverse: 5'-CCTCAGTGGGCACACACTCC-3'(110 bp). PHD2 sequence: forward: 5'-TTGATAGACTGCTGTTTTTCTGG-3'; reverse: 5'-CCTCACACCTTTTTCACCTGTTA-3' (180 bp). GAPDH sequence: forward: 5'-CCTGGAGAAACCTGCCAAGT-3'; reverse: 5'-TAGCCCAGGATGCCCTTTAG-3' (101 bp). Extraction of total RNA in retinal was followed the protocol. Dilute extracted RNA to 1000 ng/μL, add diluted 2 μL of RNA, 2 μL of oligo, 2 μL of Super PuredNTP (2.5 mmol/L each) and 8.5 μL of RNase-free ddH_2O to a nuclease-free centrifuge tube in an ice bath, heat the solution at 70℃ for 5min, immediately store it in an ice bath for 2min, add 4 μL of 5×first-strand buffer (containing DTT), 0.5 μL of RNasin and 1 μL of TIANScript M-MLV after simple centrifuging, keep it in a 42℃ warm bath for 50min, heat it at 95℃ for 5min, terminate the reaction and put it on ice for cooling.

Statistical Analysis All data is inputted to SPSS17.0 software for statistics. Measurement data is expressed with mean ±standard deviation. The comparison among groups adopts completely randomly designed one-way analysis of variance. The comparison between groups adopts t-test. $P<0.05$ means it has statistical significance.

RESULTS

Changes of Body Mass and Blood Glucose of Rats The body mass of the rats in the control group was 190±3.2 g before experiment and reached 489±13 g in the eighth week; the body mass of the rats in the experimental group was 191±3.0 g before experiment and 189±10 g after experiment. Compared to the rats in the control group, the body mass of STZ-induced diabetic rats was reduced significantly. The difference had statistical significance ($P<0.01$). During experiment, the blood glucose value of the rats in the control group was relatively stable, lower than 10 mmol/L all the time; the blood glucose value of the rats in the experiment group was similar to that of the control group before

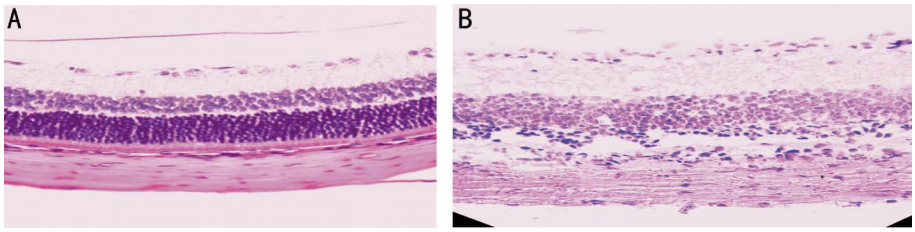

Figure 1 HE staining result of rat retina A: Normal rats; B: 8-week diabetic rats.

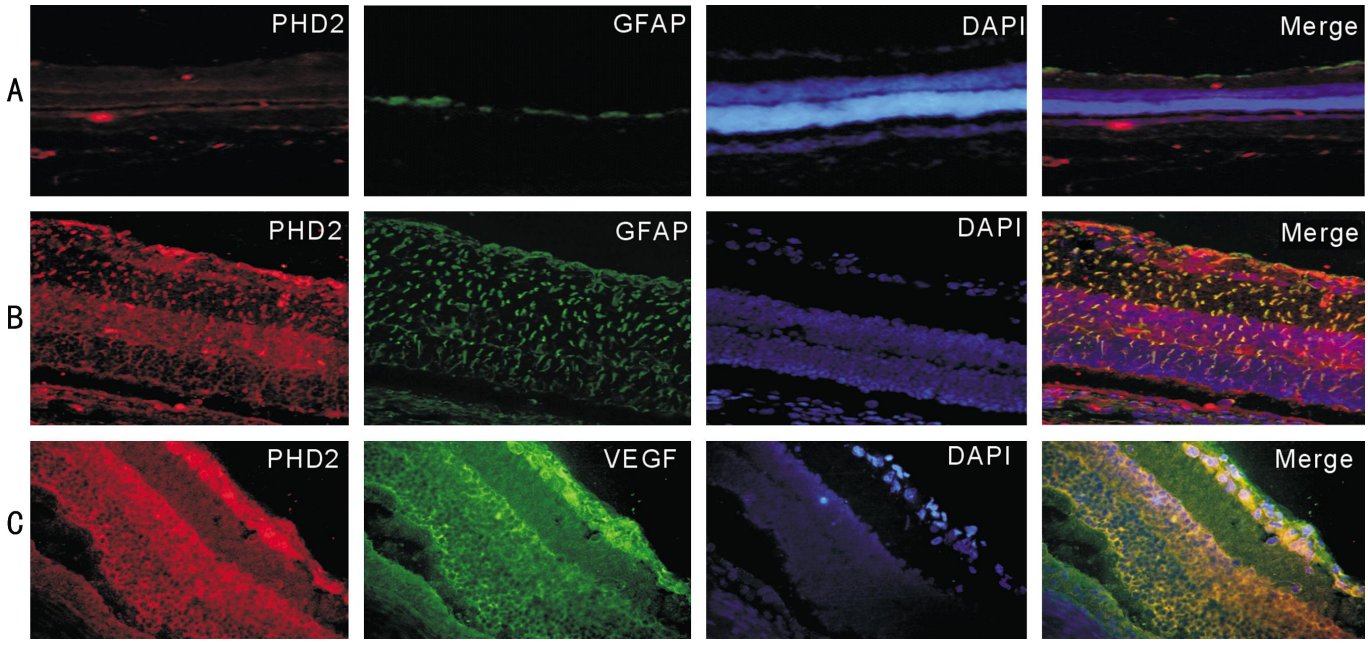

Figure 2 IF staining result of retinal PHD2, VEGF and GFAP A: Control rats; B: Diabetic rats; C: Diabetic rats. DAPI mark the nucleus, PHD2 (red), GFAP (green), VEGF (green), DAPI (blue), PHD2+GFAP (yellow), PHD2+VEGF (yellow).

injection of STZ. The blood glucose value of diabetic rats was 29.2 ±2.9 mmol/L in the 8th week after inducement. Compared to the control group, the blood glucose of STZ-induced diabetic rats rose significantly. The difference had statistical significance (P<0.01).

HE Staining Result of Retinal Issues in Pathological Examination Under an optical microscope, the retinal surface of normal rats was smooth, the structure in every layer was clear and complete and the cells were tidily arranged (Figure 1A). Eight weeks later, the retina of diabetic rats was thickened obviously, obvious proliferation of gliocytes might be seen in ganglions and nerve fiber layers, a large quantity of cells were aggregated, cell arrangement was in disorder (Figure 1B), the structural layers were unclear and the retina of normal rats was thickened obviously.

Immunofluorescence Staining Variation of Retinal Proline Hydroxylase Domain 2, Vascular Endothelial Growth Factor and Glial Fibrillary Acidic Protein Immunofluorescence (IF) result indicates PHD2 showed strong positive expression in the retina of diabetic rats. Staining showed GFAP in the positive cells expressing PHD2 had strong positive expression, suggesting that PHD2 was expressed in gliocytes. The ganglion cell layer was thickened obviously and the expression of PHD2 on it was positive. In the 8th week, GFAP expression was enhanced obviously and accompanied with obvious gliocyte proliferation (Figure 2B). Compared to the rats in the normal group, the difference in GFAP staining area has remarkable statistical significance (P<0.01). In the same period, the GFAP staining of retinal gliocytes of the rats in the control group with normal blood glucose remained unchanged. It was mainly the immune staining of astrocytes on retinal surface, the soma was small and there were many branches (Figure 2A).

At the same time, through IF staining, co-expression of PHD2 and VEGF was observed in the retina of diabetic rats. In the 8th week, the two showed co-expression in retina and PHD2 had stronger expression than VEGF in gliocytes, while VEGF was mainly expressed in the cells of ganglion cell layer as well as internal and external nuclear layers (Figure 2C).

Changes of mRNA Content of Retinal Proline Hydroxylase Domain 2, Glial Fibrillary Acidic Protein and Vascular Endothelial Growth Factor RT-PCR analysis result indicates: AGE identification result showed two clear bands: 28S (sedimentation coefficient) and 18S. The ratio of 28S/18S is about 2:1, suggesting the extracted

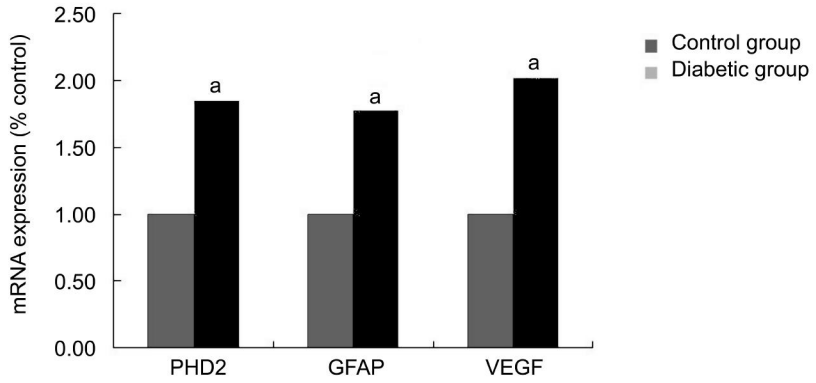

Figure 3 Analysis of PHD2, GFAP and VEGF mRNA expression levels of rat retina by RT-PCR a: P<0.01 *vs* control group.

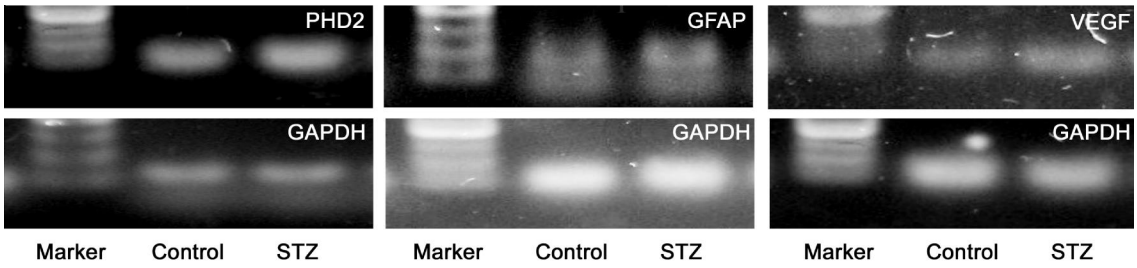

Figure 4 The mRNA expression of PHD2, GFAP and VEGF.

RNA was relatively complete and didn't have obvious degradation. Compared to the control group, the retinal mRNA level of STZ-induced diabetic rats rose obviously. The difference had statistical significance (P<0.05; Figure 3). mRNA of PHD2, GFAP and VEGF was not expressed basically or had weak expression in the control group, while the expression quantity in the diabetic rat group rose obviously. The difference between the two had statistical significance (P<0.01; Figure 4).

DISCUSSION

DR is one of the most important microvascular complications of diabetes. Its pathogenesis is still not fully known. Retinal neovascularization plays a critical role to DR progress. The recent research indicates many factors participate in the regulation and control of retinal neovascularization and HIF-1α is one of the universally accepted factors having the closest relation with neovascularization. Its expression in anoxic conditions is increased obviously and promotes neovascularization[8-9]. The key molecule regulating HIF-1 expression is prolyl hydroxylase domain (PHD). Through catalyzing HIF proline residue, thus taking hydrozylation to degrade it, it influences HIF transcription activity [10-11]. PHD2 is a new and key angiogenesis regulating factor discovered in the recent years [12-14]. It is also the currently known most critical tumor proangiogenic factor. Under the regulation of PHD2, the extracellular matrix of endothelial cells is dissolved, cells are migrated and proliferated, blood vessel lumina is formed and in the end, a new capillary network is formed [15]. Mainly because of anoxia, DR results in increase of expression of proangiogenic factors [16]. To treat neovascular diseases, appropriate methods shall be selected to reduce the expression of proliferative factors. Although STZ-induced diabetic rat model is not identical to human diabetes, but STZ-induced rat model has showed some changes in retinal vessel and function of early-stage DR patients[7].

The research result indicates: under an optical microscope, the retina of normal rats had smooth surface, the structure in every layer was clear and complete and the cells were arranged tidily. Every layer of the retina in the diabetic rat group became thinner, the arrangement was in disorder and proliferation of gliocytes and chomocytes was accompanied. IF result indicates: PHD2 showed strong positivity in the retina of early-stage diabetic rats and was expressed in various kinds of cells in the retina, particularly in retinal gliocytes. In the retina of normal rats in the control group, PHD2 was not expressed nor had weak expression; GFAP and VEGF were mainly expressed in astrocytes. Compared with the control group, PHD2, GFAP and VEGF staining showed strong positivity in all retinal layers of early-stage diabetic rats, the expression of neurogliocytes was dominated, and PHD2 was co-expressed together with VEGF and GFAP. The weak expression of PHD2 in normal tissues suggests it plays an important role in maintaining a normal and stable state of blood vessels. Research indicates that on the endothelial cells of the rats with heterozygous defects, the expression of PHD2 didn't affect the density, area, and torsion and lumen size of tumor vessels and might induce normalization of endothelial cells[5]. Compared to the control group, the mRNA levels of PHD2, GFAP and VEGF in the retinal of STZ-induced diabetic rats rose obviously (P<0.05), suggesting that the local level rise of PHD2 in retina

probably is relevant with activation of gliocytes and local autocrine. The expression of PHD2 in the retina of diabetic rats is accompanied with the high expression of VEGF, indicating that it and VEGF both play a role in DR neovascularization. Moreover, we discovered that during co-expression of PHD2 and VEGF in the retinal issues of diabetic rats, the two had slight difference in expression intensity and location, and the expression of PHD2 in gliocytes was significantly stronger than that of VEGF. The difference in expression location and strength also suggests they have different acting paths. Some researches on tumor neovascularization indicate that after inhibition of PHD2, the severity of retinopathy was alleviated, but VEGF still showed high expression possibly because the inhibition of PHD2 promoted normalization and maturity of blood vessels, but retinal neovascularization was not reduced through reduction of the total VEGF quantity [17]. It is consistent with our research result, *i.e.* PHD2 probably involves in neovascularization through some pathways[18].

The activation of gliocytes may release and generate multiple proangiogenic factors, thus promoting the activation of vascular endothelial cells and neovascularization [19]. The increase of PHD2 expression in gliocytes of diabetic retina in the early stage probably is relevant with retinal vascular endothelial cell injury protection induced by high glucose and hypoxia; on the other hand, the high expression of PHD2 has certain relation with VEGF expression and later-stage proliferative changes. The stability and activity of HIF-1α family protein are strictly regulated by PHD. With the decrease of oxygen concentration, PHD activity is inhibited and the degradation pathway of HIF-1α is interrupted, thus resulting in mass accumulation of HIF-1α. The increased HIF-1α induces expression of PHD2 and further, HIF-1α is aggregated and enters cell nuclei and may induce expression of a series of target genes, such as: VEGF and EPO, thus initiating hypoxia response reaction, forming a negative feedback regulating ring and playing a synergistic role in activating hypoxia transduction access [20-21]. Therefore, according to the discoveries of experiments, the high expression of PHD2 in gliocytes suggests that it may play a main role in the ischemia stage of DR. Probably the local ischemia in the inner layer of retina arouses increase of PHD2 expression in gliocytes, or increase of other factors, such as: nitric oxide and other substances, which indirectly promote expression of PHD2 [22-23] and eventually results in retinal neovascularization. The result of this experiment indicates retinal gliocytes of early-stage diabetic rats are in an active reactive hyperplasia and meanwhile secret PHD2, VEGF and other cellular factors. These factors probably jointly promote the occurrence and development of DR.

This research proves PHD2 expression has certain relation with retinal pathological changes of STZ-induced early-stage diabetic rats. Diabetes affects the expression of PHD2 in retina and the changes of expression will result in the occurrence and development of DR. However, further research is needed on its related action accesses in order to find new ways to treat DR.

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