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p38-MAPK pathway is activated in retinopathy of microvascular disease of STZ-induced diabetic rat model

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Abstract. – OBJECTIVE: To investigate the role of corticotropin releasing hormone (CRH) in diabetic retinopathy of microvascular disease and the potential mechanism.

MATERIALS AND METHODS: The diabetic rat model was constructed by a single intraperitoneal injection of streptozotocin (STZ). The expression of CRH in the retina of diabetic rats and wild-type rats was detected by Real-Time Polymerase Chain Reaction (RT-PCR). CRH shRNA or Scr shRNA adenovirus was injected into the eyes of diabetic rats and wild-type rats, respectively. The effect of down-regulated CRH on visual electrophysiology in rats was evaluated. Protein expressions of vascular endothelial growth factor (VEGF) and inflammatory factors that were related to the microvascular lesion after CRH downregulation were detected by Western blot. Furthermore, p38 expression was detected by Western blot to explore whether mitogen-activated protein kinase (MAPK) signaling pathway was involved in the function of retinal endothelial cells regulated by CRH.

RESULTS: The expression of CRH was significantly up-regulated in the retina of diabetic rats. RT-PCR results showed that the mRNA level of CRH in the retina of diabetic rats injected with CRH shRNA was decreased. However, no significant change in CRH level was observed in rats injected with Scr shRNA adenovirus. The down-regulated CRH could improve the diabetes-induced visual impairment and retinal inflammatory response. Moreover, the down-regulated CRH led to a decreased phosphorylation level of p38.

CONCLUSIONS: CRH improves the diabetic retinopathy of microvascular disease via the p38-MAPK pathway, which is expected to be a new target for the treatment of diabetic microangiopathy.

Key Words:
CRH, Diabetic retinopathy, MAPK.

Introduction

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes. It is a chronic progressive eye disease and has become the leading cause of blindness among working people. In patients with type 2 diabetes whose disease of course was longer than 20 years, over 60% of them may present various levels of retinopathy[1]. With the rapidly increasing number of diabetic patients worldwide, the prevalence of DR continues to elevate. It is estimated that by 2050, there will be 16 million diabetic patients over 40 years old presenting clinical features of DR in the US. Among them, about 3 million cases will suffer severe visual impairment, which will eventually bring heavy pressure to the society and economy[2]. Researches on exploring the mechanism of retinal microvascular maladjustment have been widely conducted. It is well accepted that retinal blood vessels can be directly and noninvasively observed. The independent internal environment of eyeballs makes it easier to perform drug therapy and gene intervention[3]. Although multiple studies have been focused on DR, its specific mechanism is still not fully elucidated due to the complicated pathogenesis.

The corticotropin releasing hormone (CRH) family is a kind of corticotropin releasing hormone, which has a variety of physiological and pathological functions[4]. The CRH family members include CRH, Urocortin (Ucn) Ucn I, Ucn II and Ucn III, and their biological effects are exerted mainly through two receptors, CRHR1 and CRHR2. Researchers have found that CRH and Ucn I exert their functions by binding to CRHR1, whereas

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Ucn II and Ucn III only bind to CRHR3 to exert their effects[5]. The CRH family and its receptors are widely distributed in the central nervous system and the peripheral system, including immune tissues. In the central nervous system, CRH exerts an anti-inflammatory effect by stimulating the synthesis and release of adrenocorticotrophin, as well as activating the production of glucocorticoids[6]. However, some studies have found that the CRH family distributed in the peripheral system has a significant pro-inflammatory effect[7, 8].

P38 is the most important member of the mitogen activated protein kinase (MAPK) family, which can be activated by physiological stress, lipopolysaccharide, osmotic stress and ultraviolet radiation. The activation of p38 in endogenous immune cells, such as monocytes, endothelial cells and neutrophils can be stimulated by lipopolysaccharide (LPS), tumor necrosis factor (TNF), platelet activating factor, interleukin-1 (IL-1) and ischemia/reperfusion[9, 10]. The key enzymes in the p38-MAPK pathway include MAPKK family (MKK3, MKK6) and MAPKKK family (TAK, ASK, MLK). TAK is activated by the TAK binding protein (TAB), which mediates the signal transduction of transforming growth factor (TGF-β). Moreover, TAK also activates MKK4, followed by p38 activation. Activated p38 leads to nuclear transposition, thus phosphorylating and activating multiple protein kinases and transcription factors[11,12].

In this study, siRNA was used to interfere with the expression of CRH in the fundus of diabetic rats. Retinal microangiopathy in diabetic rats were also observed to further explore the potential mechanism of CRH in the pathogenesis of DR. We aimed to investigate the role of CRH in regulating retinal microvasculature, so as to provide new ideas for the prevention and treatment of DR.

Materials and Methods

Construction of the Diabetic Rat Model

2-month-old male healthy Sprague Dawley (SD) rats weighing 180-200 g were selected. The rats were fed with grain food and water. The temperature of the feeding environment was 22-26°C, and the humidity was 50-60%. 60 experimental rats were randomly assigned into 4 groups, including the control group, single diabetes group, diabetes + CRH shRNA group and diabetes + Scr shRNA group. After fasting for 12 h, SD rats were intraperitoneally injected with streptozotocin (STZ) (dissolved in 0.1 mol/L sodium citrate buffer, pH 4.6) at a dose of 60 mg/kg. Blood sample was collected through tail vein 72 h later. The diabetic rat model was considered successful by random blood glucose level over 300 mg/de for over 1 week. The weight and blood glucose level of rats were recorded weekly. This study was approved by the Animal Ethics Committee of Heze Municipal Hospital Animal Center.

Intraocular Injection

The shRNA was injected at a dose of 3.5 mL/kg. 10% chloral hydrate was used for rat anesthesia. 5 μL of adenovirus was extracted by a micro-injector under the operating microscope after topic anesthesia. Adenovirus was then injected into the vitreous chamber from the flat of ciliary body in avoidance of retinal vessels and lenses. The antibiotic gel was applied to the surface of the eyeball and rats were put on the insulation board until revived. Postoperative fundus hemorrhage and retinal detachment were observed in the following 3 days. Antibiotic eye drops were applied 3 times a day to prevent infection.

Western Blotting

The retinal tissues were isolated for tissue lysate. 1 mL/0.1 g tissue was added in 1 mL of lysate solution containing 1 μL of phenylmethylsulfonyl fluoride (PMSF) and 1 μL of cocktail (Beyotime, Shanghai, China). After centrifugation for 10 min, the supernatant was obtained for preparing protein samples. Proteins were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of primary antibody overnight. Membranes were then incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Real-Time Polymerase Chain Reaction (RT-PCR)

We used TRIzol kit (Invitrogen, Carlsbad, CA, USA) to extract total RNA of the cryopreserved retinal tissues. According to the method of two-step Real-time PCR (RT-PCR), the extracted total RNA was reversely transcribed to complementary Deoxyribose Nucleic Acid (cDNA) and stored at -80°C for subsequent experiments. Glyce-

raldehyde 3-phosphate dehydrogenase (GAPDH) and CRH were amplified by PCR, and PCR products were retrieved in accordance with the agarose gel kit. QRT-PCR was performed following the instructions of SYBR. All experiments were repeated for 3 times.

Visual Electrophysiology

0, 2, 3 months after the construction of the diabetic rat model, visual impairment of rats in each group was evaluated by visual electrophysiology (Flash-ERG and ERG-OPs). Dark adaptation was performed 2 h before the visual electrophysiology. After light anesthesia and mydriasis, rats were lying on the platform with heads completely extended into the full field stimulator (ganzfeld). The positive pole of electrode was placed on the surface of the cornea, the negative pole was placed under the subcutaneous of the cheek on the same side, and the earth pole was placed below the ear or under the subcutaneous of the tail. After the completion of 6 individual tests, abnormal data were excluded and statistical analysis was conducted.

Statistical Analysis

We used statistical product and service solutions (SPSS 22.0, IBM, Armonk, NY, USA) Software for all statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant ($\alpha = 0.05$).

Results

Up-Regulated Expression of CRH in Diabetic Rat Model

In the present study, we explored the expression level of CRH in the diabetic rat model. RT-PCR revealed that the mRNA level of CRH in the retina of STZ-induced diabetic rats was significantly higher than that of non-diabetic rats (Figure 1A). The up-regulated expression of CRH in the diabetic retina indicated that CRH might be involved in the regulation of diabetic microvascular complications.

Construction of CRH Lentivirus

To further investigate the effect of down-regulated CRH on retinal function, Scr shRNA or CRH shRNA adenovirus was injected into the wild-type rats and diabetic rats, respectively. RT-PCR results demonstrated that CRH shRNA could significantly reduce the expression level of CRH. However, no significant difference in CRH expression was observed in Scr shRNA group (Figure 1B).

Effect of Down-Regulated CRH on the General Condition of Diabetic Rats

The diabetic rats were induced by a single intraperitoneal injection of STZ. Increased blood glucose and decreased weight of rats were observed after STZ injection. At the same time, CRH shRNA adenovirus was injected into the eyes of the wild-type and diabetic rats. The Scr shRNA adenovirus was used as negative control. Results demonstrated that down-regulated CRH did not affect the weight and blood glucose of wild-type diabetic rats injected with Scr shRNA (Table I).

Effect of Down-Regulated CRH on the Visual Function of Diabetic Rats

Visual electrophysiological examination of rats was performed after 3 months of diabetes induction. Data revealed that the amplitudes of a wave, b wave and OPs wave were significantly decreased. Meanwhile, down-regulated CRH could significantly improve the retinal function and resist the decreasing trend of a wave, b wave

Table I. The general physiological parameters of diabetic rats and non diabetic rats.

	0 month		1 month		3 month	
	Body Weight (g)	Blood glucose (mg/dL)	Body Weight (g)	Blood glucose (mg/dL)	Body Weight (g)	Blood glucose (mg/dL)
Non DR	123±15	65±6.4	201±16	72±8.2	296±20.1	82±4.8
DR	121±18	67±5.1	165±8.8*	265±12*	215±17.8*	287±10.2*
DR+Scr shRNA	129±8.9	69±7.2	172±11.8*	278±10.5*	222±19.5*	278±21*
DR+CRH shRNA	121±11	59±6.4	181±9.6*	262±9.5*	212±16.9*	269±16.4*

*indicates significant difference compared with Non DR group.

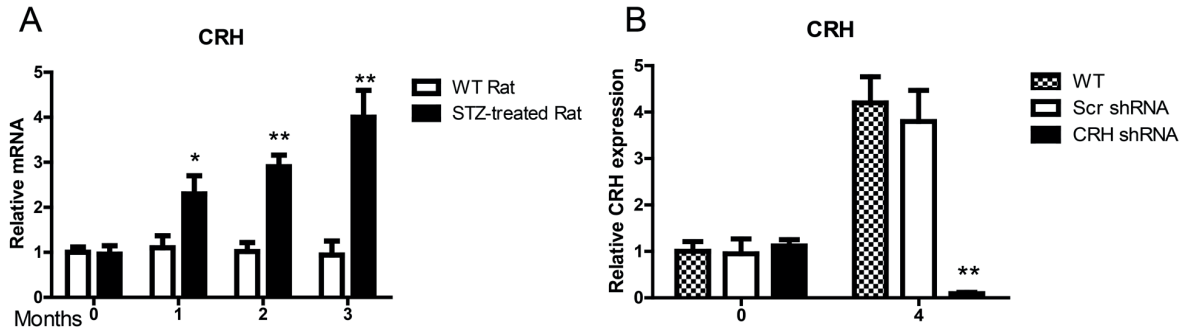

Figure 1. ***A,*** The expression of CRH in the retina of STZ-induced diabetic rats and non-diabetic rats was detected by RT-PCR. With the increase of time, the expression of CRH in the diabetic group was higher than that of the control group (mean ± SD., n=6, *p<0.05). ***B,*** The expression of CRH in rats of each group. The expression of CRH in the diabetic rats was higher than that of the wild-type rats, and the expression was decreased both in the wild-type rats or diabetic rats after CRH knockout.

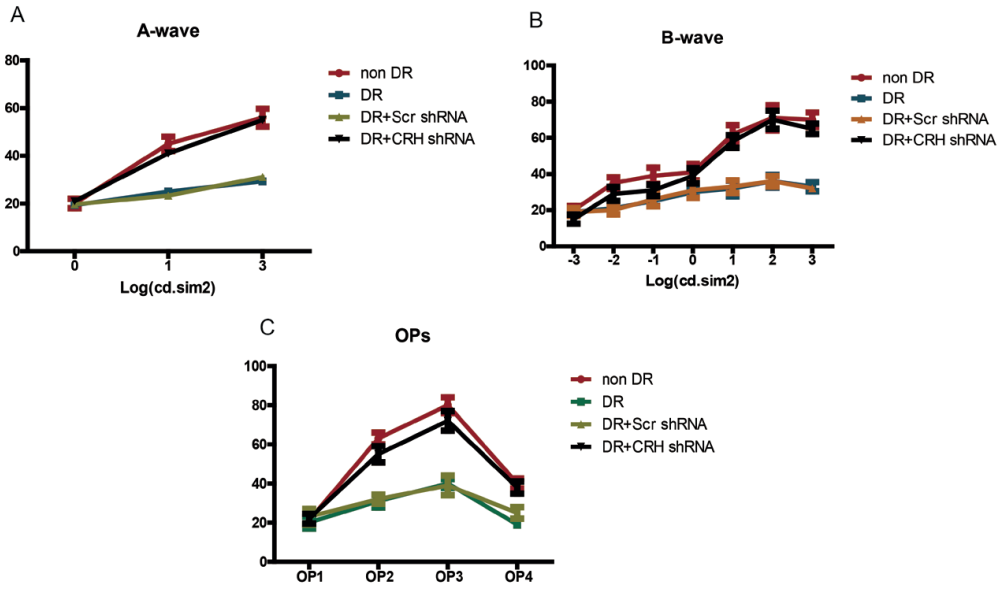

Figure 2. Visual electrophysiological examination for all the rats of each group. The down-regulated CRH could significantly improve the retinal function and resist a decreased trend of a wave (***A***), b wave (***B***) and Ops (***C***) wave.

and OPs wave (Figure 2). All these results suggested that the down-regulated CRH could prevent electroretinogram (ERG) abnormalities caused by diabetes.

Down-Regulated CRH Reduced Retina Inflammation in Diabetic Rats

CRH has been confirmed to play a vital role in the progress of diabetic microvascular syndrome. Researches have shown that the expression levels of pro-inflammatory proteins are increased during the DR progression, including ICAM-1, VEGF, IL-6 and TNF-α. Therefore, the protein expressions of ICAM-1, VEGF, IL-6 and TNF-α in the retina were detected by Western blot. Of note, the results indicated that diabetes remarkably upregulated expressions of ICAM-1, VEGF, IL-6 and TNF-α, whereas CRH knockdown led to the opposite results (Figure 3).

CRH Regulated Retinal Endothelial Cells Via p38 Pathway

Previous bioinformatics analysis indicated that MAPK signaling pathway is involved in the pathogenesis of maladjustment of retinal neovascularization. Therefore, we speculated that

MAPK signaling pathway may be involved in diabetes-induced retinal vascular injury regulated by CRH. Western blot results showed that the down-regulated CRH significantly reduced the expression level of phosphorylated p38, suggesting that the activation of the p38-MAPK pathway is regulated by CRH. The above results indicated that CRH could regulate the retinal endothelial cells through the p38-MAPK signaling pathway (Figure 4).

Discussion

CRH receptors are widely expressed in cardiac myocytes, vascular endothelial cells and vascular smooth muscle cells. Among the CRH family, only Ucn I and CRHR2 are expressed in cardiac myocytes[13]. Parkes et al[14] have found that intravenous injection of Ucn I can significantly increase heart rate and cardiac output, and reduce peripheral resistance. In the CRHR2 receptor knock-out mice, the positive inotropic and chronotropic effect, as well as vasodilation effect of Ucn I on peripheral blood were completely disappeared[15]. It is suggested that the role of Ucn I in heart and blood vessels is mediated by CRHR2. In addition, Ucn I has a significant protective effect on the ischemia/reperfusion heart. Exogenous Ucn I can significantly protect cultured cardiomyocytes, isolated hearts and *in vivo* hearts from ischemia-reperfusion injury. Studies have also suggested that the protective effect of Ucn I is related to activation of MAPK and PI3K-Akt pathway[16]. Additionally, Ucn II and Ucn III have a stronger protective effect than that of Ucn I, which is also associated with the above two signaling pathways[17].

CRH-related peptides and their corresponding receptors are also involved in the regulation of inflammatory response and vascular permeability. Novembri et al[18] have found that in the chemical inflammatory model of rat skin, CRH is abundant in inflammatory tissues, mainly including the infiltrating mononuclear inflammatory cells, fibroblasts and vascular endothelial cells. Intraperitoneal injection of CRH antibody can significantly inhibit the liquid exudation and the release of TNF-α and inflammatory cells. The effect of CRH on vascular permeability may be associated with the increased release of a variety of inflammatory factors. Multiple studies have demonstrated that CRH can increase the levels of IL-6 and TNF-α[19, 20], which may be related to the increased transcriptional activity of nuclear factor kappa B (NF-κB)[21].

RH-related peptides also activate mast cells. Subcutaneous injection of CRH or Ucn I may increase capillary permeability, inhibit mast cell degranulation and block CRHR1 transduction[22,23]. Our previous study has found that Ucn I is highly expressed in the lung tissues of rats with bronchial asthma. Ucn I promotes the degranulation of mast cells and increases the permeability of pulmonary capillary through CRHR1[24]. Moreover, Ucn I can directly regulate the permeability of vascular endothelial cells. Chouridou et al[25] have indicated that Ucn I and LPS directly increase the permeability of micro vessels. Combination of Ucn I and LPS significantly enhances the permeability of micro vessels than that of single usage.

In this study, we found that CRH regulates the function of retinal endothelial cells and the pathological growth of diabetic micro vessels by activating p38-MAPK signaling pathway. CRH is a remarkable factor involving in pathological

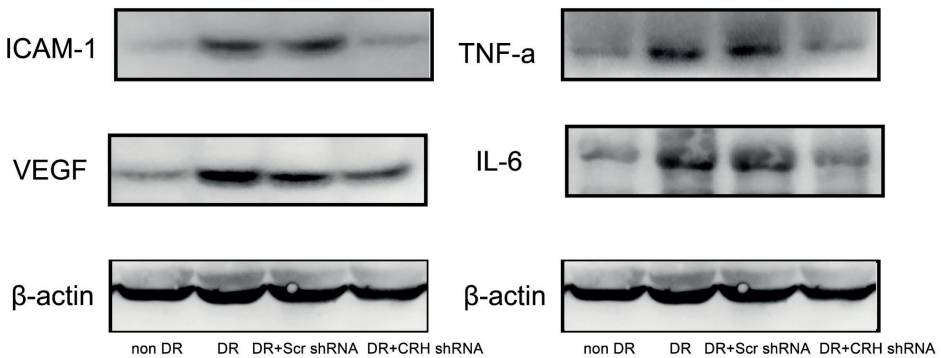

Figure 3. After 3 months of diabetes induction, the expression levels of ICAM-1, VEGF, IL-6 and TNF-α in retinal protein were detected by Western blotting. Tubulin was used as an internal control.

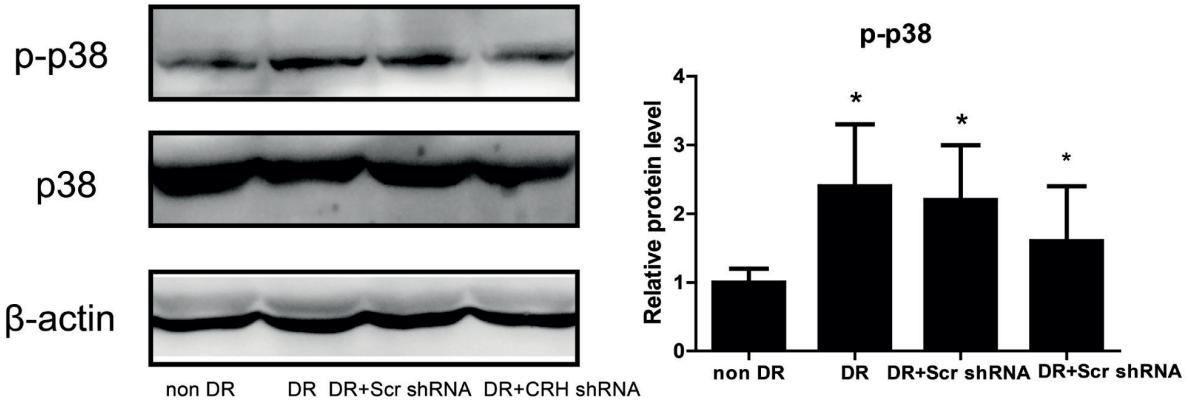

Figure 4. Protein expression of p-p38 in retina of diabetic rats.

neovascularization. Diabetic retinopathy is one of the most common vascular complications in patients with long-term diabetes[26]. The pathophysiological mechanism of DR is complex. Persistent hyperglycemia in the retinal vessels leads to the accumulation of glycosylated end products (AGEs), inflammatory response, oxidative stress, and nerve cell dysfunction[27]. These biochemical changes may cause the dysfunction of retinal microvasculature, increase retinal vascular permeability, apoptosis of vascular endothelial cells or pericytes, and retinal inflammation. Eventually, these pathological processes lead to macular edema or retinal neovascularization, further resulting in severe visual impairment[28,29]. Hyperglycemia is the most important characteristic of diabetes, which has adverse effect on vascular cells in the progress of diabetic vascular complications.

Our results demonstrated that the expression of CRH is significantly up-regulated in the retina of diabetic rats. CRH knockdown could significantly reduce the visual impairment and retinal inflammation caused by diabetes. In conclusion, our findings suggested that CRH could rescue the diabetes-induced retinal dysfunction and significantly improve visual function.

The MAPK signaling pathway can be activated by several extracellular stimulations, including high glucose stress. Moreover, the activation of MAPK signaling pathway will trigger a series of physiological effects, such as cell apoptosis, cell proliferation, cell mitosis, and gene transcription[9,11,30,31]. However, abnormal MAPK activation may lead to continuous cell proliferation or silent response to extracellular stimuli. MAPK signaling pathway is associated with the development of a variety of human diseases, such as tumor and obesity[32]. In this study, we found that the down-regulated CRH could effectively change the expression level of phosphorylated p38-MAPK. Cell proliferation induced by CRH was specifically inhibited by p38 siRNA. All the evidences revealed that there is a close relationship between the expression of CRH and the activation of p38-MAPK signaling pathway. In our study, therefore, we hypothesized that CRH could affect the function of retinal vascular endothelial cells by regulating the MAPK signaling pathway. In conclusion, the precise understanding of the molecular mechanism of CRH in vascular diseases contributes to explore new therapeutic strategies. Therefore, the inhibitory of CRH by targeted long non-coding RNA can provide a new strategy for the treatment of microvascular diseases in the future.

Conclusions

We found that the down-regulated expression of CRH improved the diabetic retinopathy of microvascular lesions in rats via activating p38-MAPK pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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