

Effects of Histone Deacetylase Inhibitor (Valproic Acid) on the Expression of Hypoxia-inducible Factor-1 Alpha in Human Retinal Müller Cells

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Purpose: To evaluate the effects of valproic acid (VPA), a histone deacetylase inhibitor (HDACI), on the expression of hypoxia-inducible factor-1 alpha (HIF-1 α) and vascular endothelial growth factor (VEGF) in human retinal Müller cells under hypoxic conditions.

Methods: Chemical hypoxia was induced in human retinal Müller cells (MIO-M1) by treatment with increasing concentrations of cobalt(II) chloride (CoCl₂). Müller cells were also treated with a set concentration of CoCl₂, along with various concentrations of VPA. The expression of HIF-1 α and VEGF in the treated Müller cells was determined by enzyme-linked immunosorbent assay.

Results: Exposure of human retinal Müller cells to increasing concentrations of CoCl₂ produced a dose-dependent increase in HIF-1 α expression. The addition of increasing concentrations of VPA lead to a dose-dependent decrease in expression of HIF-1 α and VEGF in Müller cells exposed to a set concentration of CoCl₂.

Conclusions: HDACI VPA downregulated the expressions of HIF-1 α and VEGF in human retinal Müller cells under hypoxic conditions. Using HDACI to target HIF-1 α expression in Müller cells could be a new therapeutic strategy for the treatment of retinal vascular diseases.

Key Words: Histone deacetylase inhibitors, Hypoxia-inducible factor 1 alpha, Retinal Muller cells, Valproic acid, Vascular endothelial growth factor

Diseases such as age-related macular degeneration, diabetic retinopathy, and branch retinal vein occlusion are se-

rious conditions that can decrease visual acuity and potentially lead to blindness [1,2]. An important factor in these diseases is neovascularization, which can be induced by hypoxia, ischemia, and inflammatory reactions. Vascular endothelial growth factor (VEGF), in particular, is thought to be a critical promoter of ophthalmic neovascularization [3-5]. VEGF is essential for neovascularization in the retina and choroid. During hypoxia, the expression of VEGF

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is increased to improve vascularization and vascular permeability [6,7]. Intravitreal injection of anti-VEGF has emerged as a promising treatment for neovascularization-associated ophthalmic disorders, with the drugs bevacizumab (Avastin) and ranibizumab (Lucentis) widely used in clinical settings.

In the retina, VEGF is expressed in the Müller cells, pigment epithelium, endothelium, astrocytes, and ganglion cells [8-12]. Müller cells are distributed throughout the retinal layer, perform a variety of functions, and serve as important mediators of neovascularization [13,14].

To adapt to hypoxic conditions, human cells and organs express a number of genes that affect neovascularization, metabolic processes, cell proliferation, and cell survival. The primary mediator that controls the expression of these genes is hypoxia-inducible factor-1 alpha (HIF-1 α), which regulates the production of VEGF [15,16]. HIF-1 α is composed of a β and an α subunit, the latter of which reacts to oxygen. In the presence of normal oxygen concentrations, HIF-1 α is degraded; however, in a hypoxic state, HIF-1 α is not degraded and functions to alter the expression of more than 100 genes. HIF-1 α is also involved in the regulation of vascular tone, cell proliferation, apoptosis, and other metabolic pathways [17]. Therapeutic agents that target HIF-1 α directly could provide widespread control of VEGF-induced neovascularization, an important factor in retinal hypoxic diseases, and potentially serve as alternative therapeutics to drugs targeting only individual processes in hypoxia-associated disease.

Therapeutics that target HIF-1 α have been studied thoroughly in the field of cancer treatment and many agents that target HIF-1 α directly have been developed [18-23]. Among these agents are histone deacetylase inhibitors (HDACIs), which inhibit the histone deacetylase enzyme. This allows the induction of gene transcription by hyperacetylation and reduces the activity of HIF-1 α [24]. One of the HDAC inhibitors is valproic acid (VPA), which has been widely used for the treatment of epilepsy [25]. Several studies have shown that VPA also inhibits tumor angiogenesis through suppression of angiogenic factors such as VEGF [26,27]. While HDACIs have been widely used in cancer treatment, these agents have yet to be applied to the treatment of ophthalmic disease [28-30].

In this study, the effects of the HDACI, VPA, on the expression of HIF-1 α and VEGF in human retinal Müller cells during hypoxia was evaluated.

Materials and Methods

Growth of cell lines

Human retinal Müller cells (MIO-M1) were grown in a humidified incubator at 37°C, 5% CO₂, with DMEM + GlutaMAX-I (Gibco BRL, Grand Island, NY, USA) growth media containing 10% FBS (Gibco BRL), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were provided with fresh media every 2 to 3 days.

Induction of hypoxia

Human retinal Müller cells were seeded at 5×10^3 cells per well in 96-well cell culture plates. For enzyme-linked immunosorbent assay, the cells were seeded at 1.0×10^5 cells per dish in 100 mm culture dishes. After 24 hours, the growth media was replaced with serum-free media and the cells were starved for 16 hours. Chemical hypoxia was induced by incubating cells for 24 hours in serum-free media containing various concentrations of cobalt(II) chloride (CoCl₂).

Cell viability assay

To investigate the optimal concentration of CoCl₂ for inducing chemical hypoxia, human retinal Müller cells were exposed to 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1,000 μ M CoCl₂. The cells were then washed twice with phosphate buffered saline and the cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD, USA) was used to measure cell viability. CCK-8 measures the amount of live cells by measuring formazan that is produced by dehydrogenase in cells. Ten microliters of CCK-8 and 90 μ L of serum-free media were mixed in each well. After 1 hour, optical density was measured by a microplate reader at 450 nm wavelength.

VPA treatment

VPA 1 g/mL (Sigma-Aldrich, Silver Spring, MO, USA) was diluted serially in an aqueous solution of 0.1 N sodium hydroxide (NaOH) to 10 mg/mL and added to wells containing hypoxic Müller cells that were treated with 400 μ M CoCl₂. The wells were exposed to serum-free media for 22 hours with adequate concentrations of VPA (10, 25,

50, 75, 100 µg/mL). The control group (0 µg/mL) was treated with the same amount of 0.1 N NaOH as the experimental groups.

Detection of VEGF and HIF-1α

VEGF and HIF-1α proteins were detected using the Quantikine enzyme-linked immunosorbent assay and Surveyor IC kits (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturer's instructions.

Statistical analyses

The mean ± standard deviation values were reported for the results of three independent experiments and all statistics were calculated using the IBM SPSS Statistics ver. 19.0 (IBM Co., Armonk, NY, USA). The Wilcoxon signed-rank test was used to compare differences between the experimental and control groups, with a *p*-value <0.05 considered as statistically significant.

Results

Induction of chemical hypoxia and expression of HIF-1α in human retinal Müller cells

Chemical hypoxia was induced by treating human retinal Müller cells with 0, 100, 200, 300, 400, and 500 µM of CoCl₂. A dose-dependent increase in the expression of HIF-1α was observed with increasing concentrations of

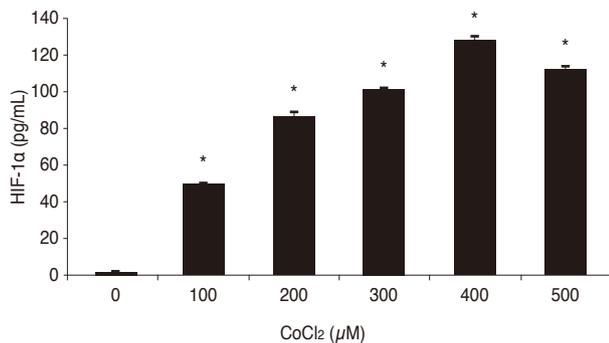


Fig. 1. Effects of cobalt(II) chloride (CoCl₂) on the expression of hypoxia-inducible factor-1 alpha (HIF-1α) in human retinal Müller cells. **p*-value < 0.05.

CoCl₂, with maximal HIF-1α expression at 400 µM CoCl₂ (Fig. 1).

Viability of human retinal Müller cells during chemical hypoxia

Müller cells were treated with 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 µM CoCl₂ and cell viability was assessed using the CCK-8 assay in order to determine the optimal concentration of CoCl₂ to induce chemical hypoxia without reducing cell viability. Cell viability decreased gradually with increasing concentrations of CoCl₂ (Fig. 2). Cell viability decreased even more at 500 µM, but the highest HIF-1α expression was observed with 400 µM of CoCl₂. Therefore, the optimal concentration of CoCl₂ to induce chemical hypoxia was selected to be 400 µM, based on the results for induction of HIF-1α expression as described above.

Effect of VPA treatment on the expression of HIF-1α and VEGF by human retinal Müller cells during hypoxia

After exposing human retinal Müller cells to 400 µM CoCl₂ to induce hypoxia, the cells were treated with 0, 10, 25, 50, 75, and 100 µg/mL of VPA. Expression of HIF-1α decreased significantly with increasing concentrations of VPA (*p* < 0.05), in a dose-dependent manner (Fig. 3). Similar results were found for the expression of VEGF (Fig. 4).

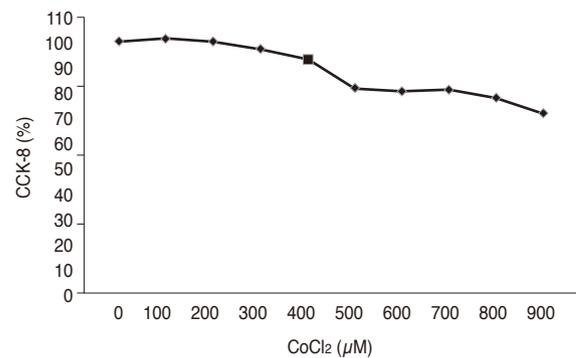


Fig. 2. Effects of cobalt(II) chloride (CoCl₂) on the survival of human retinal Müller cells. CCK-8 = cell counting kit-8.

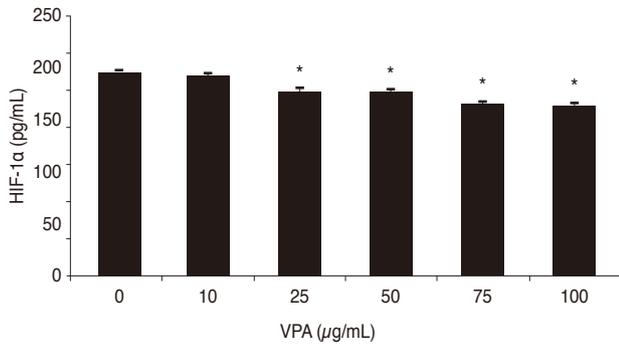


Fig. 3. Effects of increasing concentrations of valproic acid (VPA) on hypoxia-inducible factor-1 alpha (HIF-1 α) expression by human retinal Müller cells during hypoxia. * p -value < 0.05.

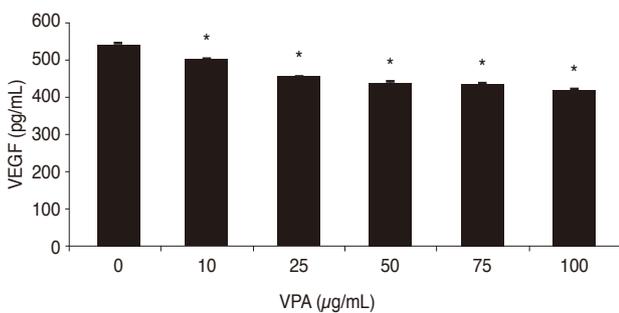


Fig. 4. Effects of increasing concentrations of valproic acid (VPA) on vascular endothelial growth factor (VEGF) expression by human retinal Müller cells during hypoxia. * p -value < 0.05.

Discussion

Neovascularization in the retina leads to vision loss and blindness in age-related macular degeneration, diabetic retinopathy, branch retinal vein occlusion, and other ophthalmic disorders. The primary cause of this neovascularization is the physiological response to hypoxia. HIF-1 α controls many of the reactions to hypoxia, as well as the expression of other genes, including placental growth factor, stromal-derived factor 1, angiopoietin 2, platelet-derived growth factor B, erythropoietin, and VEGF [31-33].

In ophthalmic diseases associated with neovascularization, improvements in vision have been demonstrated using therapeutics that target VEGF. However, these improvements appeared in only half of the patients and the effects were not long-lasting, requiring repeated, expensive treatments [34,35].

As a result, a number of studies have attempted to design therapeutics targeting mediators other than VEGF. HIF-1 α , in particular, has been one of the more promising targets. As noted, many potential mechanisms are avail-

able to suppress HIF-1 α . Studies of the pro-apoptotic, anti-angiogenic effects of HDACIs as anti-cancer therapeutics have demonstrated that HDACI-mediated suppression of HIF-1 α has a significant anti-angiogenic effect [36-38]. The HDACI VPA has been used widely in the treatment of epilepsy and bipolar disorder and this compound has been found to have neuroprotective or neuroregenerative effects on retinal ganglion cells in these patients [39-41].

This study determined that VPA suppresses HIF-1 α expression by human retinal Müller cells during hypoxia (Fig. 3), suggesting that VPA could possibly be used to target HIF-1 α in humans. However, further studies are needed to determine the precise mechanism by which HDACIs suppress HIF-1 α [42-44].

This study found that VPA suppresses VEGF expression in human retinal Müller cells during hypoxia (Fig. 4), likely due to suppression of HIF-1 α , which normally activates transcription of the VEGF gene [45].

The HIFs are heterodimeric nuclear proteins consisting of α and β subunits. There are three types of oxygen-reactive α subunits: HIF-1 α , HIF-2 α , and HIF-3 α . HIF-3 α had not been studied extensively and it is currently unknown whether HIF-1 α or HIF-2 α has a greater affect in the retina [46,47]. This study analyzed HIF-1 α , but further studies are needed to determine whether HIF-1 α or HIF-2 α is more important in human retinal Müller cells.

One of the limitations of this study is that targeting HIF-1 α is more non-specific than treatments targeting VEGF, because it controls the upper regulatory pathway. Treatments for specific targets would be better pharmacologically, but current widely used treatments that directly target VEGF have limitations, as mentioned above. Thus, controlling various hypoxia-induced pathways could be advantageous. This study identified some of the regulatory interactions between HIF-1 α and VEGF, but further studies on other factors that are controlled by hypoxia are needed. This was an *in vitro* study, so further *in vivo* studies should also be performed before making conclusions about the actual effects of VPA on human retinal Müller cells.

In this study, CoCl₂ was used to simulate the hypoxic state. One drawback of the cobalt model is that it represents only the chronic activation state of HIF-1, although it is widely established and thought to be an appropriate model for hypoxic states. With this concern in mind, experiments using CoCl₂ may not actually reflect a hypoxic state with respect to the upper regulatory level of HIF-1.

In summary, VPA was found to decrease the expression of HIF-1 α and VEGF in human retinal Müller cells during hypoxia. Using VPA or other HDACIs to target HIF-1 α in retinal Müller cells could be a potential therapeutic strategy for the treatment of retinal vascular diseases.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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