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VIP protects human retinal microvascular endothelial cells against high glucose-induced increases in TNF-α and enhances RvD1

Haoshen Shi [a], Thomas W. Carion [a], Youde Jiang [a], Jena J. Steinle [a,b], Elizabeth A. Berger [a,b,*]

[a] Department of Anatomy & Cell Biology, Wayne State University School of Medicine, 540 E. Canfield Ave., Detroit, 48201 MI, USA
[b] Department of Ophthalmology, Kresge Eye Institute, 4717 St. Antoine St., Detroit, 48201 MI, USA

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ABSTRACT

Purpose: The purpose of our study was to evaluate the therapeutic effect of VIP on human retinal endothelial cells (HREC) under high glucose conditions. Diabetes affects almost 250 million people worldwide. Over 40% of diabetics are expected to develop diabetic retinopathy, which remains the leading cause of visual impairment/blindness. Currently, treatment is limited to late stages of retinopathy with no options available for early stages. To this end, the purpose of the current study is to evaluate the therapeutic effect of vasoactive intestinal peptide (VIP) on HREC under high glucose conditions.
Methods: Primary HREC were cultured in normal (5 mM) or high (25 mM) glucose medium +/− VIP treatment. Protein levels of TNF-α, resolvin D1 (RvD1), formyl peptide receptor 2 (FPR2), G protein-coupled receptor 32 (GPR32), VEGF, and VIP receptors, VPAC1 and VPAC2 were measured.
Results: High glucose-induced changes in TNF-α and RvD1 were restored to control levels with VIP treatment. RvD1 receptors, ALX/FPR2 and GPR32, were partially rescued with VIP treatment. VPAC2 expression appeared to be the major receptor involved in VIP signaling in HREC, as VPAC1 receptor was not detected. In addition, VIP did not induce HREC secretion of VEGF under high glucose conditions.
Conclusions: Our results demonstrate that VIP's therapeutic effect on HREC, occurs in part, through the balance between the pro-inflammatory cytokine, TNF-α, and the pro-resolving mediator, RvD1. Although VPAC1 is considered the major VIP receptor, VPAC2 is predominantly expressed on HREC under both normal and high glucose conditions.

1. Introduction

Diabetic retinopathy (DR) continues as the leading cause of irreversible blindness in the United States resulting in over 10,000 new cases annually [1]. With both type 1 and type 2 diabetics at risk, over 40% of all adult diabetic patients are expected to develop this visually debilitating disease. Despite the fact that diabetes is projected to reach epidemic levels by 2030, there remains no available treatment for early stage DR, save for maintaining glycemic control. Hallmark features of DR are of both vascular and neural natures, including leukocyte adhesion to retinal vasculature, vascular occlusions, endothelial cell damage and pericyte and photoreceptor loss with underlying degenerative and inflammatory changes [2]. Inflammation has been linked to DR as early as the 1960's when it was found that diabetic patients, who were administered salicylates for rheumatoid arthritis, demonstrated a lower incidence of retinopathy [3]. However, only more recently has the inflammatory response come to the forefront as a major contributing factor to the development and progression of DR.

TNF-α is a well-characterized cytokine known to play a role in a wide spectrum of biological activities, predominately pro-inflammatory in nature. It has been reported that TNF-α levels are increased in retinas of both type 1 and type 2 diabetic rodents, as well as during the development of DR [4]. It has been indicated as a major cytokine involved in driving leukocyte adhesion. Furthermore, it has been shown that this molecule induces endothelial and pericyte cell injury and apoptotic cell death [2]; key events in the progression of DR.

In contrast, resolvins are a family of protective, pro-resolving compounds produced by docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) biosynthetic pathways [5]. RvD1 is derived

* Corresponding author at: Department of Anatomy & Cell Biology, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, 48201 MI, USA.
E-mail addresses: hshi@med.wayne.edu (H. Shi), tcarion@med.wayne.edu (T.W. Carion), youdejiang55@gmail.com (Y. Jiang), jsteinle@med.wayne.edu (J.J. Steinle), eberger@med.wayne.edu (E.A. Berger).

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from D-series ω-3 polyunsaturated fatty acids and binds to G protein-coupled receptors ALX/FPR2 and GPR32 [6], leading to reduced polymorphonuclear leukocytes (PMN) infiltration and increased nonphlogistic phagocytosis of apoptotic PMN [7]. A protective effect of RvD1 against diabetic neovascularization has been demonstrated, in part through the suppression of the pro-inflammatory cytokine TNF-α [8].

VIP is an endogenous immunoregulatory neuropeptide synthesized by neurons throughout the central and peripheral nervous systems, in addition to immune cells [9]. Focusing on the retina, VIP immunoreactivity has been detected in amacrine cells and other interneurons of the inner nuclear layer (INL) and inner plexiform layer (IPL) [10,11]. The immunoregulatory activities of VIP are mediated predominately by two G protein-coupled receptors, VPAC1/VIPR1 and VPAC2/VIPR2. VPAC1 is constitutively expressed in lymphocytes, macrophages, dendritic cells, microglia, monocytes and mast cells, whereas VPAC2 is thought to require activation [12]. VPAC1 serves as the major immunoregulatory receptor for VIP in various immune cells, while VPAC2 is thought to play a role in immune homeostasis and tissue restoration [13,14].

Recent studies have reported decreased expression of VIP and both VPAC1/VPAC2 receptors in the retina during early stage DR [14,15]. Moreover, in diabetic macular edema, activation of the protective VIP/PACAP pathway has been shown to prevent the breakdown of the outer blood retinal barrier by mediating tight junction integrity [16]. However, the modulatory mechanism and potential therapeutic effect of VIP during the development of DR is largely unknown. The current study seeks to demonstrate a potential pro-resolving role for VIP during DR by preliminarily investigating its interaction with TNF-α and RvD1 in HREC under high glucose conditions.

2. Materials and methods

2.1. Retinal endothelial cell culture

Primary HREC were acquired from Cell System Corporation (CSC, Kirkland, WA). Cells were grown in M131 medium containing microvascular growth supplements (MVGS; Invitrogen, Carlsbad, CA), 10 mg/mL gentamycin, and 0.25 mg/mL amphotericin B. All primary cells were used within six passages. Prior to experimentation, cells were transferred for three days to high (25 mM) or normal (5 mM) glucose medium (M131 medium supplemented with glucose) with MVGS and antibiotics, then quiesced by removing MVGS for 24 h. Cells were exposed to VIP (10^{-9} M) for 4 h [17,18], followed by rinsing with cold PBS and collection into lysis buffer containing protease and phosphatase inhibitors. Cellular extracts were prepared by sonication, and total protein concentration was determined for analyses as described below.

To evaluate whether VIP acts directly via VPAC2 and/or ALX/FPR2 regarding TNF-α levels, cells were treated with the VIP receptor antagonist, [D-p-Cl-Phe [6], Leu [17]]-VIP (Leu) (VPAC antagonist; R&D Systems, Minneapolis, MN) or a selective antagonist of ALX/FPR2 signaling, WRW4 (Tocris, Pittsburg, PA). Cells cultured under normal and high glucose conditions were exposed to VIP (10^{-9} M) in the presence of each antagonist (Leu at 2 μM [19] or WRW4 at 1 μM [20]) for 4 h, then processed for protein analyses as described above.

Previously, high osmolar conditions have been included as an additional control to determine whether the observed *in vitro* effects were a result of high glucose treatment or increased osmolarity of the treatment media [21]. Since it has been established that no differences were observed between high osmolarity and normal glucose, this control was omitted from the current study.

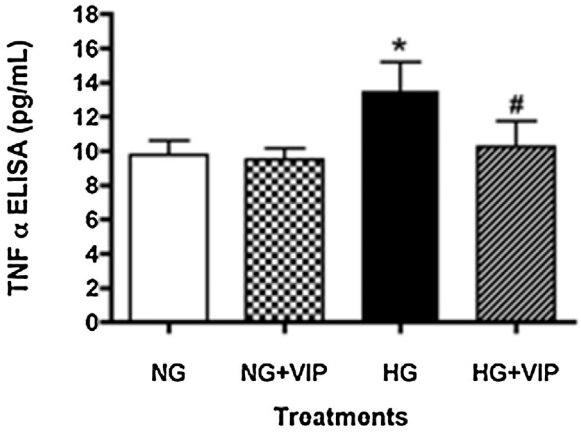

Fig. 1. TNF-α protein levels as detected by ELISA. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/− VIP treatment (10^{-9} M) for 4 h. Data shown are representative of three independent experiments in duplicate (n = 6) and are expressed as mean ± SEM. $^{*}P < 0.05$ vs NG, $^{\#}P < 0.05$ vs HG.

2.2. ELISA

Levels for TNF-α and RvD1 were determined using ELISA kits (Thermo Fisher Scientific, Waltham, MA; Cayman Chemical, Ann Arbor, MI). Cells were collected and processed as described above. All samples were centrifuged at 5000 × *g* for 5 min and an aliquot of each supernatant was assayed in duplicate or triplicate per the manufacturer's instruction. Equal protein was loaded into all wells. The reported sensitivities of these assays are as follows: <2.0 pg/mL for TNF-α and 3.3 pg/mL for RvD1.

2.3. Western blotting

Proteins were separated on 4–12% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. After blocking membranes in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA at r.t. for 60 min, membranes were incubated overnight at 4 °C with antigen-specific primary antibodies. The primary antibodies were used as follows: GPR32, ALX/FPR2 and VEGF (Abcam, San Francisco, CA); VPAC1 and VPAC2 (Santa Cruz, Santa Cruz, CA). Blots were then incubated with species-specific HRP-conjugated secondary antibodies for 2 h at r.t. Proteins were visualized by incubation with a chemiluminescence substrate kit (Thermo Fisher Scientific, Waltham, MA). Western blot images were collected (Azure Biosystem C500, Dublin, CA) and target protein levels were quantified (Image Studio Lite software) after normalizing to β-actin. One representative blot is shown. Treatment groups were normalized to β-actin levels and then compared to normal glucose, which was normalized to 1.0.

2.4. Statistical analysis

All assays were performed twice from three independent experiments and the data (n = 6/group) are presented as mean ± SEM. Data were analyzed by the Kruskal-Wallis test, followed by Dunn's testing. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. VIP reduced levels of high glucose-induced TNF-α

Changes in TNF-α protein levels were assessed under high glucose conditions and after VIP treatment, as shown in Fig. 1. As expected, TNF-α protein levels were significantly increased by